

# Antioxidants in Diabetes Management

edited by

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# Series Introduction

Oxygen is a dangerous friend. Overwhelming evidence indicates that oxidative stress can lead to cell and tissue injury. However, the same free radicals that are generated during oxidative stress are produced during normal metabolism and thus are involved in both human health and disease.

Free radicals are molecules with an odd number of electrons. The odd, or unpaired, electron is highly reactive as it seeks to pair with another free electron.

Free radicals are generated during oxidative metabolism and energy production in the body.

Free radicals are involved in:

- Enzyme-catalyzed reactions
- Electron transport in mitochondria
- Signal transduction and gene expression
- Activation of nuclear transcription factors
- Oxidative damage to molecules, cells, and tissues
- Antimicrobial action of neutrophils and macrophages
- Aging and disease

Normal metabolism is dependent upon oxygen, a free radical. Through evolution, oxygen was chosen as the terminal electron acceptor for respiration. The two unpaired electrons of oxygen spin in the same direction; thus, oxygen is a biradical, but is not a very dangerous free radical. Other oxygen-derived free radical species, such as superoxide or hydroxyl radicals, formed during metabolism or by ionizing radiation are stronger oxidants and are therefore more dangerous.

In addition to research on the biological effects of these reactive oxygen species, research on reactive nitrogen species has been gathering momentum.

NO, or nitrogen monoxide (nitric oxide), is a free radical generated by NO synthase (NOS). This enzyme modulates physiological responses such as vasodilation or signaling in the brain. However, during inflammation, synthesis of NOS (iNOS) is induced. This iNOS can result in the overproduction of NO, causing damage. More worrisome, however, is the fact that excess NO can react with superoxide to produce the very toxic product peroxynitrite. Oxidation of lipids, proteins, and DNA can result, thereby increasing the likelihood of tissue injury.

Both reactive oxygen and nitrogen species are involved in normal cell regulation in which oxidants and redox status are important in signal transduction. Oxidative stress is increasingly seen as a major upstream component in the signaling cascade involved in inflammatory responses, stimulating adhesion molecule and chemoattractant production. Hydrogen peroxide, which breaks down to produce hydroxyl radicals, can also activate NF- $\kappa$ B, a transcription factor involved in stimulating inflammatory responses. Excess production of these reactive species is toxic, exerting cytostatic effects, causing membrane damage, and activating pathways of cell death (apoptosis and/or necrosis).

Virtually all diseases thus far examined involve free radicals. In most cases, free radicals are secondary to the disease process, but in some instances free radicals are causal. Thus, there is a delicate balance between oxidants and antioxidants in health and disease. Their proper balance is essential for ensuring healthy aging.

The term *oxidative stress* indicates that the antioxidant status of cells and tissues is altered by exposure to oxidants. The redox status is thus dependent upon the degree to which a cell's components are in the oxidized state. In general, the reducing environment inside cells helps to prevent oxidative damage. In this reducing environment, disulfide bonds (S—S) do not spontaneously form because sulfhydryl groups kept in the reduced state (SH) prevent protein misfolding or aggregation. This reducing environment is maintained by oxidative metabolism and by the action of antioxidant enzymes and substances, such as glutathione, thioredoxin, vitamins E and C, and enzymes such as superoxide dismutase (SOD), catalase, and the selenium-dependent glutathione and thioredoxin hydroperoxidases, which serve to remove reactive oxygen species.

Changes in the redox status and depletion of antioxidants occur during oxidative stress. The thiol redox status is a useful index of oxidative stress mainly because metabolism and NADPH-dependent enzymes maintain cell glutathione (GSH) almost completely in its reduced state. Oxidized glutathione (glutathione disulfide, GSSG) accumulates under conditions of oxidant exposure, and this changes the ratio of oxidized to reduced glutathione; and

increased ratio indicates oxidative stress. Many tissues contain large amounts of glutathione, 2–4 mM in erythrocytes or neural tissues and up to 8 mM in hepatic tissues. Reactive oxygen and nitrogen species can directly react with glutathione to lower the levels of this substance, the cell's primary preventative antioxidant.

Current hypotheses favor the idea that lowering oxidative stress can have a clinical benefit. Free radicals can be overproduced or the natural antioxidant system defenses weakened, first resulting in oxidative stress, and then leading to oxidative injury and disease. Examples of this process include heart disease and cancer. Oxidation of human low-density lipoproteins is considered the first step in the progression and eventual development of atherosclerosis, leading to cardiovascular disease. Oxidative DNA damage initiates carcinogenesis.

Compelling support for the involvement of free radicals in disease development comes from epidemiological studies showing that an enhanced antioxidant status is associated with reduced risk of several diseases. Vitamin E and prevention of cardiovascular disease is a notable example. Elevated antioxidant status is also associated with decreased incidence of cataracts and cancer, and some recent reports have suggested an inverse correlation between antioxidant status and occurrence of rheumatoid arthritis and diabetes mellitus. Indeed, the number of indications in which antioxidants may be useful in the prevention and/or the treatment of disease is increasing.

Oxidative stress, rather than being the primary cause of disease, is more often a secondary complication in many disorders. Oxidative stress diseases include inflammatory bowel disease, retinal ischemia, cardiovascular disease and restenosis, AIDS, ARDS, and neurodegenerative diseases such as stroke, Parkinson's disease, and Alzheimer's disease. Such indications may prove amenable to antioxidant treatment because there is a clear involvement of oxidative injury in these disorders.

In this new series of books, the importance of oxidative stress in diseases associated with organ systems of the body will be highlighted by exploring the scientific evidence and the medical applications of this knowledge. The series will also highlight the major natural antioxidant enzymes and antioxidant substances such as vitamins E, A, and C, flavonoids, polyphenols, carotenoids, lipoic acid, and other nutrients present in food and beverages.

Oxidative stress is an underlying factor in health and disease. More and more evidence is accumulating that a proper balance between oxidants and antioxidants is involved in maintaining health and longevity, and that altering this balance in favor of oxidants may result in pathological responses causing functional disorders and disease. This series is intended for researchers in the basic biomedical sciences and clinicians. The potential for healthy aging and

disease prevention necessitates gaining further knowledge about how oxidants and antioxidants affect biological systems.

Diabetes (both type I and type II) has been found to be associated with indices of oxidative damage. This suggests that oxidative stress is a contributing factor in these disorders. Aging itself is known to involve the deleterious effects of oxygen and glucose, which, albeit essential for energy, may also lead to oxidative stress. Indeed, insulin insufficiency is thought to be one of the underlying factors in accelerating aging. Thus, it is logical that natural antioxidants may have beneficial implications for diabetes management and healthy aging. Vitamin E and lipoic acid are two such substances receiving attention in this regard. Therefore, *Antioxidants in Diabetes Management* is an appropriate addition to the entire series, *Oxidant Stress and Disease*, since this volume provides a comprehensive and up-to-date evaluation of the role of oxidants and antioxidants in diabetes. Chapters focusing on biochemistry, molecular biology, cell and organ physiology, as well as human clinical studies of diabetes management, are included.

*Lester Packer*  
*Enrique Cadenas*

# Preface

Diabetes and its complications present a serious medical and socioeconomic problem. Diabetes mellitus is a chronic derangement of insulin action and carbohydrate metabolism. Its major distinguishing diagnostic feature is hyperglycemia, in which blood glucose rises above 200 mg/dL within 2 h of ingestion of 75 g oral glucose. There are two major classifications: insulin-dependent diabetes (IDDM, also known as type I or juvenile-onset diabetes), which accounts for about 20% of cases, and non-insulin-dependent diabetes (NIDDM, also known as type II or adult-onset diabetes), which accounts for the rest. IDDM is characterized by lack of insulin secretion, whereas NIDDM patients generally exhibit normal or elevated insulin levels but their peripheral tissues lack insulin sensitivity. NIDDM presently affects 30 to 50% of the elderly and is also characterized by the loss of insulin-triggered glucose uptake from the bloodstream to the insulin-sensitive tissues, thus leading to elevated blood glucose levels.

The management of diabetes has changed significantly in the past half century. Diabetic coma due to uncontrolled hyperglycemia was the medical challenge 50 years ago, but with greater availability of increasingly effective insulin (e.g., recombinant human insulin), the focus of medical care has shifted toward the management of diabetic complications. Polyneuropathy, one of the most common of these, occurs in 50% of those patients who have had diabetes for 25 or more years, and leads to pain and decreased mobility and function. Nephropathy and retinopathy are also common complications, which can result in kidney dysfunction and blindness, respectively. The major cause of death among diabetic patients, cardiovascular disease, is two to four times more common in diabetic than in nondiabetic populations. In addition, peripheral vascular disease and neuropathy can cause ischemia and, in some cases, gangrene of the lower limbs leading to amputation.

The medical and socioeconomic impact of these complications is enor-

mous: diabetes is the leading cause of adult blindness, dialysis, kidney transplantation, and foot amputations. Diabetes afflicts approximately 6% of the population and is expected to rise to over 10%. Globally, there will be about 150 million diabetic patients by the year 2000, and 215 million by the year 2015. In the United States alone, the cost of diabetes mellitus and its complications increased from \$20 billion in 1987 to \$90 billion in 1997.

The current strategy to combat diabetes focuses on increasingly stringent control of hyperglycemia to prevent or modify the onset and progression of the disease and its complications. The Diabetes Control and Complication Trial (DCCT) demonstrated that intensified insulin treatment with an improvement in blood sugar control in type I diabetic patients reduced the rate of development and progression of some diabetic micro- and neurovascular complications. This clinical study supported the hypothesis that hyperglycemia is a major risk factor in the development of diabetic complications. Research over the last 10 years has shown that there are direct, potentially damaging, effects of diabetic hyperglycemia, including glucose-induced vascular abnormalities that are relevant to many diabetic complications. Cardiovascular risk, however, was not diminished by the intensified insulin treatment used in the study.

Only a minority of diabetic patients can achieve this kind of strict blood sugar control over several years in order to reach the preventive effects of hypoglycemia therapy on late diabetic complications. This is especially true for type II diabetic patients. The UKPDS study has shown that only 30% of type II diabetic patients can achieve the required glycemic control levels necessary to prevent late diabetic complications after 3 years and only 10% can do so after 9 years. Because of the therapeutic limitations of hypoglycemic therapy in practice, further interventional strategies must be developed.

Recent research indicates another promising area for inquiry and therapy—glucose (AGE)-induced vascular abnormalities, which are of relevance for all or most diabetic complications. Impaired microcirculation, capillary hypoxia, and ischemia syndrome are present in most diabetic complications. In addition to elevated blood glucose levels, increased production of reactive oxygen species (free radicals), which are known to exhibit direct tissue-damaging properties, may contribute to a number of diabetic complications and to the development of insulin resistance itself. These deleterious species can be neutralized by endogenous and exogenous antioxidants such as vitamin E, vitamin C, and thioctic (lipoic) acid. Compared to control subjects, NIDDM patients have lower plasma antioxidant vitamins (E and C) and double the lipid hydroperoxides (a measure of oxidative damage). IDDM patients also have low serum total antioxidant activity.

Increased oxidative stress in diabetic patients appears to be related to

the underlying metabolic abnormalities, and is also an early stage in the disease pathology that may contribute to the development of complications. The impaired microcirculation, capillary hypoxia, and ischemia syndrome present in most diabetic complications are associated with the production of reactive oxygen species. In addition to control of blood sugar, control of oxidative stress offers another avenue for the treatment of the disease.

This volume summarizes the current knowledge of the pathogenic role of oxidative stress in the onset and progression of diabetes and its complications, and presents results of studies aimed at modulating oxidatively induced complications through the use of antioxidants. Chapters in this volume focus on (1) basic research on oxidative stress in the development of diabetes and diabetic complications; (2) studies aimed at specific complications such as cardiovascular disease and polyneuropathy; and (3) clinical trials of antioxidants in diabetic subjects.

An overall understanding of free radical pathology and its modulation by antioxidants is central to basic research into the relationships between diabetes, oxidative stress, and antioxidants. The concept of oxidative stress and antioxidant protection is explored with emphasis on the potential synergistic effects of an interlinked antioxidant network. The significance of oxidative stress markers in diabetes, and the evidence for and against an oxidative component in the genesis of diabetes is an ongoing controversy. Oxidative stress appears to play a role not only in complications arising from the disease, but in the development of insulin resistance in NIDDM. The possibility of its modulation by the antioxidant  $\alpha$ -lipoic acid is explored. A related report focuses on oxidative stress and antioxidant treatment in animal models of both IDDM and NIDDM. The nonobese diabetic mouse model is often used in diabetic research, providing the basis for respective inquiries on basic research into diabetic mechanisms and prevention of NIDDM by antioxidant therapy.

Recently, much interest has been expressed in the possible interactions of oxidative stress induced by diabetes and cell signaling molecules in the development of diabetic complications. Studies concentrate on the tissue-damaging effects of free radicals, and also on oxidative-stress-sensitive molecular factors such as IRS-I, PI-3, PKC, and NF- $\kappa$ B, which are known to contribute to insulin resistance and diabetic complications. All of these molecular factors are redox-sensitive, which means that an imbalance between oxidative stress and antioxidant dysfunction can convert them to pathogenic factors. If one accepts the concept of oxidative stress described here as an important risk factor for diabetes and its complications, one has to consider the possible therapeutic value of antioxidants in treating this disorder. Research is presented in this volume that examines, in particular, the relationship between

oxidative stress, NF- $\kappa$ B activation, and late diabetic complications, as well as the effects of  $\alpha$ -tocopherol (vitamin E) on protein kinase C and its implications for diabetes.

Polyneuropathy, one of the most common, painful, and disabling complications of diabetes, also comes into focus. The hypothesis that ischemic reperfusion, a mechanism that induces oxidative stress, is the primary cause of diabetic polyneuropathy is explored, as is the potential for treatment with antioxidants in synergistic combination with essential fatty acids. This concept is further examined in an experimental model of polyneuropathy, using a thioctic acid ( $\alpha$ -lipoic acid)–gamma-linolenic acid conjugate for protection.

As mentioned, cardiovascular complications represent the most common cause of death among diabetics, as well as the most frequent reason for limb amputation. Potential mechanisms for antioxidant intervention are discussed. Myocardial infarction is a leading cause of death among diabetics, and oxidation of low-density lipoprotein (LDL) is now a well-established causal factor in this pathology. One chapter links protein kinase C activation with the development of diabetic vascular complications and also suggests a role for vitamin E in their prevention. Another way in which oxidative stress may be involved in vascular complications, especially microangiopathy, is explored by one group through effects on cell adhesion molecules and the related potentially protective effect of antioxidants. Antioxidant vitamins, especially vitamin E, may reduce LDL oxidation in diabetes.

The ultimate goal of research on oxidative stress and diabetes is to introduce new therapeutic possibilities and to establish the efficacy of various therapeutic regimens. Therefore, several contributions to this volume relate to clinical trials of therapeutic effects of antioxidants such as vitamin E and lipoic acid. These include an overview of clinical trials of antioxidants to reduce insulin resistance, an evaluation of the clinical evidence on antioxidants in the treatment of diabetic polyneuropathy, and a report on the clinical status of antioxidants in the treatment of diabetic vascular abnormalities. Thus, research in oxidative stress, antioxidants, and diabetes may be achieving progress in attacking the basic mechanisms of the disease and in ameliorating some of its most common complications.

The medical and socioeconomic burden of diabetes and its complications requires a successful therapeutic concept. We hope that this selection of preclinical and clinical studies will stimulate scientific discussion of the possible pathogenic role of oxidative stress in diabetes and its complications, and will help to illustrate the therapeutic potential of antioxidants for treatment of the disease.

This volume is the result of two recent workshops, “Oxidative Stress

in Diabetes and Its Complications: Implications of Antioxidant Treatment,” held in Leipzig, as a satellite of the German Diabetes Association annual meeting and in Santa Barbara at the Oxygen Club of California World Congress. We would like to acknowledge the support of the following sponsors: UNESCO-MCBN (Global Network for Molecular and Cell Biology), the American Diabetes Association, the Henkel Nutrition and Health Group, and ASTA Medica AWD GmbH.

*Lester Packer*  
*Peter Rösen*  
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# 1

## Oxidative Stress and Antioxidants: The Antioxidant Network, $\alpha$ -Lipoic Acid, and Diabetes

**Lester Packer**

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In this introductory chapter, oxidative stress in diabetes and implications of antioxidant treatment are considered. It is thought that free radicals may play a major role in aging and disease. Free radicals arise from radiation, environmental chemicals, cigarette smoke, and various other environmental sources. In addition, all through our life, we have a fire burning inside of us—our own body metabolism, which generates free radicals. Finally, many environmental substances (as well as drugs and alcohol) are metabolized in our body, generating free radicals through cytochrome P450-mediated oxidations. Many free radicals can be cytotoxic.

However, free radical reactions are also essential. They are essential for enzymes and for host defense mechanisms such as neutrophils, macrophages, and other cells of the immune system. Free radicals are important in the activation of transcription factors and in cell signal transduction and gene expression. But if free radicals are overproduced, they also can create oxidative stress and damage to molecules, cells, and tissues.

So what, then, is oxidative stress? Oxidative stress is an upset in the balance between oxidants and antioxidants. It was defined by Helmut Sies (1) in the following way: “Oxidative stress is a change in the pro-oxidant/antioxidant balance in the favor of the former, potentially leading to biological damage.” The result is molecular damage products, which are markers of oxidative stress.

## I. DEFINITION OF AN ANTIOXIDANT

What is an *antioxidant*? To find a definition, we went to the dictionary. Dorland's Medical Dictionary reports (2), "An antioxidant is one of many widely used synthetic or natural substances added to a product to prevent or delay deterioration by action of oxygen in the air." Examples of such products to which antioxidants may be added are rubber, paint, vegetable oils, and so on. But there are many other definitions of an antioxidant. For example, Halliwell and Gutteridge (3) defined an antioxidant as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate." Another definition of an antioxidant (and the one I favor) is that of a *metabolic antioxidant* (4,5): "An antioxidant is a substance which protects biological tissues from free radical damage, which is able to be recycled or regenerated by biological reductants." Thus, metabolic antioxidants have something similar to a catalytic activity, as long as they are connected, directly or indirectly, to biological reductants.

So what, then, is the antioxidant network? If antioxidants can be recycled and regenerated, then there must be some sort of coordinated network connecting them to one another and to cellular metabolic processes. The antioxidant network consists of a series of proteins and substances that provide these connections.

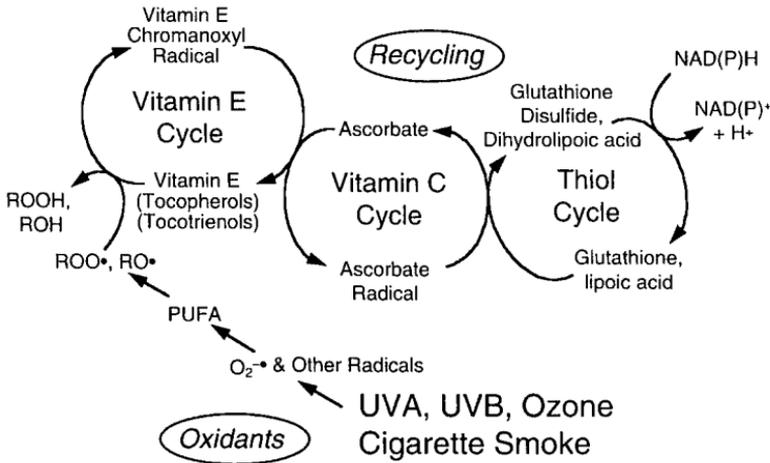
Among the proteins that are most important in antioxidant defense are superoxide dismutases to remove superoxide; enzymes that catalyze the removal of hydroperoxides; reduced thioredoxin; and a number of proteins, like transferrin and ceruloplasmin, that bind transition metals like iron and copper in such a way that they are not able to catalyze free radical reactions. Another vital antioxidant enzyme is methionine sulfoxide reductase, which repairs sulfhydryl groups of methionine residues, thus protecting cysteine residues (which are critical for biological protection against oxidation). The group of antioxidant substances, all of which are phytonutrients, is rather small: vitamin C, also known as ascorbate; vitamin E, a family of eight compounds—four tocopherols and four tocotrienols; carotenes, of which 500 different varieties may exist in nature; and flavonoids and polyphenols, of which there may be 4000–5000 different varieties. Of course, few of the carotenoids and flavonoids are common in the diet.  $\alpha$ -Lipoic acid is another antioxidant compound naturally occurring in foods and also produced by the body. Others are metals that are covalently bound to the antioxidant defense proteins to assist with the proteins' catalytic functions.

Lipoic acid is a good example of a metabolic antioxidant. It is an analogue of octanoic acid and has a dithiolane ring in its oxidized form, but the ring can be broken by reduction to form dihydrolipoic acid. Both lipoic acid and dihydrolipoic acid have unique antioxidant profiles (6). The reduced form of lipoate has somewhat more antioxidant properties than the oxidized form in that the reduced form can scavenge superoxide and peroxy radicals. Lipoic acid was found some years ago, in work from Helmut Sies's laboratory, to be taken up by the fatty acid carrier in isolated mammalian hepatocytes (7). Thus, lipoic acid can readily be taken up by cells. Three different enzymes have been identified as contributing to the reduction of lipoic acid so far: glutathione reductase (8,9) and thioredoxin reductase (10), which are NADPH-dependent enzymes, and the more abundant lipoamide dehydrogenase, an NADH-dependent enzyme. Lipoamide dehydrogenase is the E-3 component common to the  $\alpha$ -keto acid dehydrogenase complexes that exist only in the mitochondria of animal cells. After reduction, lipoic acid can be released to the extracellular compartment, so there can be a cycle of lipoic acid reduction inside the cell, its release to the outside where it is oxidized, its reuptake into cells as the oxidized form, and its reduction again, as the cycle continues (11).

## II. THE ANTIOXIDANT NETWORK

The antioxidant network is composed of redox-sensitive antioxidant substances. I like to say that the hub of the antioxidant network is vitamin C. The antioxidant network usually gets activated by vitamin E (12). After vitamin E is oxidized by oxidants or lipid free radicals, then the vitamin E free radical is formed, which in turn activates vitamin C to regenerate vitamin E nonenzymatically. Vitamin C itself becomes a radical, the vitamin C radical, in this process. Glutathione, with the aid of enzymes, can reduce the vitamin C radical (or dehydroascorbate, the completely reduced form of vitamin C). The oxidized glutathione thus produced can be reduced through enzymatic reactions that draw on cellular reducing power. There are also substances that we can obtain in our diet or that we can supplement—like flavonoids, polyphenols, and lipoic acid—that can also act in the antioxidant network (13,14). An example of how the antioxidant network works with respect to vitamin C, vitamin E, and thiol antioxidants is shown in Figure 1.

If vitamin E is made into a radical by reacting with a lipid peroxy radical, the chromanol of vitamin E becomes a chromanoxyl radical and a lipid hydroperoxide forms. If this process is induced in human low-density

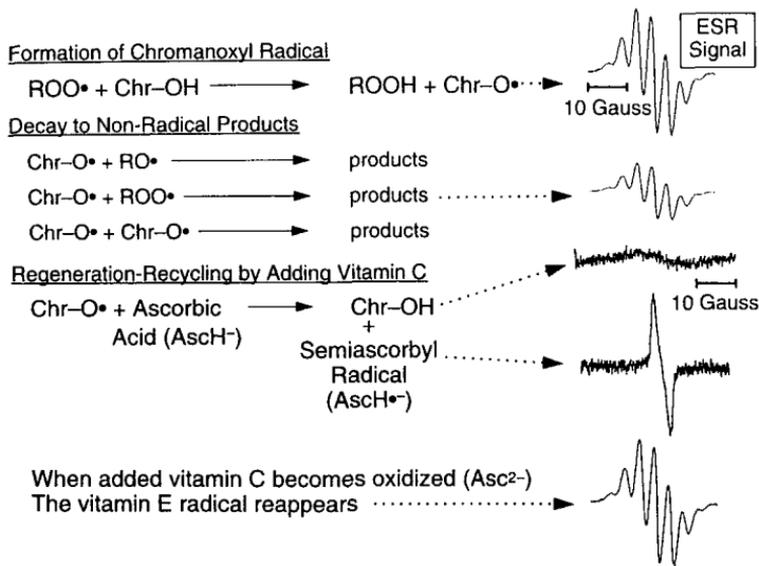


**Figure 1** Oxidative stress activates network antioxidants.

lipoproteins, there is enough vitamin E present in these lipoproteins to follow these reactions by detecting the electron spin resonance (ESR) signal of the vitamin E radical. Once vitamin E is made into a radical, it is more reactive, of course. It can react with itself or other radicals in a chain-breaking reaction. It is a slowly reacting radical because the free electron is delocalized around the chromanol ring of vitamin E. Thus, it exists for a sufficient time for ascorbic acid to react with most of the vitamin E radicals and convert them back to vitamin E, thus sparing vitamin E (15,16). When ascorbic acid does that, it becomes an ascorbyl radical (Fig. 2). The vitamin C radical can be regenerated by glutathione with the aid of enzymes or nonenzymatically by lipoic acid or certain flavonoids.

Lipoic acid is unique in this regard because it has a redox potential ( $-320$  mV) that is even lower (17) than the glutathione system ( $-280$  mV); thus, in its reduced form, it can nonenzymatically regenerate vitamin C, which in turn can regenerate vitamin E.

A dramatic example of this effect was recently observed by Podda et al. (18). When animals were placed on a vitamin E-deficient diet, they lost weight and eventually died. But when 1.65 g lipoic acid/kg of diet was fed to these animals, they did not develop the symptoms of vitamin E deficiency—weight loss and motor discoordination. Hence, lipoic acid was obviously able to take over some functions of vitamin E in these animals, presumably either



**Figure 2** Vitamin E radical reactions during lipid peroxidation.

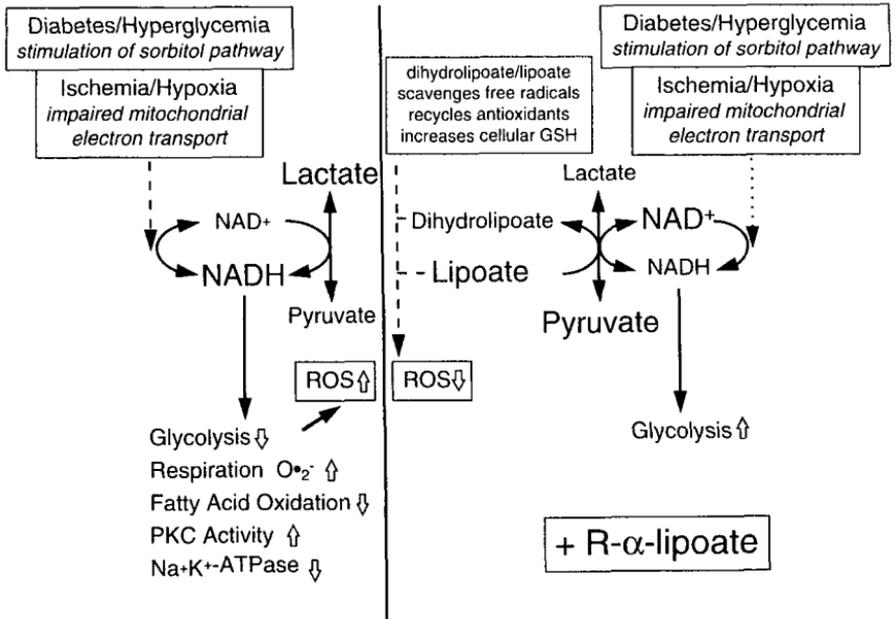
through regenerating vitamin E as described above or through directly substituting for vitamin E as an antioxidant, or some combination of both.

The vitamin E radical slowly decomposes as a result of reacting with itself or with other radicals. When ascorbic acid is added to a system in which vitamin E radical is being generated, the vitamin E radical disappears as it is reduced back to vitamin E by reaction with ascorbate and the semiascorbyl radical appears. But the vitamin E radical eventually returns as the the semiascorbyl radical disappears. From this experiment, the time that it takes for the vitamin E radical to reappear, or the lag period, can be determined. The time of the lag period is directly related to the vitamin C concentration. However, if dihydrolipoic acid is added to the reaction mixture, as well as vitamin C, and the same experiment is performed, one now observes that it takes a much longer time for the vitamin E radical to return. In this experiment, the lag period time is directly related to the concentration of the reduced lipoic acid. Reduced lipoic acid recycles the vitamin C radical (13). There are other ways in which lipoic acid can react with, and thus recycle, other antioxidants. After lipoic acid is reduced, it can regenerate oxidized thioredoxin, glutathione disulfide, or dehydroascorbate. Also, reduced lipoic acid has been reported to regenerate the semiquinone of ubiquinone (coenzyme Q10) in membranes (H.

Nohl Laboratory, Vienna). Thus, the entire antioxidant defense system can be affected by the presence of reduced lipoic acid.

### III. OXIDATIVE STRESS, THE ANTIOXIDANT NETWORK, AND DIABETES

Why are oxidative stress, antioxidants, and the antioxidant network important in diabetes? Hyperglycemia causes, as a result of stimulation of the sorbitol dehydrogenase pathway, accumulation of NADH and an increase in the lactate/pyruvate ratio. This is accompanied by decreased glycolysis, increased reactive oxygen species formation, increased protein kinase C activity, and decreased  $\text{Na}^+/\text{K}^+$  ATPase, among other effects, as shown in Figure 3. It is reasoned that the reductive imbalance that occurs in hyperglycemia, and which may also occur in ischemia/hypoxia injury (20–22), might be reversed by



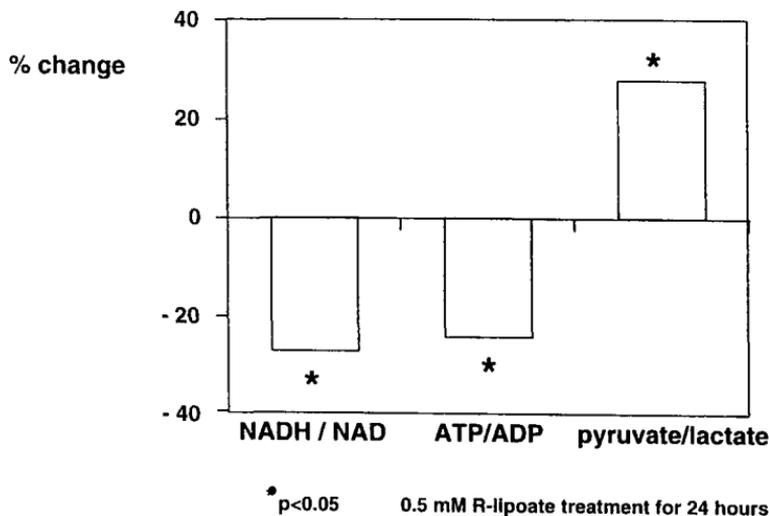
**Figure 3** Proposed mechanism of lipoate-mediated reversal of the reductive imbalance in hyperglycemia.

lipoic acid. Roy et al. (19) performed experiments to study if lipoic acid could affect the metabolic situation in hyperglycemia.

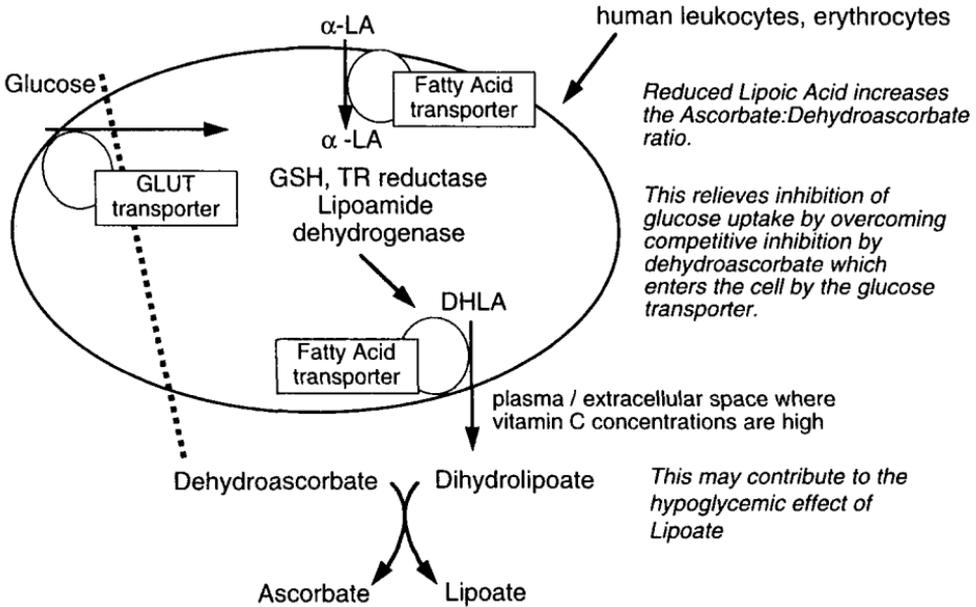
If R-lipoic acid is added to cells, it should, as a result of its reduction by mitochondrial dihydrolipoamide dehydrogenase activity, reverse the reductive imbalance in hyperglycemia and perhaps normalize the imbalance of overproduction of NADH and change the NADH/NAD<sup>+</sup> ratio toward normal. Using human T lymphocytes as a model system, we performed experiments to determine if this was the case (19). Indeed, treating these human T cells with R-lipoic acid (but not S-lipoic acid) normalized the redox status (Fig. 4).

Lipoate treatment caused the NADH/NAD<sup>+</sup> ratio to be reversed in hyperglycemia; the ATP/ADP ratio, which had fallen, was increased, and the imbalance of the pyruvate/lactate ratio was also reversed. Furthermore, the uptake of glucose by these cells was stimulated. With lipoic acid treatment, even at 100- $\mu$ M concentrations, significant increases in the uptake of glucose by these cells were observed (19).

Of course, one may wish to know how relevant these observations from a cellular system are to diabetes. We have proposed two models to link the ideas presented above and the possible therapeutic effects of lipoic acid in diabetes:

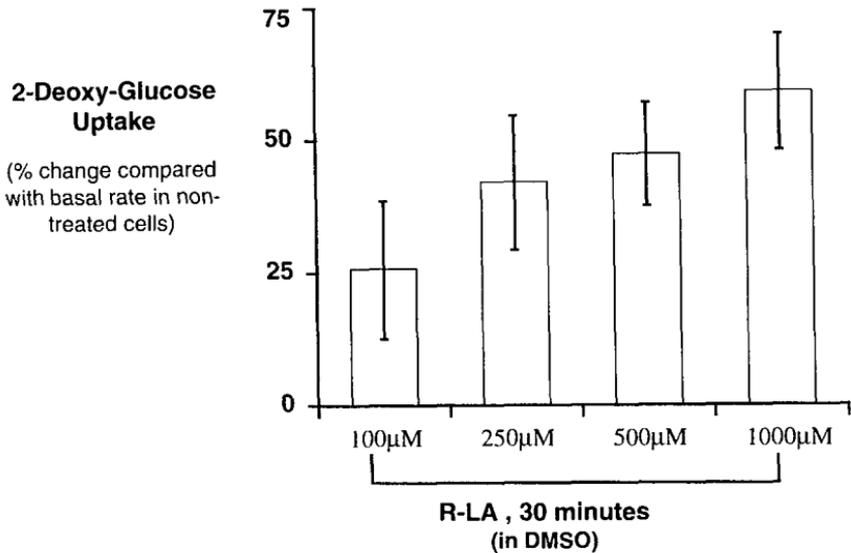


**Figure 4** Effect of R-lipoate treatment on redox status and ATP/ADP ratio in Wurzburg T cells.



**Figure 5** Proposed mechanism whereby  $\alpha$ -lipoic acid stimulates glucose uptake.

1. Lipoic acid, when it became reduced, would be exported from the cells as dihydrolipoic acid; this in turn could regenerate vitamin C radicals or dehydroascorbate, because vitamin C in the plasma becomes oxidized after reacting with radicals that are produced by neutrophils or other cells of the immune system. This maintains plasma ascorbate in its reduced form. This is important because dehydroascorbate competes with glucose for uptake by the "fast track" glucose (GLUT) transport system present in most cells. Hence, the inhibition of glucose uptake by dehydroascorbate would be overcome (23), as shown schematically in Figure 5.
2. There also may be a direct effect of lipoate on the uptake of glucose by the glucose (GLUT) transport system and thus an effect on the insulin-dependent stimulation of glucose uptake. It was of interest to investigate some of those parameters using the skeletal muscle-derived L6 myotube cell culture system. When L6 myoblasts differentiate into myotubes, they gain the ability to take up glucose. Amira Klip's laboratory has reported extensively on this system



**Figure 6** Dose-dependent effect of lipoate on glucose uptake by skeletal muscle L6 myotubes. (From Sen CK, Khanna S, Loukianoff S, Roy S, Packer L, unpublished data.)

(24,25). Using similar conditions, my colleagues Chandan Sen, Savita Khanna, Sonia Loukianoff, and Sashwati Roy have measured the cellular uptake of deoxy-*d*-glucose from a buffer system by following the uptake of the radiolabeled deoxy-glucose. A dose-dependent effect of lipoate on glucose uptake by L6 myotubes showed that under our conditions, even 100  $\mu\text{M}$  lipoic acid was sufficient to markedly stimulate glucose uptake. At higher concentrations, it continuously increased glucose uptake in a dose-dependent manner (Fig. 6).

In further experiments, pretreatment with 250  $\mu\text{M}$  lipoic acid for 30 min was used; after such treatment, a 30–40% stimulation of glucose uptake was usually observed. This is about the same extent of stimulation that has been observed under the same conditions with insulin treatment. If insulin and lipoic acid are added together, an additive, not a synergistic effect, is observed as has been reported previously (24,25).

It was of interest to determine whether the effect of lipoic acid on stimulating glucose uptake was due to the fatty acid molecular structure of lipoic

acid. Octanoic acid, an analogue of lipoic acid, had no effect on glucose uptake stimulated by 250  $\mu\text{M}$  lipoic acid, indicating that the fatty acid structure is not the cause of the stimulation.

Lipoic acid is a thiol antioxidant. Hence, it was of interest to know whether other thiol antioxidants or thiol reagents can mimic the effect of lipoic acid, one of which is the ability to increase glutathione levels or maintain levels under oxidative stress conditions (26,27). Therefore, we tested pyrrolidine dithiocarbamate (PDTC) (28), a thiol reagent, which upregulates glutathione levels in cells; diamide, which oxidizes thiol residues; and thioredoxin, which can reduce thiol residues. None of these reagents or treatments had any stimulatory effects on the lipoate-induced uptake of glucose by L6 myotubes. Next we wanted to determine if the intracellular glutathione level, which is the cell's primary preventive antioxidant, was important for glucose uptake. To find out whether modulations in the internal glutathione level was responsible for promoting glucose uptake, we treated the L6 myotubes with the inhibitor of a glutathione synthesis, butamine sulfoxamine (BSO) (29). This reagent inhibits cell glutathione synthesis. After treating cells for 24 h with BSO, glutathione levels in cells fall to very low levels. The effect of lipoic acid in stimulating glucose uptake in the presence of BSO was unchanged. So modulation of the internal glutathione level is not responsible for the stimulation of glucose uptake by lipoic acid. Confirming this, PDTC, the thiol reagent known to upregulate glutathione, also does not prevent lipoic acid from stimulating glucose uptake.

What was regulating the lipoate-dependent glucose uptake? Because calcium is an important factor in cell regulation (30), we investigated the effect of calcium-binding reagents. Two types of calcium chelators were used: EGTA, which is membrane impermeable, and the esterified form of EGTA, which is known to permeate to the inside of cells. Both reagents, when added to the L6 myotubes, inhibited the lipoic acid-stimulated glucose uptake. Further evidence was obtained from the effects of calcium channel blockers like verapamil and nifedipine; both of these reagents inhibited lipoic acid-stimulated glucose uptake. To prove that one of the effects of lipoic acid was stimulating calcium uptake, we directly followed the uptake of radiolabeled  $^{45}\text{C}$  by L6 myotubes. After 30 min, 250  $\mu\text{M}$  lipoic acid markedly stimulated ( $\sim 30\%$ )  $^{45}\text{Ca}^{2+}$  uptake.

It is known that a ryanodine-sensitive receptor is involved in calcium entry. Indeed, 25  $\mu\text{M}$  ryanodine inhibited the lipoic acid-stimulated uptake of glucose. Moreover, by adding 250  $\mu\text{M}$  4-chloro-*m*-cresol, it was possible to mimic the effect of lipoic acid. 4-Chloro-*m*-cresol is known to stimulate the

ryanodine receptor (31,32). Stimulating the ryanodine gives almost the same effect as the lipoic acid, suggesting that this receptor is involved.

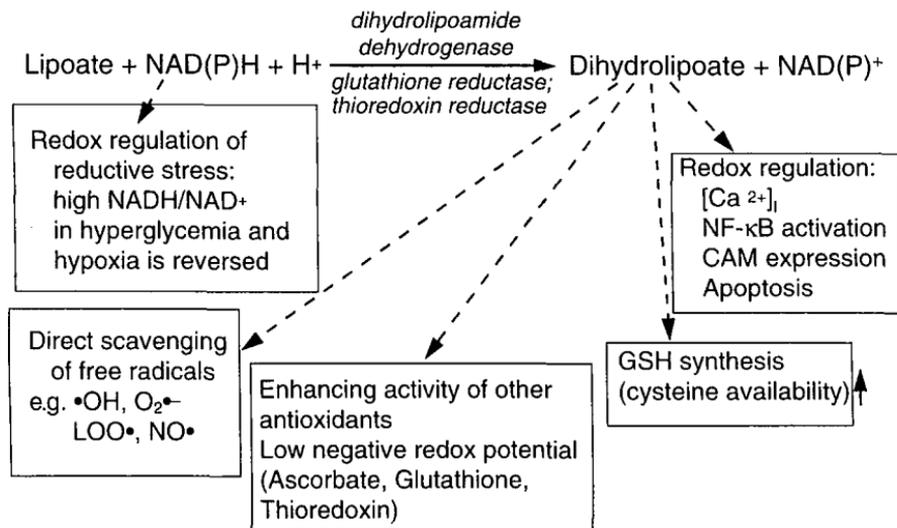
#### **IV. LIPOATE- AND INSULIN-DEPENDENT CELL SIGNALING PATHWAYS**

Insulin activates numerous metabolic and mitogenic effects by first binding to its specific transmembrane glycoprotein receptor, which has intrinsic tyrosine kinase activity. Tyrosine phosphorylation of various other substrates, particularly the insulin receptor substrate (IRS) proteins, then induces formation of a network of docking proteins that mediate insulin action of gene expression involved in its anabolic and catabolic effects (33).

From the results of the present study, it would appear that the action of lipoate in stimulating glucose uptake may also be through protein kinase activation, likely mediated by transient increases in cytosolic calcium, which is essential for the Wortmanin-sensitive, PI-3 kinase-dependent, lipoate-stimulated glucose uptake in the L6 myotube system (24,25). After upregulation of glucose transport after mobilizing GLUT transporters from the cytosolic to the plasma membrane domain, lipoate, like insulin, may recruit a broad array of kinases in target cells to activate its numerous metabolic actions.

#### **V. LIPOIC ACID, DIABETIC POLYNEUROPATHY, AND DIABETES**

Lipoic acid has been used successfully as a therapeutic agent in the treatment of diabetic polyneuropathy both in animal models and in human clinical trials (34–36). Diabetes is considered as an oxidative stress disease; evidence indicates that both insulin-dependent and noninsulin-dependent diabetes exhibit molecular markers indicative of oxidative stress. Thus, it could be anticipated that one of the most potent metabolic antioxidants known in biological systems, free  $\alpha$ -lipoic acid, should be effective in treating diabetic complications. In particular, R-lipoic acid is recognized by the mitochondrial lipoamide dehydrogenase that reduces it to dihydrolipoate, a powerful reductant that is capable of direct scavenging of radicals, regenerating vitamins E and C, increasing the potency of the entire redox antioxidant network, upregulating cellular levels of glutathione, affecting important cell regulatory activities such as nuclear factor- $\kappa$ B transcriptional activation, regulating free cytosolic calcium, and re-



**Figure 7** Redox regulation of cell functions by  $\alpha$ -lipoate: biochemical and molecular aspects. (From Ref. 37.)

versing the reductive imbalance in diabetes resulting from hyperglycemic conditions. These various effects of lipoic acid have been described previously (37) and are shown schematically in Figure 7. These properties may have therapeutic effects in oxidative stress diseases and aging.

Importantly, lipoic acid has also been demonstrated, at higher concentrations, to have hypoglycemic effects. It exhibits effects on glucose disposal, an important function of skeletal muscle, as demonstrated in animal models by Henriksen et al. (38) and in human clinical studies. It is therefore to be expected that lipoic acid somehow has a profound effect on the mechanism of glucose uptake and disposal and in regulating the glucose-dependent metabolic changes that ensue. The evidence, summarized in this chapter, provides new and interesting findings relevant to these questions.

The many molecular effects of lipoate and dihydrolipoate on receptor-mediated activity, cell signaling, transcriptional activation, and gene expression remain to be elucidated. Important among these considerations for diabetes is how it modulates insulin-dependent cell signaling system pathways.

Because the effects of insulin and lipoate in the L6 myotube experiments are additive, it is reasonable to suggest that the pathways of insulin-stimulated glucose uptake and utilization and that of lipoate-stimulated glucose uptake differ from one another. Lipoate may affect protein kinases and phosphatases,

which will modulate phosphorylation systems, and at some point may have common actions with the insulin-signaling pathways. These pathways remain to be elucidated, particularly the mechanism whereby lipoate stimulates calcium-dependent signaling pathways related to glucose transport.

## VI. SUMMARY

Oxidative stress, antioxidants, and the antioxidant network can be relevant to diabetes because diabetes appears to involve oxidative stress. One antioxidant that may have particular relevance to diabetes is lipoic acid. Reduced lipoic acid powers the antioxidant network after being taken up by cells. Lipoic acid reverses the reductive imbalance that occurs in hyperglycemia. Plausible mechanisms for this effect are as follows. First, cell reduction of lipoic acid is released into the extracellular space and maintains reduced plasma ascorbate. It can thus relieve the competitive inhibition of glucose uptake by dehydroascorbate. Second, lipoic acid stimulates glucose uptake in skeletal muscle (the main tissue responsible for glucose disposal). In L6 myotubes, used as a model system for glucose disposal, this stimulation apparently occurred by a calcium-dependent mechanism.

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# 2

## **Oxidative Stress in Diabetes: Why Does Hyperglycemia Induce the Formation of Reactive Oxygen Species?**

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There is much evidence that the formation of various markers of oxidative stress are increased in diabetes: In the plasma of diabetic patients the concentrations of lipid hydroperoxides, isoprostanes, malonic dialdehyde, and oxidized lipoproteins are elevated (1–6). The intracellular levels of antioxidants such as tocopherol and glutathione are reduced, whereas the enzymatic activity of antioxidative acting enzymes is at least partly increased (7–12). Similarly, there are many reports about the consequences of an imbalance between pro- and antioxidant actions in the cells (“oxidative stress”) and the importance of disturbances in the intracellular antioxidant network for the development of vascular complications in hypertensive or hypercholesterolemic patients. Such a pathophysiological link between oxidative stress and vascular complications is in line with many experimental observations, with large epidemiological studies, and to a lesser extent with recent clinical investigations (13–22). There is increasing evidence that the generation of reactive oxygen intermediates is also of major importance for the development of vascular complications in diabetes (23–29). However, neither the mechanisms that specifically lead to the generation of reactive oxygen intermediates (ROI) in hyperglyce-

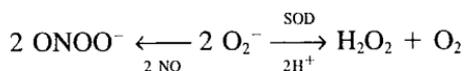
mic states nor the cascade of reactions linking the formation of ROI with the pathophysiological event are well understood.

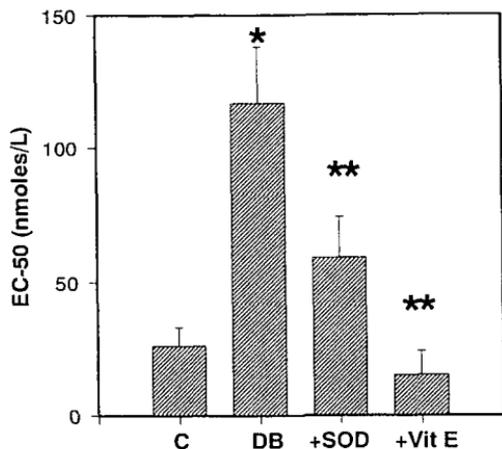
Here we present evidence that the vasculature is an important source for the formation of reactive oxygen species; that high glucose activates an endothelial NADPH-oxidase and thereby causes the release of superoxide anions; that the superoxide anions are able to react with nitric oxide, leading to the formation of peroxynitrite; and that the formation of peroxynitrite is responsible for the impaired endothelium-dependent vasodilatation and variety of cytotoxic effects on the vasculature observed in hyperglycemia, such as activation of the nuclear transcription factor kappa-B (NF- $\kappa$ B) and induction of a apoptosis.

In addition, peroxynitrite has been shown to accelerate the oxidation of low-density lipoproteins and to activate metalloproteinases. Thus, it is intriguing to suggest that the generation of ROI induced by hyperglycemia is one of the major causes for the transformation of endothelium into a proinflammatory and thrombogenic state as observed in diabetes. We assume that this endothelial activation or dysfunction is the basis for the enhancement of atherosclerosis and the development of other vascular complications in diabetes and may contribute to a destabilization of established plaques that has been shown to be one of the most decisive events for induction of myocardial infarction, angina pectoris, and cardiac death (30,31).

## I. IS THE VASCULATURE A SOURCE FOR ROIs?

We have already shown (29) that the endothelium-dependent increase in coronary flow is disturbed in isolated perfused hearts of diabetic rats. The dose-response curve for the increased coronary flow in response to 5-hydroxytryptamin is shifted to higher concentrations in diabetes, whereas the maximum coronary flow is not altered under these conditions. This defect could be prevented *in vivo* by treatment of the animals with high concentrations of vitamin E (1000 U/kg/day) and, more interestingly, under the aspect of mechanisms, by the addition of superoxide dismutase to the perfusion medium (Fig. 1). This observation suggests that the vasculature of diabetic rats releases superoxide anions spontaneously and continuously into the perfusion medium and that the generated superoxide anions are the cause for the disturbed endothelium-dependent flow regulation. We assume that nitric oxide (NO) as the main mediator of endothelium-dependent vasodilatation becomes inactivated by the simultaneously released superoxide anions.





**Figure 1** Impairment of the endothelium-dependent increase in coronary flow and its prevention by superoxide dismutase (SOD) and vitamin E. Diabetes was induced in rats by streptozotocin. After a diabetes duration of 16 weeks, the stimulation of coronary flow by 5-hydroxytryptamin was measured in the isolated heart preparation as described (29). The half-maximal concentration ( $EC_{-50}$ ) was determined and represents a measure for the sensitivity of endothelium to dilate the coronary vasculature. As can be seen, in diabetes (DB), the sensitivity of endothelium is impaired as compared with healthy controls (C), but perfusion with SOD (50  $\mu$ U/mL) or pretreatment of the animals with  $\alpha$ -tocopherol (1000 U/kg body weight) were able to improve or to restore the endothelium-dependent vasodilatation in diabetes. (From Ref. 23.)

Such an interaction between superoxide anions with NO has already been described. In a diffusion controlled reaction, both compounds react with each other under the formation of peroxynitrite (32–34).

Direct evidence for this conclusion is derived from experiments using isolated aortas from streptozotocin diabetic rats. This model enables us to directly measure the formation of superoxide anions by standard techniques as the reduction of cytochrome *c* (35). When aortas from diabetic and control animals were perfused under normoglycemic conditions, vessels from diabetic rats released significantly more superoxide anions than those from controls. In addition, the generation of superoxide anions was stimulated in both types of aortas by hyperglycemic buffers (10–30 mM glucose). The increased generation of superoxide anions could be totally reduced to control values when the endothelium was removed from the intact aortas by mechanical disruption (Rösen 1998, unpublished data). It is interesting to note that an endothelial production of ROI has also been reported for vessels isolated from hypercho-

lesterolemic and hypertonic animals (13–15). Thus, the stimulus for activation of endothelium is different in these various pathophysiological conditions, but the consequences seem to be comparable.

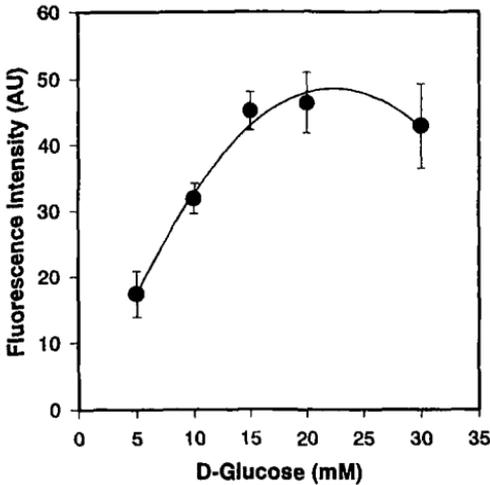
These experimental observations lead to the conclusion that endothelium is an important source of ROI and identify hyperglycemia as a stimulus for the formation of superoxide anions. Furthermore, the disturbed endothelium-dependent vasomotion in diabetes is an immediate pathophysiological consequence of the release of superoxide anions by the vasculature.

## II. WHICH MECHANISMS CONTRIBUTE TO THE ENDOTHELIAL FORMATION OF SUPEROXIDE ANIONS IN DIABETES?

To study the mechanisms of ROI generation induced by hyperglycemia in more detail, we used human umbilical vein endothelial cells (HUVECs). To identify the generation of ROI, HUVECs were loaded with dichlorodihydrofluorescein ester (DCF) (36), which is taken up by the cells and then rapidly hydrolyzed. DCF reacts with superoxide anions but presumably also other ROI under the emission of fluorescence light so that the formation of ROI can be determined in a time- and concentration-dependent manner.

Incubation of DCF-loaded cells with increasing concentrations of glucose (5–30 mM) leads to a time- and glucose-dependent increase in fluorescence (Fig. 2). A comparable increase in fluorescence was also observed if the cells were incubated with 3-*O*-methyl-D-glucose (30 mM), a glucose derivative, which is taken by the cells but not metabolized by glycolysis. These data indicate that the formation of ROI is dependent on high glucose in the culture medium but not on the synthesis of diacyl-glycerol and a glucose-dependent activation of protein kinase C. In line with this conclusion, we did not observe an alteration in DCF fluorescence by treating the cells with an inhibitor bisindolylmaleimide (BIM) or activator phorbol 12-myristate 13-acetate (PhA) of protein kinase C (23).

The formation of ROI by endothelial cells incubated with high glucose was completely inhibited by antioxidants ( $\alpha$ -tocopherol 10  $\mu$ g/mL and thiocetic acid 0.5  $\mu$ M) and by diphenyliodonium (DPI, 1  $\mu$ M), a selective inhibitor of flavoprotein containing NAD(P)H oxidases (37). The inhibitory effect of DPI is consistent with the assumption that NAD(P)H oxidases are the major source of ROI in HUVECs cultivated in hyperglycemic glucose. DPI was also reported to inhibit the NADH-dependent production of superoxide anions in bovine coronary endothelial (38).

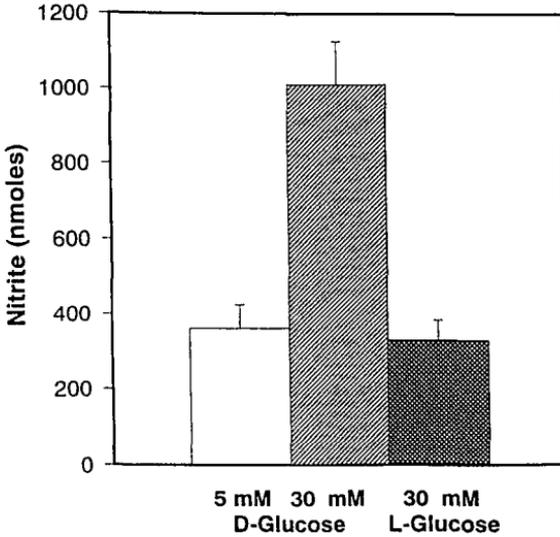


**Figure 2** Increase in the formation of ROIs by human endothelial cells in dependence of glucose. HUVECs were preloaded with the DCF (1  $\mu$ M) and dichlorodihydrofluorescein (10  $\mu$ M) for 45 min. After washing, the cells were incubated with D-glucose (5–30 mM). For control, cells were incubated with mannitol and L-glucose (25 + 5 mM). After a 15-min incubation (37°C), the fluorescence intensity as a parameter of the ROI generation was analyzed by fluorescent microscopy and quantified.

Although cyclooxygenases and lipoxygenases may also be sources of superoxide anion generation in endothelium (35,38), our data do not link these enzymes to the production of superoxide anions induced by hyperglycemia, because indomethacin and nordihydroguaretic acid did not inhibit the release of superoxide anions. Similar observations have already been reported for porcine endothelial cells (35).

Surprisingly, the DCF fluorescence was also prevented by inhibitors of NO synthase (L-nitroarginine, 100  $\mu$ M) and a chelator of intracellular calcium 1,2-bis (2-Aminoprenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). These observations indicate that the mobilization of intracellular calcium and an activation of NO synthesis are necessary steps for the formation of DCF fluorescence by hypoglycemia. In line with this conclusion, the release of nitrite (as parameter of NO synthesis) by HUVECs was stimulated by glucose (Fig. 3).

Thus, under hyperglycemic conditions, both NAD(P)H oxidase and NO synthase become activated, and both steps are a precondition for the formation of DCF fluorescence by HUVECs in hyperglycemia. This synergistic actions of NAD(P)H oxidase and NO synthase suggest that DCF fluorescence does not

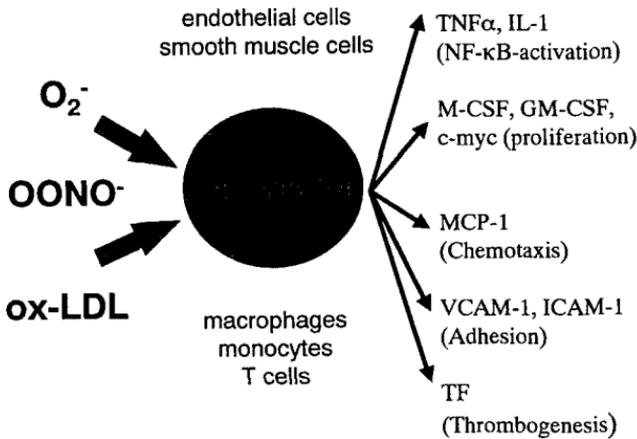


**Figure 3** Glucose stimulates the formation of NO by human endothelial cells. HUVECs were incubated with glucose (5–30 mM) for 24 h. The formation of nitrite in the supernatant was analyzed by the Gries reaction. For control, cells were incubated with L-glucose (25 + 5 mM).

specifically reflect the generation of superoxide anions but rather the reaction product of both NO and superoxide anions, presumably peroxynitrite.

Because peroxynitrite has been reported to react with tyrosine residues in proteins, leading to the formation of *o*-nitrotyrosylated proteins, *o*-nitrotyrosylation has been suggested as a long-term parameter for an enhanced formation of peroxynitrite and oxidative stress (32–34). Demonstration of nitrotyrosylated proteins in the vasculature would represent direct evidence of a preceding formation of peroxynitrite and oxidative stress. Endothelial cells were therefore incubated with high glucose and the proteins were extracted, separated by gel electrophoresis, and stained by an antibody specifically recognizing *o*-nitrotyrosylated proteins. As expected, hyperglycemia results in a dose-dependent formation of *o*-nitrotyrosylated proteins (data not shown).

There are at least two other pathways that might contribute to the generation of ROI. Giardino et al. (39) showed that the intracellular formation of advanced glycation endproducts (AGE) products is closely associated with the generation of ROI determined by DCF fluorescence and lipid peroxidation. Inhibition of lipid peroxidation also prevented the formation of AGE products,



**Figure 4** NF- $\kappa$ B-mediated pathways leading to a thrombogenic transformation of the vessel wall.

suggesting that the ROI generation is necessary for the synthesis of AGE products. On the other hand, there is some evidence that endothelium starts to produce ROI as soon as the receptor for AGE products becomes occupied (40,41). Although the exact intracellular signaling is not yet known, there is some evidence that binding of AGE products to its receptors causes an activation of NADPH oxidases (41). This AGE-mediated production of ROI is prevented by antioxidants and inhibitors of NADPH oxidases but not inhibitors of NO synthases, cyclo- and lipoxygenases, or xanthin oxidase.

Thus, the available evidence suggests that activation of NADPH oxidase by glucose or AGE is a key step for the generation of ROI by endothelium. Whether the generated ROI are transformed to peroxynitrite depends on the type of cell and the concomitant reactions. If, as in HUVECs, NO-synthase becomes activated simultaneously, peroxynitrite may be formed and represent the key mediator for the subsequent transformation of endothelium. In the absence of NO synthase activation or insufficient amounts of NO, superoxide anions may directly act as signal mediators and exert the deleterious cytotoxic effects of hyperglycemia and AGE on endothelium and other vascular cells.

There are two open questions. What are the mechanisms for the hyperglycemia-mediated increase in intracellular calcium? The formation of vascular endothelial growth factor (VEGF) as a consequence of the generation of superoxide anions is one interesting mechanism, especially because it has been shown that AGE are able to induce the expression of VEGF (42). Do changes

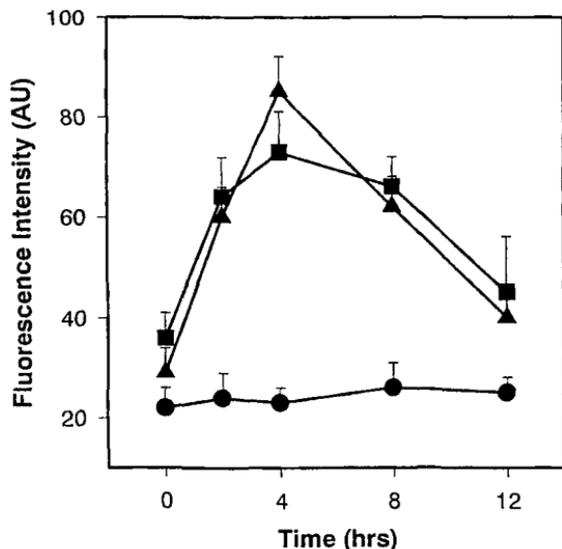
in the cytosolic NADH/NAD ratio contribute to the formation of ROI in addition to the activation of NADPH oxidases? It has been suggested that the NADH/NADPH ratio is elevated in diabetes because more glucose is metabolized by the sorbitol pathway and that the increased NADH/NAD ratio causes the formation of superoxide anions by various mechanisms (43). We do not believe that this mechanism is working in endothelium, because the formation of ROI was not inhibited by inhibitors of the sorbitol dehydrogenase, either in HUVEC or in porcine endothelial cells (35).

In summary, there is good evidence that the vasculature and more specifically the endothelium is one important source for the generation of ROIs. The formation of ROIs is specifically related to the diabetic state because it is stimulated by glucose and advanced glycation end products in a dose-dependent manner. It is interesting to note that similar observations have been reported for the vasculature in hypertension and hypercholesterolemia, suggesting that the initial processes and stimuli might be different, but that the three pathophysiological conditions finally result in an enhanced oxidative stress.

### III. WHAT ARE THE CONSEQUENCES OF OXIDANT STRESS IN DIABETES?

There is a lot of evidence that ROI and especially peroxyxynitrite are involved in activation of transcription factors such as NF- $\kappa$ B. NF- $\kappa$ B is responsible for a variety of reactions contributing to the thrombogenic transformation of endothelium (Fig. 4), (44–48): release of tumor necrosis factor  $\alpha$  and interleukin 1 $\beta$  (proinflammatory), release of growth factors (M-CSF [monocyte colony stimulating factor], GM-CSF [granulocyte-monocyte colony stimulating factor], *c-myc*), activation of the monocyte chemoattractant protein MCP-1 (chemotaxis); expression of adhesion proteins (VCAM-1 [vascular cell adhesion molecule-1], ICAM-1 [intercellular adhesion molecule-1]); and expression of tissue factor (thrombogenesis).

We used two different approaches to test whether hyperglycemia causes an activation of NF- $\kappa$ B: the electromobility shift assay (EMSA), measuring the DNA-binding activity of nuclear proteins to an NF- $\kappa$ B-specific oligonucleotide (48), and a histochemical approach using a fluorescence-labeled antibody coupled to the NF- $\kappa$ B-specific oligonucleotide (49). Using both methods we can show that hyperglycemia causes a dose- and time-dependent activation of NF- $\kappa$ B. The maximum of activation by high glucose is achieved after 4 h; after 10–12 h NF- $\kappa$ B is again completely inactivated (Fig. 5). This activation is inhibited by antioxidants (tocopherol 10  $\mu$ g/mL, thiocetic acid 0.5  $\mu$ M) and



**Figure 5** Time dependence of NF- $\kappa$ B activation by hyperglycemia in human endothelial cells. HUVECs were incubated with low glucose (5 mM), high glucose (30 mM), and 3-*O*-methyl-D-glucose (3-OMG, 25 + 5 mM glucose). After a 2-, 4-, 6-, and 12-h incubation (37 °C), the cells were fixed with paraformaldehyde and stained by the specific fluorescein isothiocyanate (FITC)-labeled consensus sequence for NF- $\kappa$ B as described. Osmotic controls (25 mM mannitol + 5 mM glucose) did not show staining above the background. ●, Controls; ■, high glucose; ▲, 3-OMG.

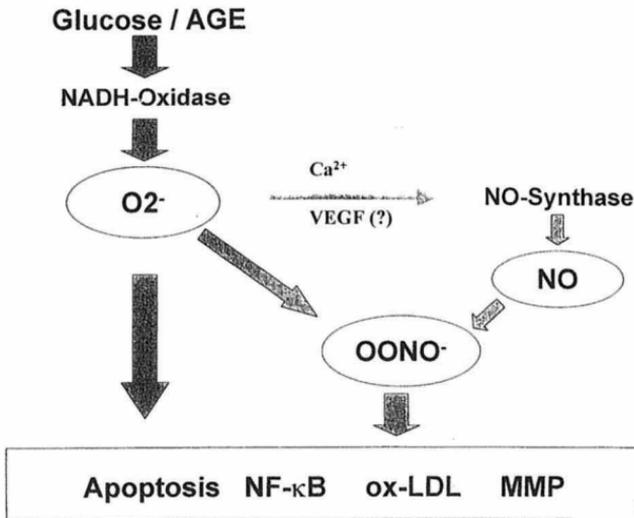
by the NO synthase inhibitor L-nitroarginine (100  $\mu$ M), whereas the modulation of protein kinase C was without any influence. These data suggest that the short-term activation of NF- $\kappa$ B by hyperglycemia is caused by peroxynitrite. It is an open question whether the recently reported long-term activation of NF- $\kappa$ B by AGE products (48) was caused by a similar mechanism. In any case, hyperglycemia seems to cause an activation of NF- $\kappa$ B by different signaling cascades: High glucose leads to a short-term activation, which might be important for the transformation of immediate and short-term variations in blood glucose into vascular reactions. Such a mechanism would also explain why not only the long-term elevation of blood glucose is cytotoxic for the vessel wall but also the spikes in blood glucose that are often observed even in patients with an overall near normoglycemic metabolic control. Activation of NF- $\kappa$ B by AGE products, on the other hand, would induce a long-term modulation of vascular functions and might be especially of importance for angiogenesis.

In addition to activation of NF- $\kappa$ B, the formation of peroxynitrite may have several other consequences that may contribute to the development of vascular complications in diabetes. First, the induction of apoptosis. We have recently reported (50) that high glucose induces the programmed cell death in HUVECs. This process was inhibited by antioxidants (thioctic acid and tocopherol), but also by inhibitors of NO synthases. The underlying mechanism is not yet fully understood at this time, but there are several lines of evidence that the induction of apoptosis is independent of the activation of NF- $\kappa$ B. The induction of apoptosis can be understood as an indicator of damage of endothelium by high glucose, as an attempt of the vasculature to get rid of damaged endothelial cells. Such a loss of endothelium would be associated with induction of angiogenesis, a process typically observed in the eye and the kidney of many diabetic patients (51,52). On the other hand, loss of endothelium would lead to an exposition of thrombogenic structures (subendothelial matrix) to the bloodstream and thereby cause an increased thrombotic risk.

Another consequence is the oxidation of low-density lipoproteins. It has been reported that peroxynitrite is a strong prooxidant and accelerates the oxidation of low-density lipoproteins (53). Because oxidized low-density lipoproteins are themselves cytotoxic for endothelium, the formation of peroxynitrite would reinforce the oxidant stress by constituting a deleterious vicious cycle. Finally, the activation of metalloproteinases has been reported to become activated by peroxynitrite *in vivo* and *in vitro* (54). Such an activation of metalloproteinases at the edge of an atherosclerotic plaque is assumed to cause a destabilization of the plaque, enhance plaque rupture, and finally a thrombotic event that is the most common cause for myocardial infarction, angina pectoris, and cardiac death (30,31).

#### IV. CONCLUSIONS

Taken together, there is good evidence that short- and long-term hyperglycemia cause an activation of NADPH oxidase and the formation of ROI. The endothelium has been demonstrated as one of the major sources of ROI generation. Experimental data from *in vitro* and *in vivo* studies clearly show that these ROIs are able to induce a thrombogenic transformation of the vessel wall and to be the cause for the endothelial dysfunction observed in diabetes but also in hypertension and hypercholesterolemia. Whether these cytotoxic effects are exerted by superoxide anions directly or are mediated by peroxynitrite depends on the local environment and the type of vasculature regarded. Our current knowledge is summarized in the hypothesis shown



**Figure 6** Current hypothesis: formation of ROI by hyperglycemia and the effects of ROI on the vessel wall as cause for the development of vascular complications in diabetes.

in Fig. 6. It is important to recognize that the consequences of these ROI-induced vascular dysfunctions might be different depending on the size of the vasculature affected: In small vessels the oxidative stress induced by hyperglycemia might be one important factor for the stimulation of proliferation of endothelium and the formation of new partially malfunctioning vessels and thereby contribute to the development of small vessel disease (retinopathy, nephropathy). In coronary vasculature, the generation of ROI might be more important for the destabilization of atherosclerotic plaques, causing an increased cardiac risk in diabetic patients. The reestablishment of the antioxidative network might therefore be a useful approach to protect the vasculature in diabetes.

## ACKNOWLEDGMENTS

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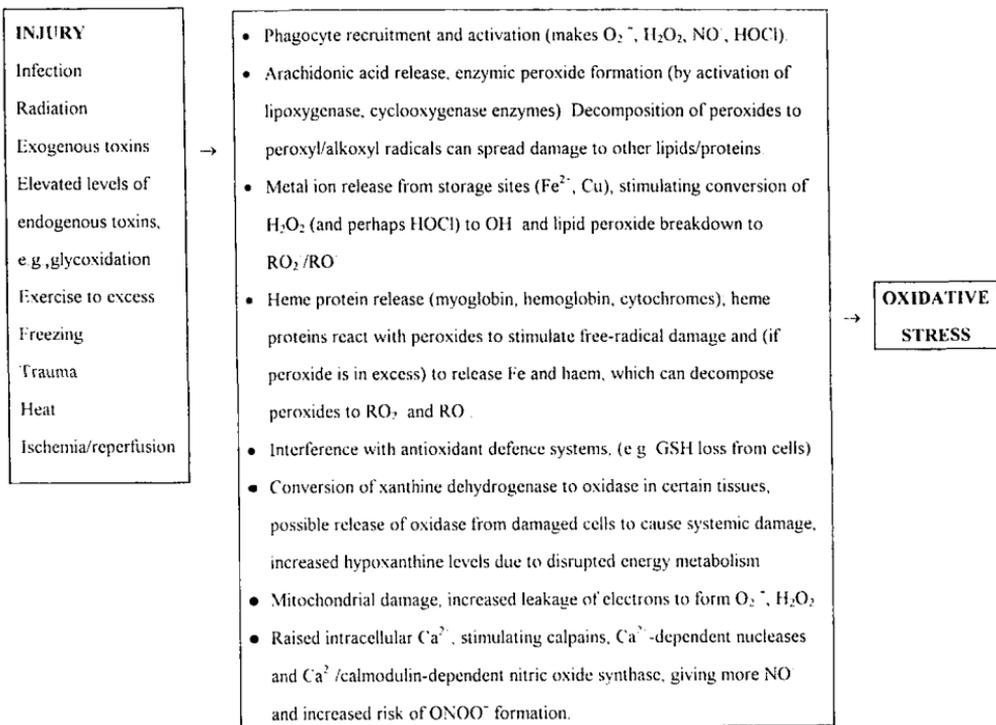
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## Oxidative Stress Markers in Human Disease: Application to Diabetes and to Evaluation of the Effects of Antioxidants

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The biomedical literature contains multiple claims that “free radicals” and other “reactive species” are involved in different human diseases. They have been implicated in over 100 disorders, ranging from rheumatoid arthritis, hemorrhagic shock, and ulcerative colitis to gastrointestinal damage by *Helicobacter pylori* and acquired immunodeficiency syndrome (reviewed in Refs. 1–4). Indeed, their importance in diabetes is widely proposed (5,6). This wide range of disorders implies that free radicals are not something esoteric but that their increased formation accompanies tissue injury in most or all human diseases (1–8), for the reasons summarized in Figure 1. Sometimes they make a significant contribution to the disease pathology; at other times they may not (7,8). Reasons for such differences are summarized in Figure 2. Establishing the real importance of reactive oxygen, nitrogen, and chlorine species (ROS/RNS/RCS) requires specific assays for their formation and the damage that they do in vivo. The lack of such assays in the past has impeded progress in our understanding of the role played by reactive species in normal physiology and in human disease. Thus, as summarized in Table 1, demonstrating that reactive species are important in diabetes (or indeed any other disease) involves much more than a mere demonstration of their formation. Table 2 lists some of the reactive species to be considered.



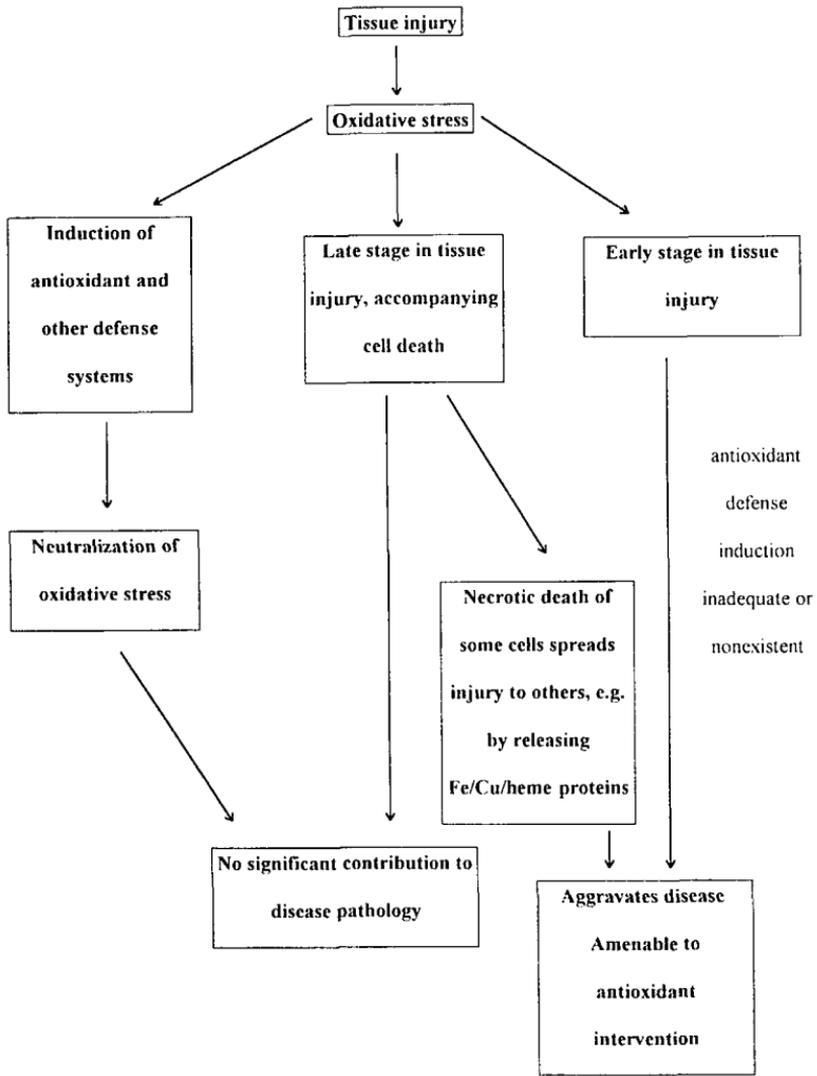
**Figure 1** Some reasons why tissue injury causes oxidative stress.

## I. OXIDATIVE STRESS IN DISEASE: DOES IT MATTER?

The term *oxidative stress* is widely used in the literature but rarely defined. In essence, it refers to the situation of a *serious imbalance* between production of ROS/RNS/RCS and antioxidant defense. Sies, who introduced the term from the title of the book he edited in 1985, introduced a somewhat vague definition in 1991 in the introduction to the second edition (9) as a disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage.

In principle, oxidative stress can result from two mechanisms:

1. Diminished antioxidants, for example, mutations affecting antioxidant defense enzymes (such as CuZnSOD, MnSOD, and glutathione peroxidase) or toxins that deplete such defenses. For example, many xenobiotics are metabolized by conjugation with GSH; high doses can deplete GSH and cause



**Figure 2** Why reactive species may be important or not important in human disease.

**Table 1** Criteria for Implicating Reactive Oxygen/Nitrogen/Chlorine Species or Any Other Agent as a Significant Mechanism of Tissue Injury in Human Disease

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1. The agent should always be present at the site of injury.
  2. Its time course of formation should be consistent with the time course of tissue injury.
  3. Direct application of the agent to the tissue at concentrations within the range found in vivo should reproduce most or all of the damage observed.
  4. Removing the agent or inhibiting its formation should diminish the injury to an extent related to the degree of removal of the agent or inhibition of its formation.
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oxidative stress even if the xenobiotic is not itself a generator of ROS or RNS. Depletions of dietary antioxidants and other essential dietary constituents can also lead to oxidative stress.

2. Increased production of ROS/RNS/RCS, for example, by exposure to elevated  $O_2$ , the presence of toxins that are themselves reactive species (e.g.,  $NO_2^+$ ) or are metabolized to generate ROS/RNS/RCS, or excessive activation of "natural" ROS/RNS/RCS-producing systems (e.g., inappropriate activation of phagocytic cells in chronic inflammatory diseases, such as rheumatoid arthritis and ulcerative colitis).

Mechanism 2 is usually thought to be more relevant to human diseases and is frequently the target of attempted therapeutic intervention but rarely is much attention paid to the antioxidant nutritional status of sick patients (e.g., Ref. 10). For example, calculations show that diabetic patients on fat-restricted diets may sometimes have a suboptimal intake of vitamin E (11). Prolonged oxidative stress can lead to depletion of essential antioxidants. For example, subnormal plasma ascorbate levels are well known in diabetics (12,13). There are conflicting views on whether diabetic patients show depleted plasma vitamin E levels, but data of Nourooz-Zadeh et al. (14) show a clear decrease in lipid standardized  $\alpha$ -tocopherol levels in diabetic patients (Table 3), although there is considerable variability between patients, as indicated by the ranges in Table 3. It is possible that variations in dietary intake can account for some of the different results reported in the literature.

In principle, the onset of oxidative stress can result in adaptation, tissue injury, or cell death. *Adaptation*, most often by upregulation of defense systems, may completely protect against damage; protect against damage, but not completely; or "overprotect," for example, the cell is then resistant to higher levels of oxidative stress imposed subsequently. As an example of not completely protecting against damage, if adult rats are gradually acclimatized to

**Table 2** Reactive Species

Radicals	Nonradicals
<b>ROS</b>	
Superoxide, $O_2^{\cdot-}$	Hydrogen peroxide, $H_2O_2$
Hydroxyl, $OH^{\cdot}$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^{\cdot}$	Hypobromous acid, HOBr
Alkoxy, $RO^{\cdot}$	Ozone, $O_3$
Hydroperoxyl, $HO_2^{\cdot}$	Singlet oxygen, $^1\Delta g$
<b>RNS</b>	
Nitric oxide (nitrogen monoxide), $NO^{\cdot}$	Nitrous acid, $HNO_2$
Nitrogen dioxide, $NO_2^{\cdot}$	Nitrosyl cation, $NO^+$
	Nitroxyl anion, $NO^-$
	Dinitrogen tetroxide, $N_2O_4$
	Dinitrogen trioxide, $N_2O_3$
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, $ONOOH$
	Nitronium (nitryl) cation, $NO_2^+$
	(e.g., as nitryl chloride, $NO_2Cl$ )
	Alkyl peroxynitrites, $ROONO$
<b>RCS</b>	
Atomic chlorine, $Cl^{\cdot}$	Hypochlorous acid, HOCl
	Chlorine $Cl_2$
	Nitronium (nitryl) chloride, $NO_2Cl$

ROS is a collective term that includes both oxygen radicals and certain nonradicals that are oxidizing agents and/or are easily converted into radicals (HOCl,  $O_3$ ,  $ONOO^-$ ,  $^1O_2$ ,  $H_2O_2$ ). RNS is also a collective term including nitric oxide and nitrogen dioxide radicals and such nonradicals as  $HNO_2$  and  $N_2O_4$ .  $ONOO^-$  is often classified as both an RNS and an ROS. "Reactive" is not always an appropriate term:  $H_2O_2$ ,  $NO^{\cdot}$ , and  $O_2^{\cdot-}$  react quickly with few molecules, whereas  $OH^{\cdot}$  reacts quickly with almost everything.  $RO_2^{\cdot}$ ,  $RO^{\cdot}$ , HOCl,  $NO_2^{\cdot}$ ,  $ONOO^-$ , and  $O_3$  have intermediate reactivities. HOCl and  $NO_2Cl$  can also be classified as "reactive chlorine species," HOBr as a "reactive bromine species."

elevated  $O_2$ , they can tolerate pure  $O_2$  for much longer than control rats, apparently due to increased synthesis of antioxidant defense enzymes and of GSH in the lung. However, the damage is merely slowed, not prevented (15). As an example of overprotection, treatment of *Escherichia coli* with low levels of  $H_2O_2$  increases transcription of genes regulated by the oxyR protein and renders the bacteria resistant to higher  $H_2O_2$  levels (16). However, few examples of this type of "overadaptation" have been reported in animals.

Oxidative stress can cause *tissue injury* to all molecular targets: DNA, proteins, and lipids (lipid peroxidation). Often, it is not clear which is the first

**Table 3** Parameters of Oxidative Stress in Healthy and Diabetic (NIDDM) Subjects

Variables	Healthy	NIDDM	<i>p</i>
Total cholesterol (mmol/L)	5.0 ± 1.1	6.0 ± 1.3	<0.002
Fasting glucose (mmol/L)	4.9 ± 0.4	12.1 ± 5.1	<0.0005
HbA <sub>1c</sub> (%)	—	11.0 ± 2.4	—
ROOH* (μmol/L)	4.1 ± 2.2	9.4 ± 3.3	<0.0005
α-Tocopherol (μmol/L)	23.8 ± 8.3 (10.6–47.0)	19.6 ± 7.5 (8.6–44.3)	<0.05
α-Tocopherol/cholesterol μmol/L/mmol/L	5.1 ± 2.3 (1.9–13.0)	3.3 ± 1.0 (1.5–6.2)	<0.0005
ROOH/α-tocopherol/cholesterol	0.9 ± 0.6 (0.1–2.7)	3.2 ± 1.6 (0.7–8.3)	<0.0005

Values are means ± SD, with ranges in parentheses.

\*Measured by FOX assay.

Source: Ref. 14.

point of attack, because injury mechanisms are interrelated in a complex way (1,17). Indeed, depending on the tissue under study and the type of reactive species causing the insult, the primary cellular target of oxidative stress can vary (18). For example, DNA is an important early target of damage when H<sub>2</sub>O<sub>2</sub> is added to many mammalian cells; increased DNA damage occurs before detectable lipid peroxidation or oxidative protein damage (19).

*Cell death* can result from multiple different insults (20). Excessive activation of poly (ADP ribose) polymerase can so deplete intracellular NAD<sup>+</sup>/NADH levels that the cell cannot make ATP and dies. This effect has sometimes been called a *suicide response*; because DNA repair is not completely efficient, a cell with extensively damaged DNA may “commit suicide” to avoid the risk of becoming an initiated cell. Cells can die from rupture of membrane blebs occurring as a result of uncontrolled increases in intracellular “free” Ca<sup>2+</sup> concentrations (20).

Cell death can be described by essentially two mechanisms, necrosis and apoptosis; both can result from oxidative stress (20). It is increasingly realized, however, that cell death can sometimes have features of both processes. In essence, during necrotic cell death, the cell swells and ruptures,

releasing its contents into the surrounding area and affecting adjacent cells. Contents can include antioxidants such as catalase or GSH and pro-oxidants such as copper and iron ions (Fig. 1). Hence, even if a cell dies by mechanisms not involving oxidative stress, necrotic cell death can impose oxidative stress on the surrounding tissues (Figs. 1 and 2). In apoptosis, the cell's own intrinsic "suicide mechanism" is activated; apoptosing cells do not release their contents and so apoptosis does not, in general, cause disruption to surrounding cells. Apoptotic cell death may be accelerated by oxidative stress, and added antioxidants often delay or prevent apoptosis induced by a range of insults (20–22). The caspases essential to apoptosis have active site cysteine residues, and so they can be inhibited by ROS/RNS/RCS at high levels (23,24). Hence, oxidative stress can induce apoptosis, but high levels of such stress can halt the apoptotic process and cause cells to die by necrosis (23).

## **II. NEED FOR "BIOMARKERS"**

To assess the importance of oxidative damage in human disease, accurate methods to measure it are essential. Before clinical trials of putative "antioxidant agents" take place, it is important to establish whether or not the planned treatment really can decrease oxidative damage in vivo. In principle, one can measure markers of oxidative damage in humans and examine how they are affected by intake of putative therapeutic antioxidants. The optimal dosage could then be determined. The same approach can be used to show not only that an antioxidant drug really is acting as an antioxidant in vivo but also to study nutritional antioxidants (25). It is important to assess all major molecular targets of damage by ROS/RNS/RCS (DNA, proteins, lipids) because an antioxidant that protects one target may fail to protect (or even exacerbate injury to) another (25). Hence, measurements of oxidative damage must accompany clinical tests of the effects of antioxidants (Fig. 3) to show that they actually did or did not affect the ROS/RNS/RCS.

## **III. WHAT BIOMARKERS ARE AVAILABLE?**

### **A. DNA**

Reactive species-mediated DNA damage can lead to cell death and, even worse, might produce initiated cells and thus facilitate cancer development (26–28). The chemistry of DNA damage by several reactive species has been well characterized in vitro (29–38), although further studies are needed with



$\text{RO}_2^\cdot$ ,  $\text{RO}^\cdot$ , and  $\text{O}_3$ . Nitric oxide ( $\text{NO}^\cdot$ ), probably via products derived from it ( $\text{NO}_2^\cdot$ ,  $\text{HNO}_2$ ,  $\text{ONOO}^-$ ,  $\text{N}_2\text{O}_3$ , etc.) can cause nitrosation and deamination of amino groups on DNA bases (34,39,40).

Whereas  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  appear not to react with DNA bases,  $\text{OH}^\cdot$  generates a multiplicity of products from all four DNA bases (31). By contrast,  $^1\text{O}_2$  appears selective for attack upon guanine (32,33). The most common base lesion, and the one most often measured as an index of oxidative DNA damage, is the nucleoside 8-hydroxy-2'-deoxyguanosine (8OHdG) (38). Measurement of 8OHdG alone can give misleading results under certain circumstances (reviewed in Ref. 40), although it is still widely used. In principle, there are two types of measurement of oxidative DNA damage. *Steady-state damage* can be measured when DNA is isolated from human cells and tissues and analyzed for base damage products; it presumably reflects the balance between damage and DNA repair. Hence, a rise in steady-state oxidative DNA damage could be due to increased damage and/or decreased repair. It is worth mentioning that the measurement of baseline levels of oxidatively modified DNA bases does not provide information as to whether this damage is in active genes or quiescent DNA.

However, it is important also to have an index of total DNA damage in the human body (i.e., the "input" side of the steady-state equation). The most common approach has been to assess the "output" side (i.e., trying to estimate the rate of repair of oxidized DNA). This is usually achieved by measuring urinary excretion of DNA base damage products. Several DNA base damage products are excreted in human urine, including 8OHdG, 8-hydroxyguanine, 8-hydroxyadenine, and 7-methyl-8-hydroxy-guanine (39,41,42), but the one most exploited is 8OHdG, usually measured by a method involving high-performance liquid chromatography (HPLC) with electrochemical detection (38). For example, in one study of 169 humans, the average 8OHdG excretion was 200–300 pmol/kg per 24 h, corresponding to 140–200 oxidative modifications of guanine per cell per day (42,43). Furthermore, smokers excrete 50% more 8OHdG than nonsmokers on average, suggestive of a mean 50% increased rate of oxidative DNA damage from smoking (42). Gas chromatography-mass spectroscopy (GC-MS) has also been used to measure 8OHdG in urine, and the limit of detection was 1.8 pmol, corresponding to a level of 8OHdG in urine of 35 nM (44).

The validity of these urinary measurements of oxidative DNA damage must be considered. The level of 8OHdG in urine is presumably unaffected by the diet because nucleosides are thought not to be absorbed from the gut. However, this question, and the question as to whether any 8OHdG is metabolized to other products in humans, has not been rigorously addressed in the

literature. In addition, it is possible that some or all of the 8OHdG excreted in urine may arise not from DNA but from dGTP in the DNA precursor pool of nucleotides. An enzyme has been described that hydrolyzes dGTP containing oxidized guanine, presumably to prevent its incorporation into DNA (45).

### *1. Relevance to Diabetes*

Both steady-state levels of 8OHdG in blood monocytes (46) and urinary excretion of 8OHdG (47) have been reported as elevated in diabetic patients. Gas chromatography/mass spectrometry has recently shown elevated levels of a wide range of base oxidation products in DNA from white blood cells of diabetic patients. The pattern of base damage was diagnostic of increased OH<sup>•</sup> formation in vivo (48).

## **B. Lipid Peroxidation**

Lipid peroxidation is important in vivo for several reasons, in particular because it contributes to the development of atherosclerosis (49–51), a process known to be accelerated in diabetic patients (6). Lipid peroxides and other end products of the peroxidation process may be toxic to vascular endothelium in diabetics (6,52). Many assays are available to measure lipid peroxidation, but the simpler ones, such as the TBA test and diene conjugation, are notoriously unreliable when applied to human tissues and body fluids (reviewed in Ref. 53), although the TBA test can be improved by linking it to HPLC and adding antioxidants with the TBA reagents to prevent peroxidation during the assay procedure (54). Levels of lipid peroxides in human plasma, as measured by reliable analytical methods (54–59), seem to be low, usually <0.1 μM. Human body fluids also contain low levels of F<sub>2</sub>-isoprostanes, compounds isomeric to prostaglandins that are thought to arise by free radical oxidation of phospholipids containing arachidonic acid (60). It has been suggested that one of the F<sub>2</sub>-isoprostanes, 8-epiPGF<sub>2</sub>α, can be generated by cyclooxygenase in human platelets (61), although this does not appear to be a significant contributor to total body production of 8-epiPGF<sub>2</sub>α (62). Isoprostanes appear to exist in human plasma largely esterified to phospholipids rather than “free,” and sensitive assays to measure them have been described (60–65). Families of F<sub>3</sub>- and F<sub>4</sub>-isoprostanes, derived from eicosapentaenoic and docosahexaenoic acids, respectively, have recently been described (66).

### *1. Relevance to Diabetes*

There seems to be general agreement that lipid peroxidation is elevated in diabetes, although its relationship to disease progression is uncertain. Some

investigators have reported an association between plasma or serum TBA-reactive substances (TBARS) or diene conjugates and diabetic complications, whereas others have not (67–70). However, TBARS and diene conjugation assays should be interpreted with caution (53). MacRury et al. (70) compared different methods (conjugated dienes, TBARS, and chemiluminescence) of assessing free radical activities in diabetic subjects. In each case, diabetes was associated with elevated levels of different indirect measurements of lipid peroxidation. However, they did not find a relationship between diabetic complications and plasma measures of oxidative stress. More convincingly, elevated levels of plasma 8-epi PGF<sub>2</sub>α have been reported in diabetics, although its association with disease progression was not discussed (71). Another study, using the ferrous oxidation with xylenol orange (FOX) assay to measure lipid peroxides, found higher lipid-standardized peroxides in plasma from diabetic patients (Table 3). This elevated level was not influenced by sex, age, smoking habit, or diabetic complications and was taken to suggest that the elevated levels of plasma hydroperoxide in patients are associated with the diabetes itself rather than consequent tissue injury (e.g., nephropathy, neuropathy). The reliability of the FOX assay as a measure of lipid peroxides in human plasma remains to be established, however. As Table 3 indicates, “basal” peroxide levels in plasma from healthy subjects seem higher than the 0.1 μM or less measured by more chemically robust assays (see above).

## 2. *Measuring “Total” Lipid Peroxidation in the Human Body*

Peroxide levels in body fluids or tissues represent a balance between peroxide formation and peroxide metabolism or decomposition (i.e., they are essentially a “steady-state” measurement). Can some measure of total body lipid peroxidation be obtained?

This has most often been attempted by measuring hydrocarbon gases (ethane, pentane) in exhaled air (72) and urinary excretion of MDA (more properly called TBA-reactive material) (73). The latter assay is probably confounded by diet: Most lipid-related TBARS appearing in urine seems to arise from lipid peroxides or aldehydes in ingested food, which are presumably largely generated during cooking (74,75). For example, Brown et al. (75) showed that a diet rich in cooked meat promoted urinary TBARS excretion, to an extent depending on the temperature at which the meat was cooked. Hence, urinary TBARS is not a suitable assay to assess whole body lipid peroxidation, although it could theoretically be used to look at effects of antioxidant supplementation of people on a controlled diet (74). In any case, HPLC must be used to separate the real (TBA)<sub>2</sub>MDA adduct; much TBARS in urine is not even lipid derived (76) or arises from aldehydes other than MDA (77).

Breath excretion of ethane and pentane, minor end products of lipid peroxidation, is difficult [but not impossible (78)] to measure in humans because of the problem of contamination of the atmosphere by these gases, leading to their partitioning into body fat stores (79). Particular problems with pentane include the fact that it is metabolized by cytochromes P450 (78,80) and that GC columns frequently used to separate 'pentane' for measurement can fail to separate it from isoprene, a hydrocarbon also excreted in exhaled air (79,81,82). Indeed, the levels of excreted real pentane seem close to zero in most humans (79,81,82). Perhaps further evaluation of the technique of hydrocarbon gas exhalation should focus on ethane (72), but in general the technique would seem difficult to use reliably in human studies except where subjects are confined to controlled environments, breathing air of minimal hydrocarbon content. The possible effect of dietary changes on hydrocarbon gas production by gut flora is another potential confounding factor.

Isoprostanes and their metabolites can be measured in human urine (60,62,64), and this may prove to be a valuable assay of whole body lipid peroxidation if a confounding effect of diet can be ruled out.

### C. Protein Damage

Damage to proteins may be important *in vivo* both in its own right [affecting the function of receptors, enzymes, transport proteins, etc., and perhaps generating new antigens that provoke immune responses (83)] and also because it can contribute to secondary damage to other biomolecules (e.g., by inactivation of antioxidant defense enzymes or repair enzymes). Attack of various RNS ( $\text{ONOO}^-$ ,  $\text{NO}_2^*$ ,  $\text{NO}_2\text{Cl}$ , and possibly some other species) upon tyrosine (both free and within proteins) leads to production of 3-nitrotyrosine, which can be measured immunologically or by HPLC or GC-MS techniques (reviewed in Refs. 84 and 85). RCS can produce chlorinated products (e.g., 3-chlorotyrosine), and these have been detected in human atherosclerotic lesions (50).

The chemical reactions resulting from attack of ROS/RNS/RCS on proteins are complex. Free radical attack can generate protein peroxides, which may decompose to generate free radicals (reviewed in Ref. 86). Several assays to measure damage to specific amino acid residues in proteins by ROS/RNS/RCS have been developed. They include assays of L-DOPA (produced by tyrosine hydroxylation), valine hydroxides (produced from valine hydroperoxides), tryptophan hydroxylation and ring-opening products, 8-oxohistidine, di-tyrosine, and *ortho*- and *meta*-tyrosines, products of attack of  $\text{OH}^*$  upon phenylalanine (86). The levels of any one (or, preferably, of more than one) of these products in proteins could in principle be used to assess the balance

between oxidative protein damage and the removal of damaged proteins. The only products exploited to date have been the hydroxylated phenylalanines. For example, levels of *ortho*-tyrosine and dityrosine in human lens proteins have been reported in relation to age (87). These products were also measured in hair from "Alpine Man," *Homo tirolensis* (88).

### 1. Carbonyl Assay

More use has been made of the carbonyl assay, a general assay of oxidative protein damage (89), to assess steady-state levels of such damage in human tissues and body fluids. The carbonyl assay is based on the ability of several ROS to attack amino acid residues in proteins (particularly histidine, arginine, lysine, and proline) to produce carbonyl functions that can be measured after reaction with 2,4-dinitrophenylhydrazine (89,90). The carbonyl assay has become widely used, and many laboratories have developed individual protocols for it. Sometimes the assay procedures used in a particular laboratory are not specified precisely in published papers and often differ from those used originally by the group of Stadtman et al. This point is important because there is a considerable variation in the "baseline" levels of protein carbonyls in certain human tissues, depending on how the assay is performed (reviewed in Ref. 91). By contrast, most groups seem to obtain broadly comparable values for protein carbonyls in human plasma, of  $<1$  nmol/mg protein, so plasma protein carbonyls should be a useful assay of oxidative protein damage. However, protein glycation and covalent binding of certain aldehyde end products of lipid peroxidation to proteins can also generate carbonyls (89).

### 2. Relevance to Diabetes

Glycooxidation seems to play an important role in the vascular endothelial dysfunction detected in diabetic patients (92,93) and perhaps in the nephropathy (94). Elevated glucose may cause increased generation of  $O_2^{\cdot-}$  by endothelium, antagonizing the action of  $NO^{\cdot}$  and perhaps forming peroxynitrite,  $ONOO^-$  (92,93). All these effects should be amenable to treatment by appropriate antioxidants (92).

## IV. CONCLUSION

Despite the arguments that can be raised about the validity of some individual biomarkers, the sum of evidence from biomarkers reporting oxidative damage shows that such damage is increased in diabetes, affecting DNA, lipids, and

proteins (glycooxidation), supporting the concept of increased oxidative stress in diabetes. Indeed, the newly introduced drug troglitazone may exert some of its protective effects by its antioxidant capacity (95). Further work is required using modern biomarkers to evaluate the extent to which agents beneficial in the treatment of diabetes, including lipoic acid (see other chapters in this volume), act by suppressing oxidative stress. Another exciting area is the prevention of the teratogenic effects of hyperglycemia: Studies in rats have shown that vitamin E (96) and overexpression of CuZnSOD (97) can be beneficial.

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# 4

## Plasma Lipid Hydroperoxide and Vitamin E Profiles in Patients with Diabetes Mellitus

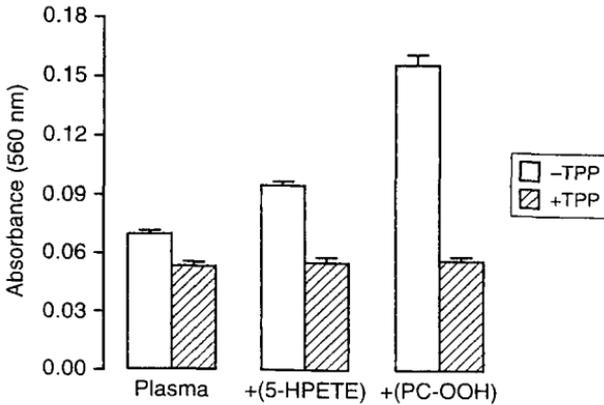
**Jaffar Nourooz-Zadeh**

*University College London, London, England*

Patients with non-insulin-dependent diabetes mellitus (NIDDM) are at increased risk of developing vascular and other complications. This excess risk is only partially explained by the traditional risk factors, including smoking, hypertension, and dyslipidemia (1–3). Therefore, oxidative stress has been proposed as a possible explanation for the accelerated complications in NIDDM (4–7). A major hypothesis is that low-density lipoprotein (LDL) modification by oxidation or glycosylation contributes to tissue damage through cytotoxic reactions with endothelial cells or through further reactions to generate “modified” LDL that is selectively accumulated by “scavenger” receptors (8).

Despite the biochemical importance of oxidative stress, its measurement *in vivo* has been difficult (9). Common approaches to assess oxidative stress in biological fluids are measurement of lipid peroxidation products, oxidatively modified DNA, or protein damage and measurement of the depletion of antioxidants.

Enhanced lipid peroxidation in diabetics has been reported using thiobarbituric acid reactive substances (TBARS) as an assay (10–14). The simple TBARS assay in fact measures many substances in addition to products of lipid peroxidation and is affected by the lipid content of the sample (15). Therefore,



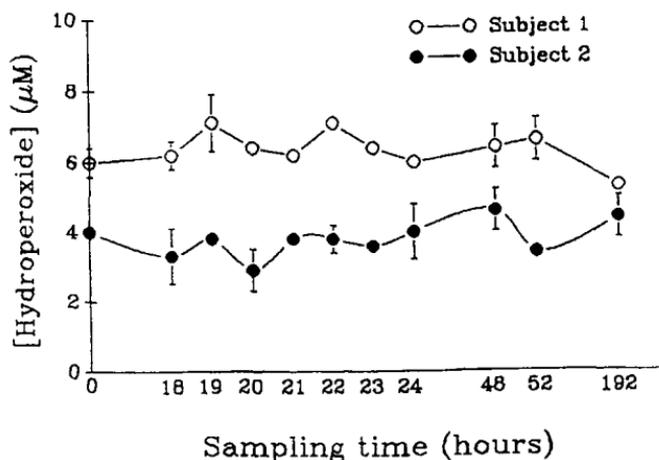
**Figure 1** Detection of authentic ROOHs in plasma. PC-OOH, phospholipid hydroperoxides; 5-HPETE, 5-hydroperoxyecotetraenoic acid.

it is unclear to what extent plasma lipoprotein peroxidation assessed by this method accounts for biological changes associated with oxidative stress.

We have used the ferrous oxidation with xylenol orange (FOX) assay coupled with triphenylphosphine (TPP) to determine plasma lipid hydroperoxide (ROOH) levels in health and disease (16,17). TPP is used to reduce ROOHs. This maneuver is necessary to generate a proper control for each individual plasma sample because plasma contains interfering components, mainly ferric ions that are detected by xylenol orange (Fig. 1). Other advantages of the FOX2 assay over existing techniques are kinetics of the reaction are independent of the chemical structure of the ROOHs and no extraction step is normally needed because the use of the 90% methanol–25 mM H<sub>2</sub>SO<sub>4</sub> denatures proteins sufficiently allow access of the ferrous ions to available ROOHs. The coefficient of variation for individual plasma samples using this method is typically less than 5%, whereas that for the interassay coefficient of variation is <10% (16–19).

## I. DIURNAL VARIATION OF PLASMA ROOHs

No information is available on the effect of diurnal variation on plasma lipid peroxidation products. Using the FOX assay, we examined this issue in two subjects (one woman, one man, aged 30 and 36, respectively) under fasting

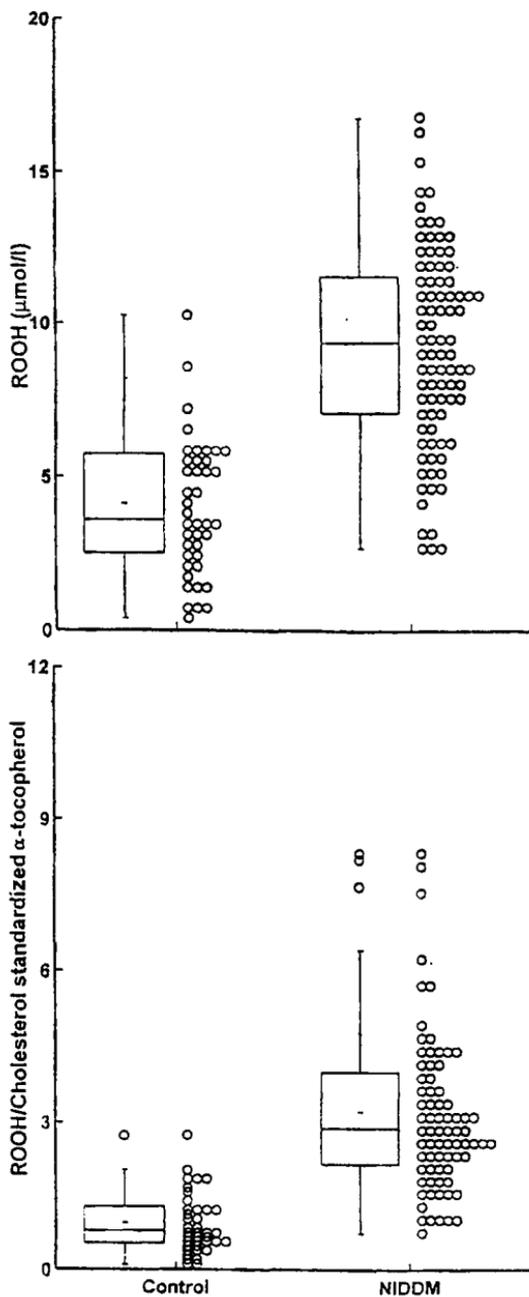


**Figure 2** Diurnal change in plasma ROOHs. (From Ref. 18.)

and nonfasting conditions (17). As shown in Figure 2, little fluctuation around the mean ROOH value was associated with either the fasted or fed state. These data suggest that dietary input of ROOHs appears to be small by comparison with metabolic production of ROOH.

## II. PLASMA ROOH LEVELS IN HEALTHY AND DIABETIC SUBJECTS

Mean levels of ROOH of  $9.04 \pm 4.3$  and  $9.4 \pm 3.3$   $\mu\text{mol/L}$  were detected in freshly prepared plasma from type II diabetics from two different studies ( $n = 22$  and  $87$ , respectively) (17,18). The corresponding levels for healthy volunteers from three different studies were  $3.02 \pm 1.85$ ,  $3.76 \pm 2.48$ , and  $4.1 \pm 2.2$   $\mu\text{mol/L}$  ( $n = 23$ ,  $21$ , and  $41$ , respectively) (16–18). Data spread for plasma ROOHs and ratio of ROOHs/cholesterol standardized  $\alpha$ -tocopherol in NIDDM and control subjects are shown in Figure 3. Clinical characteristics of NIDDM and control subjects are summarized in Table 1. Similar plasma ROOH concentrations have also been reported by other investigators using the FOX2 assay (20–22). These data together provide the evidence for the reliability of the FOX2 assay for the measurement of plasma ROOHs.



**Figure 3** Data spread for ROOHs (top) and ROOHs/cholesterol-standardized  $\alpha$ -tocopherol (bottom) in control and type II diabetic subjects.

**Table 1** Clinical Characteristics of Healthy and NIDDM Individuals

Variables	Healthy	NIDDM	<i>p</i>
Numbers	41	87	—
Sex (F/M)	24/17	40/47	—
Age (yr)	38.2 ± 12.3 (21–69)*	58.4 ± 14.7 (17–86)	<0.0005
Diabetes duration (yr)	—	12.0 ± 8.3 (0.0–44)	—
Total cholesterol (mmol/L)	5.0 ± 1.1 (1.4–6.9)	6.0 ± 1.3 (3.3–9.9)	<0.002
Triglycerides (mmol/L)	0.9 ± 0.5 (0.3–2.5)	2.8 ± 1.8 (0.6–3.5)	<0.0005
Fasting glucose (mmol/L)	4.9 ± 0.4 (4.2–5.8)	12.1 ± 5.1 (1.9–28.9)	<0.0005
HbA1c (%)	— (5–8)**	11.0 ± 2.4 (5.9–17.8)	—
ROOH (μM)	4.1 ± 2.2 (0.4–10.3)	9.4 ± 3.3 (2.7–16.8)	<0.0005
α-Tocopherol (μmol/L)	23.8 ± 8.3 (10.6–47.0)	19.6 ± 7.5 (8.6–44.3)	<0.05
α-Tocopherol/cholesterol (μmol/L)/ (mmol/L)	5.1 ± 2.3 (1.9–13.0)	3.3 ± 1.0 (1.5–6.2)	<0.0005
ROOH/α-tocopherol/cholesterol	0.9 ± 0.6 (0.1–2.7)	3.2 ± 1.6 (0.7–8.3)	<0.0005

\*Data in brackets are ranges.

\*\*Data in parentheses are normal ranges.

Source: Ref. 18.

### III. PLASMA ROOH IN DIABETICS WITH AND WITHOUT COMPLICATIONS

The association between diabetic complications and plasma lipid peroxidation as measured by nonspecific techniques (e.g., thiobarbituric acid- or ultraviolet-absorbing diene conjugates) has been examined by a number of investigators but has yielded contradictory results (10–12). We have shown that the elevated level of plasma ROOHs in diabetic subjects was not influenced by diabetic complications (Table 2). These data suggest that oxidative stress is an early stage in the disease pathology and not simply a consequence of the complications.

**Table 2** Clinical Characterization for NIDDM Subjects With and Without Complications

Variables	No complications	Complications	<i>p</i>
Numbers	38	49	—
Age (yr)	53.3 ± 13.7 (17–82)*	62.3 ± 14.1 (31–86)	<0.005
Total cholesterol (mmol/L)	5.6 ± 1.2 (3.3–9.9)	6.4 ± 1.1 (4.2–9.9)	<0.05
Triglycerides (mmol/L)	2.5 ± 1.5 (0.7–6.2)	3.1 ± 1.9 (0.5–9.5)	NS
Fasting glucose (mmol/L)	11.4 ± 5.4 (1.9–28.9)	12.7 ± 5.1 (2.3–28.5)	NS
HbA1c (%)	10.9 ± 2.5 (5.9–16.8)	11.1 ± 2.3 (6.9–17.8)	NS
ROOH (μmol/L)	9.5 ± 3.3 (2.7–15.5)	9.4 ± 3.4 (2.7–16.8)	NS
α-Tocopherol (μmol/L)	18.6 ± 5.6 (9.2–30.9)	20.4 ± 8.7 (8.6–44.3)	NS
α-Tocopherol/cholesterol (μmol/L)/(mmol/L)	3.4 ± 0.9 (1.9–5.0)	3.2 ± 1.0 (1.5–6.2)	NS
ROOH/α-tocopherol/cholesterol	3.0 ± 1.4 (0.6–6.1)	3.3 ± 1.6 (0.9–8.3)	NS

\*Data in brackets are ranges.

NS, not significant.

Source: Ref. 18.

#### IV. PLASMA VITAMIN E STATUS IN HEALTHY AND DIABETIC SUBJECTS

Data are conflicting on the α-tocopherol status in diabetic subjects. Some studies report no changes, others a decrease, and still others an increase (23–25). One problem with the previous studies was a failure to standardize α-tocopherol for lipid concentration, which would produce misleading results in hyperlipidemic patients. We have found that absolute plasma α-tocopherol levels in diabetic subjects were slightly, but significantly, lower than those of the control subjects (Table 1). Plasma α-tocopherol levels between the two groups differed markedly when α-tocopherol levels were expressed per unit of cholesterol. No difference was found in absolute plasma or cholesterol-standardized α-tocopherol in the diabetic patients with and without complications (Table 2). These findings have recently been confirmed by Borcea et al. (22). Further

studies are needed to address the question whether the low plasma  $\alpha$ -tocopherol in the diabetic patients is related to increased oxidative stress or chronic low dietary intake of vitamin E.

## **V. RELATIONSHIP BETWEEN ROOH AND GLYCEMIC CONTROL**

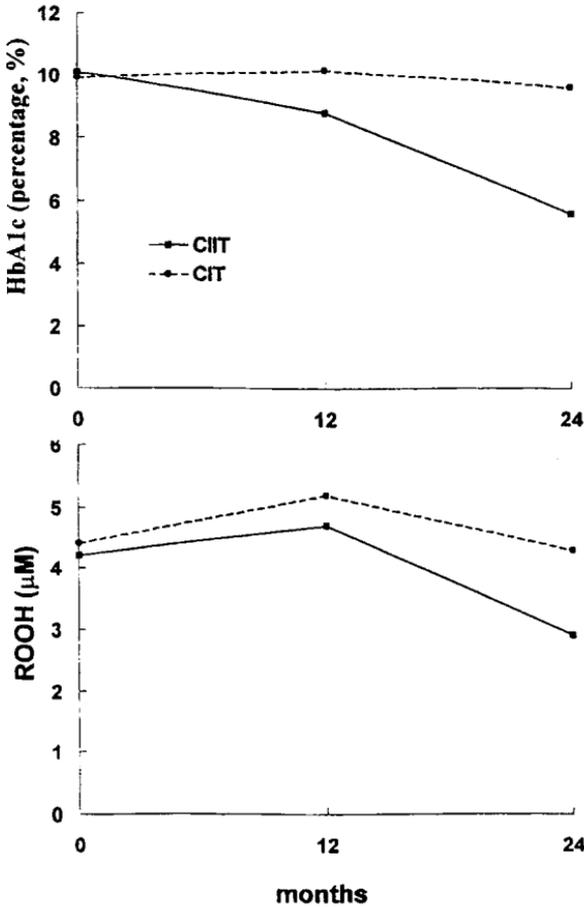
Two independent studies from this laboratory have shown no correlation between plasma ROOHs and HbA1c (17,18). On the other hand, there was a scatter association between ROOH and fasting blood glucose ( $r = 0.2$ ,  $p < 0.05$ ) in the diabetic subjects but not in the control group (17,18). ROOH/cholesterol-standardized  $\alpha$ -tocopherol ratio also showed a weak association with fasting blood glucose in the diabetic subjects but not in the control group ( $r = 0.23$ ,  $p < 0.05$ ) (18).

## **VI. INFLUENCE OF INSULIN THERAPY ON PLASMA ROOHs**

Little information is available on the effect of glycemic control on plasma markers of oxidative stress. Berg et al. (26) compared the effect of continuous intensified insulin treatment (CIIT) and conventional insulin treatment (CIT) on plasma lipid peroxides as measured by the FOX assay. Plasma ROOHs in patients receiving CIIT fell by 31% as compared with baseline over a period of 24 months. HbA1c fell by 15% during the same period (Fig. 4). By contrast, no difference was seen in patients receiving CIT over the same period. Faure et al. (27) also examined the effect of CIIT on plasma lipid peroxides using the TBA assay. They too reported a marked reduction in TBARs after CIIT as compared with the baseline level ( $2.42 \pm 0.25$  vs.  $3.03 \pm 0.27$   $\mu\text{mol/L}$ ;  $n = 16$ ) over a period of 7 years (27). These observations provide further support for the hypothesis of a beneficial effect of insulin therapy on lipid peroxidation brought about by decreasing circulating HbA1c levels

## **VII. EFFECT OF ANTIOXIDANT TREATMENT ON PLASMA ROOHs**

To the best of our knowledge, there is one study addressing the effect of antioxidant therapy on plasma markers of oxidative stress in diabetic patients.



**Figure 4** Change in plasma ROOHs and HbA1c during continuous intensified insulin therapy (CIIT) and conventional insulin therapy (CIT).

Borcea et al. (22) studied the effect of the antioxidant  $\alpha$ -lipoic acid on plasma ROOHs in diabetic patients ( $n = 33$ ) receiving  $\alpha$ -lipoic acid for 12 weeks (600 mg/day). Diabetics treated with  $\alpha$ -lipoic acid had markedly lower levels of plasma ROOHs than the control group. A trend toward higher  $\alpha$ -tocopherol concentration was seen in the  $\alpha$ -lipoic acid-treated subjects as compared with control subjects.  $\alpha$ -Lipoic acid exists naturally in physiological systems as a cofactor for enzymatically catalyzed acyl transfer reactions (28).  $\alpha$ -Lipoic acid and its intracellularly reduced form, dehydrolipoate, have been shown to scav-

enge a variety of reactive species such as HO<sup>•</sup>, ROO<sup>•</sup>, HOCl, and peroxynitrite; to regenerate both  $\alpha$ -tocopherol and ascorbate; and to raise intracellular glutathione levels. Thus, these data provide the first direct evidence for the hypothesis that treatment with the antioxidant  $\alpha$ -lipoic acid reduces accumulation of ROOHs in the circulation.

## VIII. CONCLUSION

Diabetes mellitus has been proposed to be associated with a high risk of atherosclerosis and kidney and nerve damage. Preliminary work from this laboratory has shown that plasma from individuals with diabetes mellitus contains elevated levels of ROOHs. The diabetic subjects also had lower levels of plasma  $\alpha$ -tocopherol as compared with control subjects, which was unrelated to dyslipidemia. Another important point from our data is that ROOH levels were similar in diabetics with and without complications, suggesting that oxidative stress occurs at an early stage in the disease pathology. It predates the complications, not simply a consequence of the complications. In addition, we have shown that insulin therapy and antioxidant therapy have a beneficial effect on oxidative stress.

## ACKNOWLEDGMENT

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# 5

## Concentrations of Antioxidative Vitamins in Plasma and Low-Density Lipoprotein of Diabetic Patients

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Several recent studies indicate that oxidative stress is increased in diabetic patients. Oxidative stress is a major component in the development of late complications in diabetic patients (1–4). It is mainly based on hyperglycemia. During the cellular metabolization of glucose, superoxide anions can be formed that shift the pro/antioxidative balance in blood (5,6). Intracellular activation of the polyol pathway produces an imbalance in the ratio of NADH/NAD<sup>+</sup>. Elevated blood glucose concentrations also cause increased glycation of lipoproteins. Because of these factors, reactive oxygen species and lipoperoxides are formed in the blood of diabetic patients (7–12) and their lipoproteins are more prone to in vitro oxidation (13,14). Glucose can act oxidatively on low-density lipoprotein (LDL) in vitro (1,6,15,16). Impaired protection of lipid membranes against damage by free radicals is important in insulin-dependent diabetes mellitus (IDDM) because islet cell destruction by leukocytes may be mediated through the generation of toxic oxygen radicals (17).

There are many defenses to protect the organism from free radical processes (18). Antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are preventive antioxidants because they eliminate species involved in the initiation of free radical chain reactions. Some small molecule antioxidants, such as ascorbate, the tocopherols, ubiquinol, urate,

and glutathione, are able to repair oxidizing radicals directly and therefore are chain-breaking antioxidants.

## I. VITAMIN E IN DIABETES

Vitamin E (RRR- $\alpha$ -tocopherol) is the most important lipid-soluble antioxidant, which protects lipoproteins and cell membrane lipids from oxidative damage. This ability is coupled to other antioxidant systems (vitamin C, glutathione, lipoic acid) that can recycle the vitamin E radical (19). In the absence of such systems, vitamin E can behave as an oxidant (20). Dietary vitamin E is transported to the liver and secreted from the liver within very-low-density lipoproteins (VLDL). It is distributed among VLDL and LDL during the transfer and metabolism of the lipoprotein lipids (21). Thermodynamic partitioning also permits some transfer into high-density lipoproteins (22). An important part of vitamin E is constituent of cell membranes where it protects the lipid moiety against peroxidation (23).

About one-half of the total plasma vitamin E is a constituent of circulating LDL. Interindividual variations in plasma vitamin E are closely related to those in LDL (24,25). The concentration of vitamin E per LDL particle is rather low (i.e., in the order of 5–9 molecules compared with 2200 molecules of cholesterol and 170 molecules of triglycerides) (26). Nevertheless, the level of vitamin E in LDL is an independent factor that influences susceptibility of LDL to oxidation. The lagtime of *in vitro* LDL oxidation was found to be related to the level of vitamin E in LDL when diabetic patients were supplemented with vitamin E (27,28). For persons with usual nutritional habits, the corresponding relationship was observed in two studies (29,30) but not in others (3,31,32).

There are several publications on vitamin E concentrations in the plasma of diabetic patients. The assumption that the oxidative stress in diabetes is due to deficient vitamin E in plasma could not be confirmed. In most studies, no statistically significant differences in the concentrations between diabetic and control persons were observed. Two studies exhibited even significantly higher levels in diabetes (Table 1). The weighted mean values (control subjects  $26.4 \pm 1.9 \mu\text{mol/L}$ , IDDM  $24.4 \pm 1.6 \mu\text{mol/L}$ , NIDDM  $22.4 \pm 2.0 \mu\text{mol/L}$ ) are only marginally different for the plasma of control persons, IDDM, and non-IDDM (NIDDM).

Published data on vitamin E concentrations in LDL (Table 2) show very similar mean values for diabetic persons and control subjects (control subjects  $7.5 \text{ mol/mol}$ , IDDM  $7.0 \text{ mol/mol}$ , NIDDM  $6.8 \text{ mol/mol}$ ). One study demon-

**Table 1** Plasma (Serum) Vitamin E Concentrations in Diabetes

Group	Vitamin E ( $\mu\text{mol/L}$ )	<i>n</i>	Sign.	Ref.
Control subjects	21.4 $\pm$ 0.6	29		
IDDM	23.7 $\pm$ 0.8	27	*	33
Control subjects	24.9 $\pm$ 1.4	20		
IDDM	24.6 $\pm$ 1.6	15		3
Control subjects	26.5	62		
IDDM	22.7	77		34
NIDDM	21.4	81		
Control subjects	24.8 $\pm$ 2.3	180		
NIDDM	21.1 $\pm$ 1.5	164	*	35
Control subjects	23.9 $\pm$ 1.4	20		
IDDM	28.3 $\pm$ 1.5	20		36
Control subjects	17.0 $\pm$ 2.1	12		
NIDDM	16.2 $\pm$ 2.9	9		37
Control subjects (vs. NIDDM)	20.1 $\pm$ 2.7	15		
Control subjects (vs. IDDM)	18.0 $\pm$ 1.9	10		30
NIDDM	22.6 $\pm$ 6.5	53		
IDDM	21.0 $\pm$ 4.1	10		
Control subjects	27.8 $\pm$ 2.4	47		
NIDDM	25.8 $\pm$ 2.1	59		38
Control subjects	20.6 $\pm$ 0.7	40		
NIDDM	24.2 $\pm$ 1.0	40	*	39
Control subjects	27.9 (16.3–39.5)	28		
NIDDM	27.5 ( 8.7–46.3)	21		40
Control subjects (M/F)	28.6/27.9*	210/240		
IDDM (M/F)	23.6/26.2	60/63	*	41
Control subjects	23.8 $\pm$ 1.3	41		
NIDDM	19.5 $\pm$ 0.8	87		42
Control subjects (vs. NIDDM)	33.3 $\pm$ 3.3	20		
Control subjects (vs. IDDM)	27.5 $\pm$ 1.4	24		43
NIDDM	32.0 $\pm$ 1.8	24		
IDDM	25.2 $\pm$ 1.4	28		

\*Median.

**Table 2** LDL Vitamin E Concentrations in Diabetes

Group	Vitamin E (mol/mol)	<i>n</i>	Sign.	Ref.
Control subjects (vs. NIDDM)	7.5 ± 1.0	20		31
Control subjects (vs. IDDM)	7.1 ± 1.3	20		
NIDDM	8.5 ± 3.5	20		
IDDM	7.7 ± 2.1	20		
Control subjects (vs. NIDDM)	8.1 (1.20)*	15		30
Control subjects (vs. IDDM)	7.4 (1.33)	10		
NIDDM	6.1 (1.33)	53	*	
IDDM	5.7 (1.32)	10		

\*Geometric mean and geometric standard deviation (in parentheses).

strated significantly lowered levels in NIDDM; vitamin E in LDL was inversely related to HbA1c and positively related to the lagtime of ex vivo oxidation of LDL. This means that vitamin E in LDL is a better marker of antioxidant deficiency in diabetes than vitamin E in plasma (30).

## II. PROBLEM WITH LIPID STANDARDIZATION OF VITAMIN E

There are close correlations between the concentrations of vitamin E and lipids (triglycerides, cholesterol, and phospholipids) in plasma. They are due to the lipophilic properties of vitamin E, and moreover they reflect that the antioxidative capacity of lipids is regulated in progression with the lipid mass.

Epidemiological studies revealed that the mathematical relationship between vitamin E and lipid concentrations (44) has the feature that it does not pass the origin. Rather it has a large ordinate section. This means that low lipid concentrations are associated with relatively more vitamin E and high lipid concentrations with relatively less vitamin E. Lipid standardization has the purpose to make vitamin E concentrations comparable irrespective of the corresponding lipid concentrations. The ratio of vitamin E to lipid concentration does not fulfill this condition. It can be shown that this ratio is inversely related to the lipid concentrations. Therefore, the widely used division of vitamin E by cholesterol (3,37,39,41,42,45) or cholesterol plus triglyceride (34, 40,45) concentrations is inadequate. By this procedure, most persons with above-normal lipid concentrations appear vitamin deficient. An alternative is

a correction formula derived from the multiple regression of vitamin E on cholesterol and triglyceride concentrations. The constants in this formula should be derived from the population under study. For this purpose, 15–20 complete data sets may be sufficient (44). Recently, the formula was published with constants as follows (vitamin E in  $\mu\text{mol/L}$ ) (46):

$$\begin{aligned}\text{Standardized vitamin E} &= \text{measured vitamin E} \\ &\quad -2.9 (\text{cholesterol} - 5.2) \\ &\quad -1.5 (\text{triglycerides} - 1.3)\end{aligned}$$

The multiple regression on cholesterol and triglyceride concentrations was used for vitamin E standardization by several authors (44,46–48). No statistically significant difference of standardized values between diabetic patients and control persons was observed.

### III. VITAMIN A IN DIABETES

Vitamin A (*trans*-retinol) is required for normal growth, vision, and resistance to radical-mediated processes during infections. The fat-soluble vitamin is taken up with animal food and partly formed from other carotenoids. After absorption, vitamin A is transported in chylomicrons from the gut via the lymph duct and blood to the liver. Although the liver secretes a specific retinol-binding protein (38,49) into the bloodstream, plasma vitamin A correlates with plasma lipids almost as strongly as vitamin E does (50).

The level of vitamin A in diabetes has found much attention in recent literature. Vitamin A deficiency may cause blindness. Poorly controlled diabetes mellitus is attributed to a decreased availability of retinol carrier protein and subsequently to depressed vitamin A levels in blood. The impaired vitamin A status may not be improved by vitamin A supplementation but by insulin administration (51). In persons with well-controlled NIDDM without insulin deficiency, the metabolism of vitamin A appears not to be impaired. Interestingly, persons with impaired glucose tolerance show increased ( $2.5 \mu\text{mol/L}$ ) vitamin A versus persons with normal glucose tolerance ( $2.1 \mu\text{mol/L}$ ) (46). Most studies demonstrated lowered levels in IDDM, the difference being significant in six studies. Significantly lowered levels in NIDDM were found in two studies (Table 3). The weighed mean values are similar for control subjects and NIDDM and lower for IDDM (control subjects  $1.95 \pm 0.23 \mu\text{mol/L}$ , IDDM  $1.52 \pm 0.46 \mu\text{mol/L}$ , NIDDM  $2.29 \pm 0.23 \mu\text{mol/L}$ ).

**Table 3** Plasma (Serum) Vitamin A (Retinol) Concentrations in Diabetes

Group	Vitamin A ( $\mu\text{mol/L}$ )	<i>n</i>	Sign.	Ref.
Control subjects	2.32 $\pm$ 0.36			52
IDDM	1.68 $\pm$ 0.83		*	
Control subjects	1.86 $\pm$ 0.35			53
IDDM	1.63 $\pm$ 0.33		*	
Control subjects	1.82 $\pm$ 0.45			
IDDM	1.45 $\pm$ 0.36		*	54
Control subjects	1.95 $\pm$ 0.11	20		
IDDM	1.55 $\pm$ 0.07	15	*	3
Control subjects	1.89 $\pm$ 0.56	20		
IDDM	1.71 $\pm$ 0.99	20		36
Control subjects	2.14 $\pm$ 0.10	47		
NIDDM	2.23 $\pm$ 0.10	59		38
Control subjects	2.3 $\pm$ 0.18	180		
NIDDM	2.2 $\pm$ 0.11	164	*	35
Control subjects	1.7 $\pm$ 0.1	47		
NIDDM	1.5 $\pm$ 0.1	46	*	39
Control subjects	2.7 (0.8–4.4)	28		40
NIDDM	2.6 (1.6–3.6)	21		
Control subjects (M/F)	1.86/1.54*	210/240		
IDDM (M/F)	1.46/1.28	60/63	*	41
Control subjects (vs. NIDDM)	2.23 $\pm$ 0.18	20		
Control subjects (vs. IDDM)	1.94 $\pm$ 0.10	24		43
NIDDM	2.23 $\pm$ 0.14	24		
IDDM	1.30 $\pm$ 0.05	28	*	
Control subjects	2.9 $\pm$ 0.6	35		
IDDM	3.3 $\pm$ 1.0	10		51
NIDDM	3.2 $\pm$ 0.9	53		

\*Median.

#### IV. VITAMIN C IN DIABETES

Vitamin C (ascorbic acid) is a powerful antioxidant and a cofactor in collagen biosynthesis, which affects platelet activation, prostaglandin biosynthesis, and the polyol pathway. Vitamin C acts as an antioxidant both *in vitro* and *in vivo* and protects plasma lipids and lipid membranes. It has the power to spare and to increase plasma-reduced glutathione (56). The antioxidative ability of vitamin E can be continuously restored through its recycling by other antioxidants, mainly vitamin C (18,19).

**Table 4** Plasma Vitamin C Concentrations in Diabetes

Group	Vitamin C ( $\mu\text{mol/L}$ )	<i>n</i>	Sign.	Ref.
Control subjects	82.9 $\pm$ 30.9	22		
NIDDM	55.6 $\pm$ 20.0	21		
NIDDM + Complic.	42.1 $\pm$ 19.3	20		57
Control subjects	60.0 $\pm$ 5.5	180		
NIDDM	48.8 $\pm$ 2.3	164	*	
NIDDM + Complic.	29.9 – 41.2	163	*	35
Normal persons	61.3 $\pm$ 9.7	20		
Borderline FBG	47.7 $\pm$ 7.4	10	*	
NIDDM	28.4 $\pm$ 5.7	30	*	58
Control subjects	71.6 (37.5–105.6)	28		
NIDDM	57.9 (51.7–64.2)	21		40
ND, PVD	47.1 (7.4–86.9)	21	*	
NIDDM, PVD	48.3 (15.3–80.1)	11	*	
Control subjects A = 28 yr	87.5 $\pm$ 4.9	24		
IDDM	63.6 $\pm$ 6.0	28	*	43
Control A = 63 yr	58.5 $\pm$ 5.3	20		
NIDDM	38.6 $\pm$ 5.7	24	*	

Complic., diabetic complications; FBG, fasting blood glucose; PVD, peripheral vascular disease.

Abnormalities of vitamin C metabolism have been reported in experimentally induced diabetes and in diabetic patients. Hyperglycemia may be directly responsible for a vitamin C deficit. Exposure to glucose may inactivate antioxidant enzymes and impair the intracellular regeneration of vitamin C by removing reducing equivalents in the form of NADPH for the polyol pathway (59).

Nearly all studies demonstrated significantly diminished levels of vitamin C in the plasma of NIDDM. The reduction was also observed in IDDM and in borderline fasting blood glucose, and it was more pronounced when diabetic complications occurred in addition (Table 4). The weighed mean values are very different for control subjects and NIDDM (control subjects  $65.0 \pm 7.8 \mu\text{mol/L}$ , NIDDM  $46.8 \pm 4.6 \mu\text{mol/L}$ ).

## V. CONCLUSION

The epidemiological data of this chapter refer to diabetic patients and control persons who are not supplemented with antioxidant vitamins and who do not

take antioxidative medications. There are many reports showing that increased dietary intake or supplementation with vitamins E, A, and C can largely increase the plasma levels of the antioxidant vitamins, with positive consequences concerning metabolic control and late complications of diabetes.

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# 6

## Oxidative Stress in Diabetes

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### I. DEFINING OXIDATIVE STRESS

The first step in addressing the role of oxidative stress (OxS) in diabetic complications is to define OxS. It is often defined as a shift in the pro-oxidant–antioxidant balance in the pro-oxidant direction. This definition of OxS is more descriptive than quantitative and chemical in nature. Philosophically, it implies a null point, a balance point at which there is no OxS—OxS occurs only when the balance is shifted toward the pro-oxidant direction. There is a conceptual flaw in this definition because it fails to recognize that OxS is a constant feature of biological systems. Peroxides, superoxide, hydroxyl radicals, and other reactive oxygen species (ROS), the mediators of OxS, are being formed continuously in the body and always exist at some steady-state concentration. The resulting oxidative damage to protein, DNA, and other biomolecules is a ubiquitous and universal consequence of life under aerobic conditions.

OxS might be better defined as “a measure of the prevailing level of ROS in a biological system.” This definition acknowledges the continuous presence of ROS in biological systems, at some level determined by the relative rates of their formation and consumption. It accepts OxS as a normal feature of cellular metabolism rather than a disturbance in an equilibrium—OxS waxes and wanes but never disappears from the biological scene. Like metabolites, levels of ROS may differ at different stages in the feeding–fasting and diurnal cycles, among different subcellular compartments, among different cell types, in a cell at different stages in its growth and development, and

even among cells of the same type but in different regions of a tissue. ROS are mediators of hormone action and growth factor and cytokine activity, and variations in ROS concentrations and OxS in intracellular and extracellular environments appear to be a central feature of regulatory biology (1).

From a quantitative viewpoint, OxS may be considered the sum of the products of the concentration and reactivity of numerous ROS in the cell. Should cells in which redox coenzyme systems are off-balance or in which reduced glutathione (GSH) is depleted also be considered oxidatively stressed? This is not a trivial question because alterations in ascorbate or GSH homeostasis are often cited as evidence of OxS. However, even a poor defense may be adequate in the absence of an oxidative challenge. Persons with glucose 6-phosphate dehydrogenase deficiency, for example, are asymptomatic until they are challenged by drugs or infection, leading to hemolytic anemia. Thus, a shift in the set point or concentration of redox coenzymes in a cell may predispose to oxidative stress, but the perturbation per se does not necessarily indicate that the cell is oxidatively stressed.

At this time it is not possible to quantify OxS, but this may eventually be achievable. The total radical antioxidant potential (TRAP) of plasma can now be estimated, for example, as the sum of a variety of antioxidant concentrations in plasma, including ascorbate, tocopherols, uric acid, and protein (2). Plasma TRAP is commonly expressed relative to that of a concentration of an antioxidant standard, such as Trolox (3). It may eventually be possible to develop a standard, such as "H<sub>2</sub>O<sub>2</sub> equivalents" for assessing OxS in cells and tissues or actually to measure the concentration of specific oxyradicals by electron paramagnetic resonance spectroscopy (4).

Because of the many components and factors affecting OxS (Fig. 1), it is difficult, if not impossible, to assess the overall status of OxS in a biological system by measurement of the status of an individual or even several enzymes or antioxidant systems. Indeed, the interpretation of these data is often gratuitous. A low level of antioxidant enzyme is often interpreted as evidence of OxS, but high levels of superoxide dismutase are associated with OxS in the lungs in response to hyperbaric oxygen. Similarly, high plasma levels of uric acid are associated with inflammation in gout, whereas high levels of ascorbic may be pro-oxidant in the presence of free or heme iron.

In the absence of unambiguous assays or standards for measuring OxS, measurement of the consequences of OxS has been used as a surrogate. One approach for assessing the status of OxS is to measure the rate of excretion of products of oxidation of DNA, such as thymi(di)ne glycol or 8-oxodeoxy-guan(os)ine (5,6). Another is to measure the level of activation or expression of protein kinases, activator protein-1 or nuclear factor kappa B, growth factors (transforming growth factor- $\beta$ , insulin-like growth factor-1, and vascular en-

**Oxidative Stress**

**a measure of the steady state level  
of reactive oxygen species**



**Pro-Oxidant**

- Hyperbaric oxygen
- Metals overload
- decompartmentalization
- Metabolic
  - hyperglycemia
  - glycation & AGEs
  - polyol pathway activity
- Immunological
  - inflammation
  - Complement activation
  - autoimmune disease
  - phagocytosis
  - NADPH oxidase
  - myeloperoxidase
- Drugs & Xenobiotics
  - smoking
  - alcohol

**Anti-Oxidant**

- Antioxidant enzymes
  - SOD, CAT, GPx
- Antioxidant vitamins
  - A, C, E
- Other antioxidants
  - bilirubin
  - glutathione
  - taurine
  - ubiquinol
  - urate
- Metal sequestration
  - albumin
  - transferrin
  - ferritin
  - hemopexin
- Dietary factors
  - flavonoids
  - micronutrients
  - selenium

**Figure 1** Some factors determining the status of OxS in biological systems.

dothelial growth factor) or heme oxygenase, all of which are involved in the response to oxidative stress (1,7) but also respond to other stresses such as reductive, thermal (heat/cold), and osmotic shock. A third approach, the focus of our research and of this article, is the measurement of the extent of oxidative damage to long-lived proteins, such as collagen, which integrates the time-averaged ambient level of OxS.

**II. NATURE OF OXIDATIVE DAMAGE**

Oxidative damage to protein may be divided chemically into primary and secondary damage (Table 1). Primary damage results from direct reaction of pro-

**Table 1** Biomarkers of Oxidative Stress

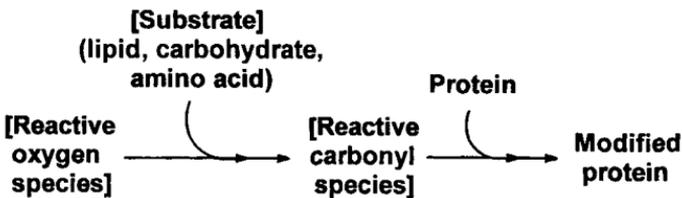
Precursors: Reactive Oxygen Species			
H <sub>2</sub> O <sub>2</sub> , O <sub>2</sub> <sup>-•</sup> , OOH <sup>•</sup> , OH <sup>•</sup> , HOCl, ONOOH, metal:oxo complexes			
Primary Products of Oxidation of Protein			
Class	Example		
Aromatic	<i>o</i> -Tyrosine, dityrosine, chlorotyrosine nitrotyrosine, dityrosine, dihydroxyphenylalanine		
Sulfhydryl	Protein disulfides, methionine sulfoxide		
Amino acid hydroperoxides	Valine, leucine, isoleucine		
Protein carbonyls	Oxohistidine, adipic semialdehyde		
Secondary Products of Oxidation of Protein			
	Lipid	Carbohydrate	Mixed
Precursor	MDA-Lys	Pentosidine	CML, CEL
Products	HNE-(Lys, His, Cys)	Crosslines	GOLD, MOLD
	Pyrroles	Vesperlysines	Argpyrimidine

teins with ROS. Products of these reactions include stable compounds, isolable by acid hydrolysis of proteins, such as *o*-tyrosine and methionine sulfoxide (MetSO). Some products, such as nitrotyrosine and chlorotyrosine, provide insight into the source of the damage, peroxyxynitrite and HOCl, respectively. Other products, such as protein carbonyls, may be derived from several sources, are unstable to acid hydrolysis, and may be transient but important intermediates in the cell, e.g., in marking proteins for turnover (8).

Secondary oxidative damage results from reaction of proteins with products of oxidation of small molecules, including lipids, carbohydrates, and amino acids. The intermediates in this process are reactive carbonyl and dicarbonyl compounds, such as malondialdehyde,  $\alpha$ ,  $\beta$ -unsaturated and hydroxyaldehydes, glyoxal, and methylglyoxal (MGO), which react with nucleophilic groups on protein to form lipoxidation (9) and glycoxidation (10,11) products (Table 1). Lipoxidation products require oxidation (peroxidation) for their formation from lipids, whereas glycoxidation products are a subclass of advanced glycation end products (AGEs), requiring autoxidation chemistry (oxidation by molecular oxygen) for their formation from reducing sugars or ascorbate. Some AGEs (e.g., pyrroline and imidazolones formed by reaction of 3-deoxyglucosone [3DG] with lysine and arginine residues in protein) do not require oxidation for their formation from reducing sugars. These AGEs are useful indicators of nonoxidative chemical modification of proteins.

Some secondary oxidation products, such as carboxymethyllysine (CML), carboxyethyllysine (CEL), pentosidine (12,13), and vesperlysines (14), are stable to acid hydrolysis, whereas others, such as crosslines (15) and the malondialdehyde and 4-hydroxynonenal adducts to lysine (MDA-lysine, HNE-lysine) (16), are labile. Some are characteristic of lipid peroxidation, such as MDA-Lys and HNE adducts to Lys, His, and Cys, whereas pentosidine, vesperlysines and crosslines are derived exclusively from carbohydrates. CML and CEL are more general markers that may be formed after oxidation of carbohydrates, lipids, or amino acids (17,18). Some of these compounds, such as CML, CEL, and the imidazolium salts, glyoxal- and methylglyoxal-lysine dimer (GOLD, MOLD) (19), are end products, whereas others, such as MDA and HNE adducts to lysine, may progress to form crosslinks and pyrrole adducts in protein.

Both primary and secondary oxidation products are measured as indicators of OxS. In the several cases in which stable end products are formed, such as *o*-tyrosine, MetSO, CML, CEL, pentosidine, GOLD, and MOLD, the products accumulate with age in long-lived proteins, such as collagens and crystallines (12,13,17,19,20). Because they are oxidation products, these compounds are biomarkers of oxidative damage to protein and should provide insight into levels of oxidative stress. Primary oxidation products are formed directly by reaction of ROS with protein, and their concentration in tissue proteins should provide a direct index of OxS. In contrast, secondary oxidation products should be second-order products of OxS (Fig. 2), that is, their levels in tissues are determined by both the prevailing level of OxS and the ambient concentration of oxidizable substrates.



**Figure 2** Reaction pathway illustrating the role of both prevailing oxidative stress (ROS concentration) and ambient substrate concentration in formation of secondary oxidation products. According to this scheme, the rate of production of reactive carbonyl species, intermediates in the formation of secondary oxidation products, is first order in ROS, first order in substrate, and second order overall. Increases in either ROS or substrate may increase the rate of formation of reactive carbonyl species and secondary oxidation products.

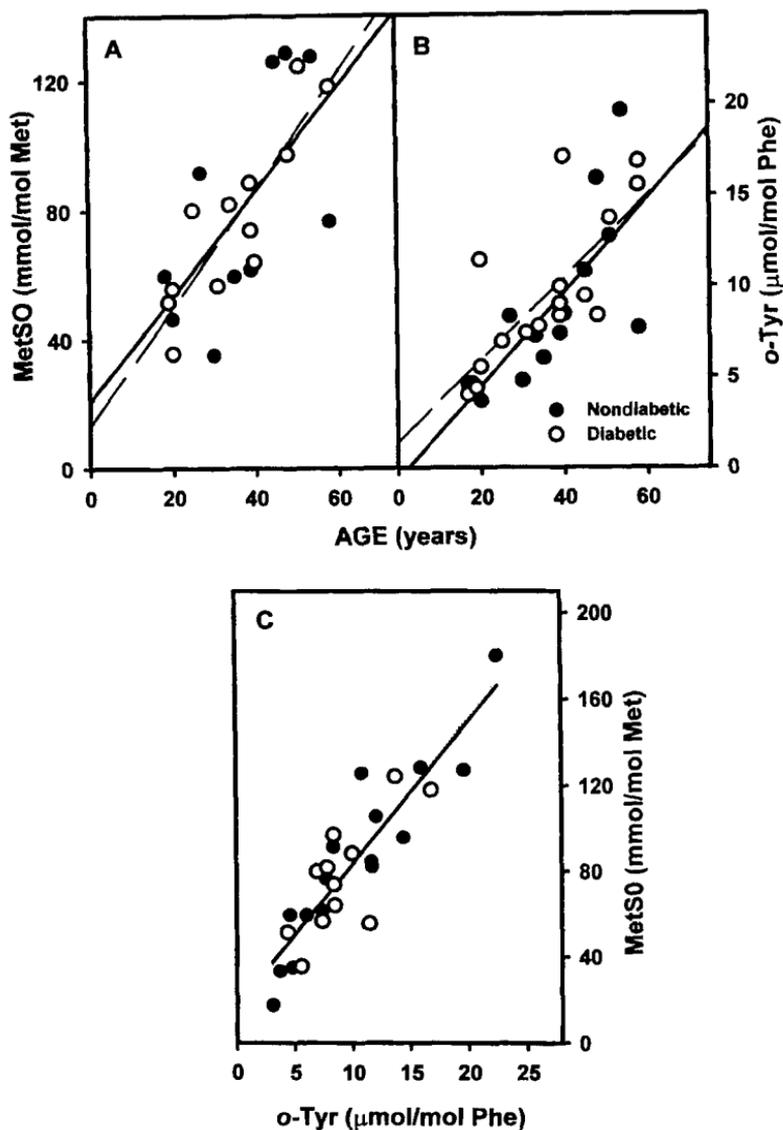
### III. IS OxS INCREASED IN DIABETES?

OxS is a feature of all chronic diseases. Free Radicals in Biology and Medicine (21), one of the early monographs in this field, lists a full page of diseases in which OxS is implicated as a pathogenic agent. OxS is a sign of cellular stress, injury, and apoptosis. At sites of overt pathology, such as in the kidney in diabetes and in plaque formed in atherosclerosis, Alzheimer's disease, and dialysis-related amyloidosis, both primary and secondary biomarkers of OxS are detected together by immunohistochemical techniques (22–28). Although these observations indicate a broad spectrum of oxidative damage to tissue proteins in chronic disease, it is unlikely that OxS is a primary pathogenic mechanism in most chronic diseases. This is most obvious in autoimmune diseases where an errant immunological response underlies the development of pathology. Even in the case of disease induced by environmental agents, such as chronic pulmonary disease, OxS associated with phagocytosis and complement activation is probably the major source of tissue damage. The question about the role of OxS in diabetes is, therefore, not whether OxS is increased but whether OxS is a primary pathogenic mechanism in diabetes.

### IV. WHAT DO CHEMICAL BIOMARKERS OF OxS TELL US ABOUT THE STATUS OF OxS IN DIABETES?

As summarized below and presented in greater detail elsewhere (11), there are several lines of evidence that OxS is not a primary pathogenic mechanism underlying diabetic complications.

1. Age-adjusted levels of the primary oxidation products, *o*-Tyr and MetSO, in skin collagen are not increased in diabetes (Fig. 3) (20).
2. Increases in secondary oxidation (lipoxidation and glycoxidation) products in plasma and tissue proteins and in urine can be explained by increases in substrate concentrations alone, without invoking an increase in oxidative stress (11,13).
3. Increases in lipoxidation and glycoxidation products in plasma are more closely associated with the presence of vascular (29,30) and renal (31) complications rather than diabetes itself, suggesting that OxS is apparent only when advanced tissue damage has occurred.
4. Increased levels of 3DG, a nonoxidative AGE precursor, are ob-



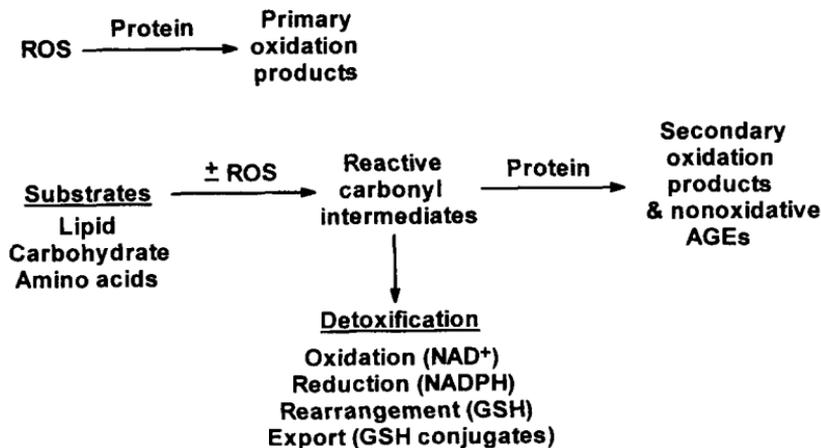
**Figure 3** Age-dependent increase in (A) MetSO and (B) *o*-tyrosine in human skin collagen from diabetic and nondiabetic subjects. Despite significant differences in the absolute concentrations of these biomarkers, there is a strong correlation (C) between levels of these primary oxidation products in skin collagen. (From Ref. 20.)

served in diabetic serum (32,33), suggesting a more generalized increase in carbonyl stress (11) in diabetes.

## V. CARBONYL STRESS IN DIABETES

The increase in lipoxidation and glycoxidation products in diabetes is the direct result of an increase in carbonyl precursors; however, not all of these intermediates are derived from oxidative reactions. 3DG, for example, is formed nonoxidatively from Amadori compounds (34) or fructose-3-phosphate (35) and is also increased in diabetes, along with increases in both pyrrolidine and 3DG-arginine imidazolone (36) adducts that are derived from 3DG by nonoxidative mechanisms. MGO adducts to protein, including CEL and MOLD, are also increased in diabetes (37,38). Like 3DG, MGO is formed by anaerobic mechanisms, either enzymatically as an intermediate in amino acid catabolism or by  $\beta$ -elimination reactions of triose phosphates (39,40). However, MGO may also be produced during the oxidative chemistry of both lipids and carbohydrates, so that its precise origin *in vivo* is unknown. In any case, the increase in 3DG, and possibly MGO, and their adducts to proteins suggests that limitations in the detoxification of reactive carbonyl compounds, produced by both oxidative and nonoxidative mechanisms, underlie the increased chemical modification of proteins in diabetes (Fig. 4).

There are three major routes for detoxification of reactive carbonyl compounds:  $\text{NAD}^+$ -dependent oxidation of aldehydes to carboxylic acids, NADPH-dependent reduction of aldehydes to alcohols, and rearrangement of ketoaldehydes to hydroxyacids. The first pathway is illustrated by the oxidation of HNE to hydroxynonenic acid (41,42) or of 3DG to 2-keto-3-deoxygluconic acid (43), the second by reduction of 3DG to 3-deoxyfructose (44,45), and the third by the GSH-dependent glyoxalase pathway (40). Conjugation of reactive aldehydes to GSH thiohemiacetals by GSH S-transferases is also important in presenting substrates for oxidation by dehydrogenases (42,46), for metabolism of MGO in the glyoxalase pathway (38), or for export of the GSH conjugates from cells (42,47). There is widespread evidence that these pathways are compromised in diabetes as a result of shifts in redox coenzyme systems or that they are overwhelmed by an excess of carbonyl substrates. The shift in the redox potential of the  $\text{NAD}^+ \parallel \text{NADH}$  couple during pseudohypoxia (48,49) may limit  $\text{NAD}^+$ -dependent oxidation reactions, contributing to the increase in diacylglycerol concentration and activation of protein kinase C. Shifts in the  $\text{NADP}^+ \parallel \text{NADPH}$  system as a result of polyol pathway activity



**Figure 4** Reaction scheme illustrating the role of impaired detoxification pathways in increased formation of lipoxidation and glycoxidation products and nonoxidative AGEs in tissues in diabetes. The increase in substrate concentrations and impairment of detoxification systems, rather than increased oxidative stress, is considered the most significant factor contributing to the increased chemical modification of proteins in diabetes.

may limit the efficiency of NADPH-dependent reduction reactions (50), explaining the increase in both 3DG (30,31) and 3-deoxyfructose (51) in plasma of diabetic patients, and decreases in GSH (52). Similarly, the parallel increases in MGO, D-lactate and S-lactoylglutathione (40), in diabetic blood suggests an overload on the glyoxalase pathway in diabetes resulting from a combination of increased substrate flux, rate-limiting GSH production, and excessive GSH-conjugate efflux from tissues.

The combination of increased concentration of oxidizable substrates and decreased efficiency of detoxification pathways can cause an increase in the concentration of reactive carbonyl intermediates, even in the absence of an increase in OxS. The resultant increase in secondary oxidation products (lipoxidation and glycoxidation products) in tissues may appear, at first glance, to be the result of an increase in OxS. However, the increase in secondary oxidation products (lipoxidation and glycoxidation products) (13) without a corresponding increase in primary oxidation products (MetSO and *o*-tyrosine) (20) suggests that this is not the case in diabetes. At least in the early stages of the disease, before the appearance of overt complications, the increase in

secondary oxidation products can be attributed to an increase in oxidizable substrates, coupled with insufficient detoxification activity, without invoking an increase in oxidative stress.

## VI. IS THERE A RATIONALE FOR ANTIOXIDANT THERAPY IN DIABETES?

In principle, all chronic disease should respond to antioxidant therapy. In fact, however, antioxidant therapy has had limited impact on the progress of chronic diseases, such as chronic pulmonary disease, or autoimmune diseases, such as lupus erythematosus or rheumatoid arthritis. There is epidemiological evidence that antioxidants may limit atherogenesis, but alternative therapies, such as antihypertensive and lipid-lowering drugs, are substantially more effective than antioxidant therapy. Antioxidant therapy in autoimmune diseases is also symptomatic therapy, directed at limiting the damage rather than suppressing the pathogenic mechanism. Likewise, antioxidant therapy to address the fulminating OxS characteristic of advanced complications in diabetes may have limited effect in retarding the development of early diabetic complications. However, “antioxidant” therapy with agents such as *N*-acetylcysteine and lipoic (thioctic) acid, which yield an increase in cellular GSH, may provide protection against tissue damage by enhancing detoxification systems for reactive carbonyl compounds. GSH is a bifunctional coenzyme—it supports both antioxidant and other detoxification activities in the cell. It is oxidized (to GSSG) when it functions as a coenzyme for antioxidant reactions (e.g., in reduction of protein disulfides or in detoxification of hydrogen or organic peroxides) catalyzed by GSH peroxidase. However, GSH is regenerated intact when it participates in the glyoxalase pathway or as a substrate for GSH S-transferases. In these instances, it acts as a coenzyme, not as an antioxidant. The therapeutic benefits of *N*-acetylcysteine and lipoic acid therapy may therefore be attributable to their role in enhancing GSH-dependent detoxification rather than antioxidant pathways. At the same time, other antioxidants, such as ascorbate and vitamin E, may spare GSH for use in detoxification functions, providing some protective advantage to cells challenged by increase rates of reactive carbonyl formation in diabetes. The interplay between the antioxidant and carbonyl detoxification roles of GSH is critical for protecting the cell against both oxidative and carbonyl stress.

## VII. SUMMARY AND CONCLUSION

In the foregoing discussion we have tried to define the nature of OxS and then, using that definition, to assess the status of OxS in diabetes. Based on analysis of various biomarkers of OxS in long-lived proteins, we conclude that OxS is not overtly or systemically increased in diabetes, except at later stages in the development of complications. Metabolic derangements in diabetes lead to an increase in concentration of oxidizable substrates and compromised detoxification pathways. The resulting increase in reactive carbonyls in tissues, known as carbonyl stress, leads directly to increased chemical modification of proteins in diabetes. Efforts directed at decreasing substrate concentration (maintenance of euglycemia and normolipidemia), bolstering detoxification pathways (GSH precursors or enhancers), and trapping reactive carbonyl species (AGE inhibitors, carbonyl traps) represent reasonable therapeutic approaches for limiting the chemical modification and crosslinking of proteins in diabetes and inhibiting the development of diabetic complications. Antioxidant vitamins and drugs may spare coenzymes for detoxification pathways during early stages of diabetes and may be useful as supportive therapy at later stages of the disease.

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# 7

## **Antioxidative Defense in Diabetic Peripheral Nerve: Effects of DL- $\alpha$ -Lipoic Acid, Aldose Reductase Inhibitor, and Sorbitol Dehydrogenase Inhibitor**

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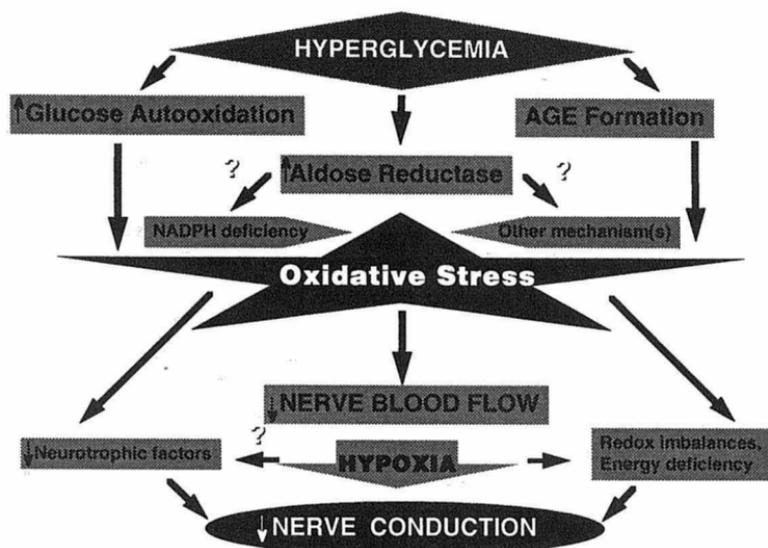
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Diabetes-induced oxidative stress in target tissues for diabetic complications, including peripheral nerve, results from at least three mechanisms (Fig. 1), including glucose autooxidation, formation of advanced glycation end products, and increased aldose reductase (AR) activity (1). The contribution of oxidative stress to peripheral diabetic neuropathy has been well established (1–6). Diabetes-induced oxidative stress leads to decreased endoneurial blood flow with resulting endoneurial hypoxia (1–4,6,7). Increased formation of reactive oxygen species (ROS) impairs neurotrophic support (8) and causes redox imbalances (9), energy deficiency (9), and perhaps defects in ion-transport mechanisms, which theoretically can be both mediated by and be independent of the corresponding changes in nerve blood flow.

Although the role of oxidative stress in diabetes-induced nerve vascular dysfunction and nerve conduction deficits is no longer a subject for debate, there is still a number of questions that need to be addressed. It is still unclear



**Figure 1** Mechanisms and pathogenetic consequences of oxidative stress in diabetic peripheral nerve.

(and no consensus has been reached so far) what the relative contribution of oxidative stress-linked defects in endoneurial blood flow, metabolism, and neurotrophic support to nerve conduction deficits is. It remains to be established whether ROS-induced neurovascular dysfunction and resulting endoneurial hypoxia mediate the diabetes-induced decrease in neurotrophic support as they, at least partially, mediate metabolic defects (10), considering that the levels of substance P (a product of nerve growth factor [NGF]-influenced gene in primary afferents) in the nerve are decreased under hypoxic conditions (11). Mechanisms leading from hyperglycemia to increased ROS formation require further studies as well. In particular, the relative contribution of the three aforementioned ROS-generating mechanisms to diabetes-induced nerve free radical damage remains to be identified. Little information is available on the changes in antioxidative defense enzymes (1) and mechanisms of their downregulation in diabetic peripheral nerve and a possibility of modulation of their activity with antioxidants and other pharmacological interventions. In addition, the role for AR in diabetes-induced changes in nerve antioxidant status needs further studies because reports (2,12) indicate an inconsistency between marked depletion of nerve total glutathione (TG) versus very minor increase in oxidized glutathione (GSSG) in diabetes and restoration of TG levels with

AR inhibitor (ARI) treatment, which points to the contribution of other AR-dependent mechanism(s) in addition to or instead of AR-mediated NADPH deficiency to nerve antioxidant deficit. Also, it remains to be established whether there is any role for the second enzyme of the sorbitol pathway, sorbitol dehydrogenase, in diabetes-induced nerve oxidative injury.

Some of the aforementioned questions were addressed in the present study, which was designed to identify diabetes-related deficits of peripheral nerve antioxidative defense enzymes; to evaluate a role of oxidative stress in impairment of protective mechanisms against superoxide, hydrogen peroxide, and semiquinone radical-induced oxidative injury by assessing a possibility of preventing downregulation of superoxide dismutase, catalase, and total quinone reductase by antioxidant DL- $\alpha$ -lipoic acid; and to compare the effects of ARI and sorbitol dehydrogenase inhibitor (SDI) on parameters of oxidative stress and antioxidative defense in diabetes and thus to identify the role for two enzymes of the sorbitol pathway in diabetes-induced nerve oxidative injury.

## I. MATERIALS AND METHODS

Experiments were performed in accordance with regulations specified by the National Institutes of Health 1985 revised version of the principles of laboratory animal care and University of Michigan Protocol for Animal Studies.

### A. Animals

Barrier-sustained cesarean-delivered male Wistar rats (Charles River, Wilmington, MA), body weight 250–300 g, were fed a standard rat chow diet (ICN Biomedicals, Cleveland, OH) and had ad libitum access to water. Diabetes was induced by a single intraperitoneal injection of streptozotocin (Upjohn, Kalamazoo, MI, 55 mg/kg body weight, IP, in 0.2 mL of 10 mM citrate buffer, pH 5.5) to animals that were fasted overnight. Blood samples for measurements of glucose were taken from the tail vein ~48 h after streptozotocin injection and the day before they were killed. The rats with blood glucose  $\geq 250$  mg/dL were considered diabetics, and the treatments were started ~48 h after streptozotocin injection. Three experiments were performed. In experiment 1, the experimental groups included control, 6-week diabetic, and 6-week diabetic rats treated with DL- $\alpha$ -lipoic acid (Sigma, 100 mg/kg body weight/day, IP, 5 days a week). Experiment 2 was performed in control, 3-week diabetic, and 3-week diabetic rats treated with the SDI-157 (Hoechst

Marion Roussel, 100 mg/kg body weight/day, in the drinking water). In experiment 3, the groups included control, 6-week diabetic, and 6-week diabetic rats treated with the ARI sorbinil (Pfizer, 65 mg/kg body weight/day, in the diet, for 2 weeks, after 4 weeks of untreated diabetes).

## B. Reagents

Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma Chemical (St. Louis, MO). Methanol (high-performance liquid chromatography grade), perchloric acid, hydrochloric acid, and sodium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Ethyl alcohol (200 proof dehydrated alcohol, U.S.P. punctilious) was purchased from Quantum Chemical Company (Tiscola, IL).

## C. Experimental Procedure

Rats from each group were sedated with carbon dioxide and subsequently killed by cervical dislocation. Both nerves were rapidly dissected, carefully blotted with fine filter paper to remove any accompanying blood, and frozen in liquid nitrogen for subsequent biochemical analyses. In experiment 1, we measured levels of sorbitol pathway intermediates (glucose, sorbitol, fructose) and activities of antioxidative defense enzymes, including superoxide dismutase (SOD), catalase, glutathione reductase (GSSGRed), glutathione transferase (GSHTrans), total quinone reductase (TQR), and DT-diaphorase. In experiment 2 and 3, in addition to the above-mentioned sorbitol pathway intermediates and antioxidative defense enzyme activities (except DT-diaphorase), measurements of total MDA and 4-hydroxyalkenal and reduced glutathione (GSH) levels and glutathione peroxidase (GSH-Px) activities were performed.

## D. Biochemical Measurements

### 1. Measurements of Sorbitol Pathway Intermediates

Nerve segments (~20 mg) were weighed and homogenized in 0.8 mL 0.9% NaCl. A 100- $\mu$ L volume of 0.3 M zinc sulfate, followed by an equivalent of barium hydroxide, was then added to homogenate for protein precipitation. The samples were centrifuged at  $4000 \times g$  for 10 min (Sorvall MC 12V), and aliquots of the supernatant were taken for spectrofluorometric measurements of glucose, sorbitol, and fructose by enzymatic procedures, using hexokinase/glucose 6-phosphate dehydrogenase (13), sorbitol dehydrogenase

(14), and fructose dehydrogenase (15). In brief, the analytical mixture for glucose contained 0.9 mL of 1 mM  $MgCl_2$ , 0.5 mM dithiothreitol, 300  $\mu M$  ATP, 5  $\mu M$  NADP, and 0.02 U/mL glucose 6-phosphate dehydrogenase in 25 mM Tris-HCl buffer, pH 8.1, and deproteinized extract (0.1 mL for control and 0.02 plus 0.08 mL  $H_2O$  for diabetic nerves). The reaction was initiated by addition of  $\sim 0.14$  U of hexokinase. Initial and final readings were taken at  $\lambda$  excitation 340 nm,  $\lambda$  emission 460 nm, slits 20 and 15 (spectrofluorometer Perkin-Elmer LS-5B), and were compared with the corresponding glucose standards ( $0.5\text{--}10 \times 10^{-9}$  M) processed in the same run. The analytical mixture for sorbitol contained 0.8 mL 0.5 mM NAD in 0.1 M glycine-NaOH buffer (pH 9.5) and deproteinized extract (0.2 mL for control and 0.05 mL plus 0.15 mL  $H_2O$  for diabetic nerves). The reaction was started by addition of  $\sim 0.8$  U of sorbitol dehydrogenase. Initial and final readings were taken at  $\lambda$  excitation 340 nm,  $\lambda$  emission 460 nm, slits 20 and 15. The analytical mixture for fructose contained 0.9 mL of rezasurin-containing 150 mM citrate buffer, pH 4.5 (1 aliquot of rezasurin [5 mg–10 mL  $H_2O$ ] was mixed with 100 aliquots of citrate buffer), and deproteinized extract (0.1 mL for control and 0.05 mL plus 0.05 mL  $H_2O$  for diabetic nerves). The reaction was started by addition of  $\sim 0.5$  U of fructose dehydrogenase. Initial and final readings were taken at  $\lambda$  excitation 572 nm,  $\lambda$  emission 585, slits 5 and 5. The differences in initial and final readings for tested samples were compared with those with corresponding sets of standards for glucose, sorbitol, and fructose ( $0.1\text{--}10 \times 10^{-9}$  M) processed in the same run.

## 2. Measurements of GSH

Nerve segments ( $\sim 15\text{--}20$  mg) were weighed, homogenized in 1 mL of ice-cold 6%  $HClO_4$ , and centrifuged at  $4000 \times g$  for 10 min. After centrifugation, the samples were immediately neutralized with 5M  $K_2CO_3$  to pH 6–7 and were centrifuged again at  $4000 \times g$  for 5 min to precipitate insoluble  $KClO_4$ . A total of 0.1 mL of extract was mixed with 0.89 mL of 20 mM EDTA in 1.0 M Tris-HCl buffer (pH 8.1), and the reaction was initiated by addition of 0.01 mL of *o*-phthaldialdehyde (10 mg–1 mL methanol). Initial and final readings were taken at  $\lambda$  excitation 345 nm,  $\lambda$  emission 425 nm, slits 5 and 5. The differences in initial and final readings were compared with those in corresponding GSH standards ( $1\text{--}10 \times 10^{-9}$  M) processed in the same run.

## 3. Measurements of Total MDA and 4-Hydroxyalkenal

Measurements of total malondialdehyde (MDA) and 4-hydroxyalkenal levels were performed using kits from Oxis International, Inc. (LPO-586 assay). The

method is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole, with MDA and 4-hydroxyalkenals at 45 °C. The samples were prepared by homogenization of preweighed nerve segments (~40 mg) in 1 mL of 20 mM Tris buffer, pH 7.4, containing 5 mM butylated hydroxytoluene a total of 200  $\mu$ L of homogenate was used for measurements of total MDA and 4-hydroxyalkenal levels according to the procedure described in detail in the kit. The absorbance of chromogenic product was measured at 586 nm (spectrophotometer Beckman DU 640) and was compared with the absorbance in corresponding 4-hydroxyalkenal standards.

#### 4. Measurements of Antioxidative Defense Enzyme Activities

For measurements of antioxidative defense enzyme activities, ~30 mg of frozen sciatic nerves were homogenized in 2 mL of ice-cold 0.1 M sodium-phosphate buffer, pH 6.5 (16). Homogenates were centrifuged at  $20,000 \times g$ , and supernatant fraction was used for assays of enzymatic activities and protein content. Protein levels were quantified with the Pierce BCA protein assay kits (Rockford, IL). SOD activity was measured by following spectrophotometrically (at 480 nm) the autooxidation of (–)-epinephrine at pH 10.4 (17). The reaction mixture contained 0.8 mL 50 mM glycine buffer, pH 10.4, and 0.2 mL supernatant. The reaction was started by addition of 0.02 mL of (–)-epinephrine (due to poor solubility of (–)-epinephrine in water, the solution was prepared by suspending 40 mg of the compound in 2 mL water and then by adding two to three drops of 2 N HCl). SOD activity was expressed as nmol of (–)-epinephrine protected from oxidation after addition of the sample compared with the corresponding readings in the blank cuvette. The molar extinction coefficient of  $4.02 \text{ mM}^{-1} \text{ cm}^{-1}$  was used for calculations. Catalase activity was measured by following spectrophotometrically (at 240 nm) for 5 min the decrease in absorbance of hydrogen peroxide after addition of 0.1 mL of supernatant to 0.9 mL of  $\text{H}_2\text{O}_2$ -containing 50 mM phosphate buffer, pH 6.8. The enzyme activity was calculated using  $2.04 \text{ mM}^{-1} \text{ cm}^{-1}$  as molar extinction coefficient. GSHTrans activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was measured according to Habig et al. (18). A total of 0.8 mL of the reaction mixture contained 0.1 M sodium-phosphate buffer, pH 6.5, 1 mM GSH, 1 mM CDNB (preliminary dissolved in ethanol), and 1 mM EDTA. The reaction was started by addition of 0.2 mL supernatant and was monitored spectrophotometrically at 340 nm for 5 min. Calculations were performed using extinction coefficient of  $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ . GSSGRed activity was measured spectrophotometrically at 340 nm by monitoring NADPH oxidation coupled to reduction of GSSG to GSH; 0.8 mL of the reaction mixture contained 0.1 M

potassium-phosphate buffer, pH 7.0, 2.5 mM GSSG, and 125  $\mu$ M NADPH. The reaction was started by addition of 0.2 mL of the sample and was monitored for 5 min. The calculations were performed by using a molar extinction coefficient of 6.22  $\text{mM}^{-1} \text{cm}^{-1}$ . Total quinone reductase and DT-diaphorase activities were measured with *p*-benzoquinone as a substrate and Twin-20 as an activator as described in detail by Ernster et al. (19).

## E. Statistical Analysis

The results are expressed as means  $\pm$  SD. In experiment 1, differences among experimental groups were determined by ANOVA, and the significance of differences between these groups assessed by the Student-Newman-Keuls multiple range test. Significance was defined at  $p = 0.05$ . In experiments 2 and 3, analysis of the parameters was performed (on natural logarithm transformed data) with the SAS general linear models procedure. Overall differences among experimental groups for each parameter were first assessed by the Van der Waerden test; individual pairwise comparisons were evaluated by least-square means analysis only if the Van der Waerden test was significant at  $p < 0.05$  for a given parameter. A nonparametric Blom transformation of all data was performed before assessment of individual pairwise group differences. Uncorrected  $p$  values based on two-tailed tests of significance are shown for the relevant comparisons.

## II. RESULTS

The body weights were lower in diabetic rats compared with those in controls (experiment 1,  $327.3 \pm 50.9$  vs.  $420.4 \pm 27.0$  g,  $p < 0.01$ ; experiment 2,  $313.6 \pm 25.7$  vs.  $372.8 \pm 20.8$  g,  $p < 0.05$ ; and experiment 3,  $300.1 \pm 44.1$  vs.  $428.3 \pm 17.9$  g,  $p < 0.01$ ). The initial body weights were similar in control and diabetic groups in all three experiments (not shown). No statistically significant difference was found between body weights in diabetic rats treated with DL- $\alpha$ -lipoic acid ( $305.5 \pm 32.0$  g), SDI ( $308.5 \pm 32.6$  g), or ARI ( $307.8 \pm 65.2$  g) and the corresponding untreated groups.

Blood glucose levels were markedly increased in diabetic rats compared with those in controls (experiment 1,  $340.0 \pm 60.3$  vs.  $66.8 \pm 9.3$  mg/dL; experiment 2,  $334.1 \pm 62.3$  vs.  $71.4 \pm 10.5$  mg/dL; and experiment 3,  $326.3 \pm 42.7$  vs.  $58.2 \pm 5.0$ ;  $p < 0.001$  for all three comparisons). Blood glucose levels in diabetic rats were not affected by DL- $\alpha$ -lipoic acid ( $322.6 \pm 43.5$  mg/dL), SDI ( $348.5 \pm 87.0$  mg/dL), or ARI ( $332.7 \pm 38.5$  mg/dL).

**Table 1** The Levels of Glucose, Sorbitol, and Fructose ( $\mu\text{mol/g}$  wet weight) in the Sciatic Nerve of Control and Diabetic Rats Treated With/Without DL- $\alpha$ -Lipoic Acid (LA) ( $n = 7-8$ )

	Control	Diabetic	Diabetic + LA
Glucose	$3.54 \pm 0.69$	$10.46 \pm 2.32^*$	$19.44 \pm 3.79^\ddagger$
Sorbitol	$0.260 \pm 0.091$	$1.34 \pm 0.51^*$	$2.14 \pm 0.88^\ddagger$
Fructose	$2.00 \pm 0.42$	$7.47 \pm 1.23^*$	$10.12 \pm 1.20^\ddagger$

\* Significantly different compared with those in controls ( $p < 0.0001$ ).

†‡ Significantly different compared with those in untreated diabetics ( $p < 0.01$  and  $0.001$ , respectively).

Nerve glucose, sorbitol, and fructose levels in control and diabetic rats treated with/without DL- $\alpha$ -lipoic acid are presented in Table 1. Glucose, sorbitol, and fructose levels in diabetic rats were increased 3.9-, 19.6-, and 6.4-fold, respectively, compared with those in controls. DL- $\alpha$ -Lipoic acid treatment further increased levels of glucose, sorbitol, and fructose (1.9-, 1.6-, and 1.4-fold, respectively, compared with those in untreated diabetics).

Nerve glucose, sorbitol, and fructose levels in control and diabetic rats treated with/without SDI are presented in Table 2. Glucose, sorbitol, and fructose levels in diabetic rats were increased 3.6-, 11.1-, and 5.5-fold, respectively, compared with those in controls. Glucose levels were indistinguishable in diabetic rats treated with/without SDI. Sorbitol levels in the SDI-treated diabetic rats were increased 4.4-fold compared with those in untreated diabetics, whereas fructose levels were markedly reduced but not completely normalized.

**Table 2** The Levels of Glucose, Sorbitol, and Fructose ( $\mu\text{mol/g}$  wet weight) in the Sciatic Nerve of Control and 3-Week Diabetic Rats Treated With/Without SDI ( $n = 7-8$ )

	Control	Diabetic	Diabetic + SDI
Glucose	$3.24 \pm 0.94$	$11.78 \pm 4.17^*$	$11.32 \pm 2.78^\ddagger$
Sorbitol	$0.149 \pm 0.034$	$1.65 \pm 0.36^*$	$7.34 \pm 1.77^\ddagger$
Fructose	$1.22 \pm 0.15$	$6.66 \pm 2.10^*$	$1.73 \pm 0.77^\ddagger$

\* Significantly different compared with those in controls ( $p < 0.0001$ ).

†‡ Significantly different compared with those in untreated diabetics ( $p < 0.01$  and  $0.001$ , respectively).

**Table 3** The Levels of Glucose, Sorbitol, and Fructose ( $\mu\text{mol/g}$  wet weight) in the Sciatic Nerve of Control and 6-Week Diabetic Rats Treated With/Without ARI ( $n = 7-8$ )

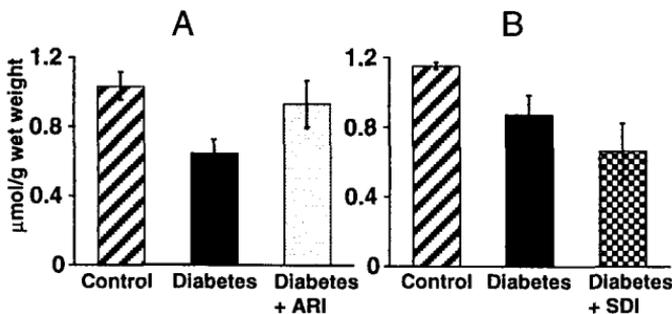
	Control	Diabetic	Diabetic + ARI
Glucose	$3.58 \pm 0.44$	$13.43 \pm 2.44^*$	$13.54 \pm 1.78^\dagger$
Sorbitol	$0.128 \pm 0.045$	$1.36 \pm 0.37^*$	$0.062 \pm 0.042^\ddagger$
Fructose	$1.29 \pm 0.40$	$6.54 \pm 0.19^*$	$1.21 \pm 0.33^\ddagger$

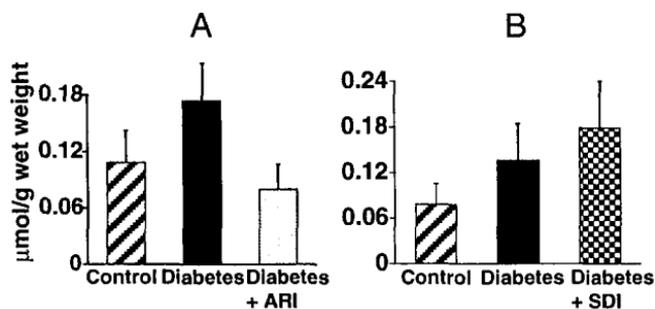
\* Significantly different compared with those in controls ( $p < 0.0001$ ).

‡ Significantly different compared with those in untreated diabetics ( $p < 0.01$  and  $0.001$ , respectively).

Nerve glucose, sorbitol, and fructose levels in control and diabetic rats treated with/without ARI are presented in Table 3. Glucose, sorbitol, and fructose levels in diabetic rats were increased 3.8-, 10.6-, and 5.1-fold over those in controls. Glucose levels in diabetic rats were not affected by the ARI treatment, whereas sorbitol levels were decreased below those in nondiabetic controls and fructose levels were normalized.

Nerve total MDA and 4-hydroxyalkenal levels in control and diabetic rats treated with/without ARI and SDI are presented in Fig. 2, A and B. Total MDA and 4-hydroxyalkenal levels were increased in both 3-week diabetic rats and 6-week diabetic rats vs. controls ( $p < 0.02$  and  $< 0.001$ , respectively). The increase in 6-week diabetic rats was completely corrected by the ARI treatment ( $p < 0.001$  vs. untreated diabetics), whereas the increase in 3-week

**Figure 2** Total MDA and 4-hydroxyalkenal levels in sciatic nerve of control and diabetic rats treated with or without ARI (A) or SDI (B) (mean  $\pm$  SD,  $n = 8-12$ ).



**Figure 3** GSH levels in sciatic nerve of control and diabetic rats treated with or without ARI (A) or SDI (B) (mean  $\pm$  SD,  $n = 8-12$ ).

diabetic rats further progressed with the SDI treatment ( $p < 0.03$  vs. untreated diabetics)

Nerve GSH levels in control and diabetic rats treated with/without ARI and SDI are presented in Fig. 3, A and B. GSH levels were decreased by 23.4% and 37.6% in 3-week and 6-week diabetic rats vs. corresponding controls ( $p < 0.002$  and  $< 0.001$ , respectively). The decrease in 6-week diabetic rats was completely corrected by the ARI treatment ( $p < 0.001$  vs. untreated diabetics), whereas the decrease in 3-week diabetic rats further progressed with the SDI treatment ( $p < 0.02$  vs. untreated diabetics).

Antioxidative defense enzyme activities in control and diabetic rats treated with/without DL- $\alpha$ -lipoic acid are presented in Table 4. SOD, catalase,

**Table 4** Antioxidative Enzyme Activities in the Sciatic Nerve of Control and 6-Week Diabetic Rats Treated With/Without DL- $\alpha$ -Lipoic Acid (LA) (nmol/mg protein per min,  $n = 5-8$ ).

	Control	Diabetic	Diabetic + LA
SOD	95.9 $\pm$ 18.9	70.5 $\pm$ 9.8*	136.8 $\pm$ 55.8†
Catalase	109.7 $\pm$ 28.6	77.4 $\pm$ 19.8*	122.9 $\pm$ 31.5‡
GSHTrans	39.6 $\pm$ 15.9	57.7 $\pm$ 12.9	56.3 $\pm$ 19.8
GSSGRed	9.0 $\pm$ 2.6	10.5 $\pm$ 3.0	12.0 $\pm$ 3.6
TQRed	174.9 $\pm$ 29.7	131.2 $\pm$ 32.8*	170.4 $\pm$ 44.2†
DT-diaphorase	119.7 $\pm$ 25.8	123.8 $\pm$ 30.8	119.7 $\pm$ 30.9

\* Significantly different compared with those in controls ( $p < 0.05$ ).

†‡ Significantly different compared with those in untreated diabetics ( $p < 0.05$  and  $< 0.01$ ).

**Table 5** Antioxidative Enzyme Activities in the Sciatic Nerve of Control and 3-Week Diabetic Rats Treated With/Without SDI (nmol/mg protein per min,  $n = 8-10$ )

	Control	Diabetic	Diabetic + SDI
SOD	50.8 ± 17.8	73.0 ± 26.4	93.0 ± 31.8*
Catalase	194.6 ± 56.6	195.2 ± 50.2	300.1 ± 82.3*†
GSHTrans	39.3 ± 4.9	31.5 ± 2.8‡	30.4 ± 3.6§
GSSGRed	21.6 ± 3.5	18.3 ± 2.6	16.5 ± 2.5‡
GSH-Px	13.7 ± 3.6	9.8 ± 4.6	17.1 ± 8.6†
TQRed	313.9 ± 52.3	236.8 ± 22.5	193.5 ± 39.2‡¶

\*‡§|| Significantly different compared with those in controls ( $p < 0.05$ ,  $<0.01$ ,  $<0.001$ , and  $<0.0001$ , respectively).

†¶ Significantly different compared with those in untreated diabetics ( $p < 0.05$  and  $<0.01$ , respectively).

and TQRed activities were decreased in 6-week diabetic rats vs. controls, whereas GSHTrans, GSSGRed, and DT-diaphorase activities were indistinguishable between the two groups. DL- $\alpha$ -Lipoic acid treatment prevented diabetes-induced downregulation of SOD, catalase, and TQRed and did not affect GSHTrans, GSSGRed, and DT-diaphorase.

Antioxidative defense enzyme activities in control and 3-week diabetic rats treated with/without SDI are presented in Table 5. SOD, catalase, GSH-

**Table 6** Antioxidative Enzyme Activities in the Sciatic Nerve of Control and 6-Week Diabetic Rats Treated With/Without ARI (nmol/mg protein per min,  $n = 8-10$ )

	Control	Diabetic	Diabetic + ARI
SOD	89.7 ± 22.5	64.7 ± 8.5*	76.9 ± 11.6†‡
Catalase	102.0 ± 37.1	71.6 ± 13.4*	76.8 ± 17.5*
GSHTrans	33.8 ± 4.5	44.5 ± 10.8	36.2 ± 4.4
GSSGRed	13.1 ± 1.9	12.2 ± 2.5	12.1 ± 2.5
GSH-Px	11.2 ± 2.2	13.0 ± 4.8	11.3 ± 1.7
TQRed	197.8 ± 64.4	134.6 ± 38.2*	160.4 ± 25.3§

\*†‡ Significantly different compared with those in controls ( $p < 0.05$ ,  $<0.01$ , and  $<0.001$ , respectively).

‡|| Significantly different compared with those in untreated diabetics ( $p < 0.05$  and  $<0.01$ , respectively).

Px, and GSSGRed activities were similar in control and 3-week diabetic rats. SOD, catalase, and GSH-Px were increased in SDI-treated diabetic rats compared with untreated diabetic group, whereas GSSGRed activity was slightly decreased. TQRed and GSHTrans were decreased in 3-week diabetic rats vs. controls, and TQRed but not GSHTrans activity was further decreased by the SDI treatment.

Antioxidative defense enzyme activities in control and 6-week diabetic rats treated with/without ARI are presented in Table 6. Similar to experiment 1, SOD, catalase, and TQRed activities were decreased in the diabetic group vs. controls, whereas GSH-Px, GSSGRed, and GSHTrans remained unaffected. The ARI treatment partially corrected SOD and TQRed but not catalase activity. GSH-Px, GSSGRed, and GSHTrans activities were indistinguishable between the SDI-treated and untreated diabetic groups.

### III. DISCUSSION

The findings of the present study are indicative of increased vulnerability of diabetic peripheral nerve to free radical-induced oxidative damage and are consistent with the studies of Low and colleagues (1,20,21), demonstrating accumulation of conjugated dienes and lipid peroxide, reduction of GSH levels and Cu,Zn-SOD activity, and changes of other markers of oxidative stress in nerve in the model of streptozotocin-induced diabetes. Comparison of Tables 5 and 6 suggests that antioxidative defense enzyme activities, especially those of SOD, catalase, GSSGRed, and TQRed, are age-dependent [consistent with observations of age dependence for other parameters related to oxidative stress (20)]. Also, it is important to point out that diabetes-induced changes in most parameters in the present study revealed strong dependence on the duration of diabetes. For example, depletion of GSH, the major biological antioxidant, progressed with the duration of diabetes, being more advanced in 6-week than in 3-week diabetic model. SOD, catalase, and TQRed activities remained within the normal range in 3-week diabetic rats but were markedly reduced in 6-week diabetic rats, whereas, on the contrary, GSHTrans deficit appeared to be transient and was present in the 3-week but not in the 6-week model of streptozotocin diabetes. Another important characteristic of diabetes-induced impairment of antioxidative defense is its selectivity, manifested by deficits in SOD, catalase, and TQRed coexisting (in 6-week diabetes) with normal GSSGRed, GSHTrans, and diaphorase activities. Although the significance of these changes for diabetes-induced increased ROS production and antioxidative defense against certain free radical species still remains to be established,

the finding of the diabetes-induced deficit in TQRed together with normal DT-diaphorase activity [DT-diaphorase represents a cytoplasmic component of TQRed [19]] is probably indicative of increased attack of semiquinone free radicals (16) (very strong reducing agents that can also rapidly react with molecular oxygen generating the superoxide anion radical,  $O_2^-$ ) in mitochondria and is consistent with the concept that mitochondria are a primary target for oxidative damage (1). It is also interesting that only those enzymes that are affected by diabetes respond to antioxidant (DL- $\alpha$ -lipoic acid) treatment.

The latter observation is consistent with the report of Maitra et al. (22) that demonstrated restoration of buthionine sulfoximine-induced catalase and GSH-Px deficits with  $\alpha$ -lipoic acid treatment and with studies in other models of oxidative stress (23,24), suggesting a possibility of posttranslational down-regulation of some antioxidative defense enzymes by oxidative modification of their proteins. The concept of posttranslational regulation of antioxidative defense enzymes in diabetic peripheral nerve is supported by recent unpublished findings of Phillip Low's laboratory demonstrating the lack of any effect of streptozotocin-diabetes of different duration on Cu,Zn-SOD, Mn-SOD, GSSGRed, and GSH-Px mRNAs. The finding of a marked upregulation of SOD by  $\alpha$ -lipoic acid in diabetic peripheral nerve in the present study is different from reports for other tissues in other models of oxidative stress (22,25), demonstrating the lack of correcting effect of  $\alpha$ -lipoic acid on SOD activity. Based on this apparent discrepancy, it is possible to suggest the indirect modulation of SOD by  $\alpha$ -lipoic acid in diabetic peripheral nerve, probably via NGF, which is known to both upregulate and replace SOD in some types of oxidative injury (26,27). This assumption is supported by the findings of decreased NGF levels in diabetic peripheral nerve and of partial prevention of diabetes-induced deficit in neurotrophic support with DL- $\alpha$ -lipoic acid treatment (28).

The elevated levels of total MDA and 4-hydroxyalkenals (products of lipid peroxidation) are consistent with the presence of peripheral nerve oxidative injury in both 3-week and 6-week streptozotocin-diabetic rat models. As MDA levels were reported (20) to be similar in control and diabetic rats (regardless of duration of diabetes), the increase in lipid aldehyde level in the present study probably reflects the accumulation of 4-hydroxy-2,3-transnonenal (HNE), a major toxic product of lipid peroxidation (29). Interestingly and surprisingly, the lipid aldehyde level is normalized by ARI despite the fact that AR has been reported to be involved in HNE metabolism (29). A possible explanation can be derived from a comparison of the results of the present study with the recent report of Srivastava et al. (30) for isolated perfused hearts from nondiabetic rats, indicating that the major metabolic transformations of HNE involve conjugation with glutathione and oxidation to 4-hydroxy-2-non-

enoic acid and providing evidence that sorbinil does not affect formation of glutathione-HNE conjugates (at the same time, preventing their further AR-mediated reduction to glutathione-1,4-dihydroxy-2-nonene) and stimulates HNE oxidation to 4-hydroxy-*trans*-2-nonenoic acid. Based on these findings and assuming that the pathways of HNE metabolism are similar in heart and peripheral nerve, one would expect to find an accumulation of HNE in peripheral nerve under diabetic conditions [characterized by depletion of GSH (2, and in the present study) and by a decrease in the free cytosolic NAD<sup>+</sup>/NADH ratio (9,31) that may affect NAD-dependent oxidation of HNE to 4-hydroxy-*trans*-2-nonenoic acid by aldehyde dehydrogenase], which is consistent with the results of the present study. Also, taking into consideration that ARI treatment corrects both GSH levels (12, and in the present study) and the redox state of free cytosolic NAD-couple (31), the metabolic basis for prevention of total MDA and 4-hydroxyalkenal accumulation by sorbinil treatment becomes understandable.

The importance of GSH for neutralization of toxic products of lipid peroxidation is confirmed by the experiments with SDI, which effectively (by ~90.8%) inhibited increased flux through sorbitol dehydrogenase in the 3-week streptozotocin-diabetic rat model. GSH depletion in 3-week streptozotocin-diabetic rats was further exacerbated by the SDI treatment, which is consistent with a further accumulation of HNE over the level in the untreated diabetic group. A more advanced GSH depletion in the SDI-treated diabetic rats compared with the corresponding untreated group is consistent with the findings of Geisen et al. (32) for diabetic lens. These findings implicate sorbitol accumulation-linked osmotic stress in the mechanisms underlying increased vulnerability of diabetic peripheral nerve to oxidative injury and are in accordance with other reports demonstrating the inconsistency between a substantial (~40%) depletion of GSH versus a very minor (2) or an absent (12) depletion of GSSG, which calls into question the importance of glutathione redox cycling mechanism in diabetes-induced GSH depletion. In addition, total glutathione depletion in the diabetic nerve and correction of both total and reduced glutathione levels by ARIs (12, and in the present study) points to the involvement of other AR mediated mechanisms in addition to (or instead of) NADPH deficiency to nerve GSH deficit. It is important to point out that involvement of the osmotic factor in GSH depletion in the diabetic nerve does not necessarily mean increased GSH leakage from the endoneurium (although some studies for the lens (33) implicated decreased amino acid uptake and increased GSH efflux under hypersmotic conditions in the GSH deficit). We suggest that osmotic stress can be involved in diabetes-induced energy deficiency

(9,10) and, considering that ATP is required for both  $\gamma$ -glutamyl cysteine synthetase and glutathione synthetase reactions, contributes to diabetes-related GSH deficit through the impairment of cofactor supply of GSH biosynthesis. The assumption regarding the link between sorbitol accumulation-linked osmotic stress and energy deficiency in diabetic peripheral nerve is supported by observations in DL- $\alpha$ -lipoic acid-treated diabetic rats, demonstrating an increased sorbitol accumulation and the lack of prevention of nerve energy deficiency despite correction of both mitochondrial and cytosolic redox state of NAD couple (9,34) and by the findings of exacerbated decrease of nerve phosphocreatine/creatine ratio (a marker of free cytosolic ATP/ADP ratio) in diabetic rats treated with SDI (CP-166,572, Pfizer, 200 mg/kg/day in the drinking water), which had nerve sorbitol levels exceeding those in untreated diabetics  $\sim$ 6.3-fold (Obrosova, 1996, unpublished observations).

It has been suggested (35) that diabetes-induced increase in flux through sorbitol dehydrogenase causes increase in free cytosolic NADH/NAD<sup>+</sup> ratio (so called "pseudohypoxia"), which in turn contributes to increased ROS formation due to activation of NADH oxidase, a superoxide generating enzyme [studies of NADH oxidase in diabetic tissues reported contradictory results (36–38)]. If this was true, one would expect the SDI treatment to be beneficial on parameters of oxidative injury and antioxidative defense. However, the present study demonstrates that the SDI treatment resulted in an opposite effect, because total MDA and 4-hydroxyalkenal accumulation, GSH depletion, and total quinone reductase deficit in the diabetic nerve were exacerbated with the SDI treatment. Interestingly, nerve SOD, catalase, and GSH-Px activities (which were similar in control and 3-week streptozotocin-diabetic rats) were increased in 3-week streptozotocin-diabetic rats treated with SDI, a phenomenon probably indicative of a compensatory response to the increased attack of ROS of superoxide and hydrogen peroxide due to exacerbated GSH and total quinone reductase deficits.

In conclusion, the increased vulnerability of diabetic peripheral nerve to oxidative injury results from impairment of antioxidative defense mechanisms manifested by decreased GSH levels and SOD, catalase, and total quinone reductase activities. Downregulation of these enzymes under diabetic conditions is caused by ROS (probably posttranslational regulation) and is prevented by antioxidant DL- $\alpha$ -lipoic acid treatment. AR is an important, although not the only, mechanism of diabetes-induced oxidative injury, whereas sorbitol dehydrogenase has a protective role, and its inhibition exacerbates oxidative damage. These findings implicate sorbitol accumulation-linked osmotic stress in nerve antioxidant deficit in diabetes.

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# 8

## Pathways of Glucose-Mediated Oxidative Stress in Diabetic Neuropathy

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Diabetic distal symmetric sensorimotor polyneuropathy (DPN), the most common peripheral neuropathy in developed countries (1–3), affects up to 60–70% of diabetic patients (4) and is the leading cause of foot amputation (5). The typical slowing of nerve conduction and the advancing distal symmetrical sensorimotor deficits are thought to reflect an underlying slowly progressive distal axonopathy of the dying-back type primarily affecting sensory nerve fibers (6). Improved blood glucose control substantially reduces the risk of developing DPN in insulin-dependent (type 1) diabetes (7,8), thereby strongly implicating hyperglycemia as a causative factor.

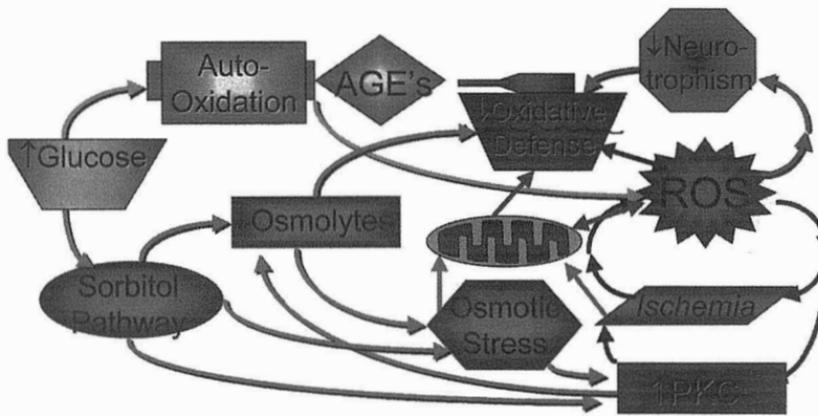
Animal and in vitro experiments have implicated a variety of enzymatic and nonenzymatic metabolic mechanisms in the initiation of glucose-induced neurotoxicity. These “metabolic initiators” include nonenzymatic glycation of proteins with subsequent chemical rearrangements, yielding complex protein adducts known as advanced glycation end products (AGEs) (9,10); auto-oxidation of glucose (11); increased aldose reductase (AR) activity leading to sorbitol and fructose accumulation, NADP-redox imbalances, and alterations in signal transduction (12–14); and activation of protein kinase C (PKC), perhaps due to increased de novo synthesis of diacylglycerol (DAG) from glucose

and inhibition of DAG kinase (15–18). These metabolic initiators are compartmentalized within the rich anatomical complexity and cellular heterogeneity of the peripheral nervous system (PNS) and its supporting vasculature and connective tissue elements. This compartmentalization channels and shapes the physiological response to metabolic initiators into the specific nerve fiber damage and loss underlying DPN. The intervening physiological mediators include interruption of nerve blood flow (NBF) (19–22), mitochondrial dysfunction (19,23), impaired neurotrophic support (24), osmolyte derangements (14), and induction of neuronal and/or Schwann cell apoptosis (25). Combinations and permutations of metabolic initiators, cellular and subcellular compartmentalization, and physiological mediators give rise to the current spectrum of pathogenetic hypotheses for DPN (Table 1).

The intellectual challenge to basic and clinical scientists exploring the pathogenesis of DPN is the identification and characterization of the important initiators, compartments, and mediators common to various pathogenetic hypotheses. These common elements may serve as a linchpins around which to array and perhaps unify otherwise competing pathogenetic mechanisms. Glucose-induced generation of reactive oxygen species (ROS) may subserve this purpose (Fig. 1). Autooxidation of glucose, catalyzed by trace amounts of free transition metals such as iron and copper (26), generate ROS in vitro (27), and metal chelating agents to preserve normal nerve conduction velocity

**Table 1** Putative Metabolic Initiators and Physiological Mediators of Glucose Toxicity in Experimental Diabetic Neuropathy

Metabolic initiators	Tissue compartments	Physiological mediators
Nonenzymatic glycation	Endoneurial microcirculation	Interruption of nerve blood flow
Sorbitol pathway	Perineurial/epineurial vessels	Mitochondrial dysfunction
Glucose autooxidation	Dorsal root/anterior horn neurons	Reduced neurotrophic support
Protein kinase C activation	Myelinated/ unmyelinated axons Schwann cells Perineurial cells Distal motor/sensory projections	Osmolyte derangements



**Figure 1** Generation of ROS from elevated levels of glucose may occur by multiple putative metabolic pathways: nonenzymatic glucose autooxidation and nonenzymatic formation of AGEs (yellow) and activation of the enzymes of the sorbitol pathway by mass action (red). Sorbitol pathway activation produces osmolyte and NADPH depletion that diminish antioxidative defense and produce osmotic stress. Osmotic stress may trigger cellular stress-response mechanisms such as PKC that have been linked to endothelial dysfunction, vasoconstriction, and ischemia. Ischemia and osmotic stress impair mitochondrial function and integrity, which can limit mitochondrial contribution to oxidative defense. Ischemia/reperfusion and impaired oxidative defense further magnify ROS accumulation, vascular function, and mitochondrial integrity. Neurotrophic support that upregulates oxidative defense mechanisms may be impaired by ROS, further compromising oxidative defense.

and NBF in diabetic rats (28) [transition metal handling may be impaired in experimental diabetes (29)] (Fig. 1, yellow). Furthermore, ROS may interconnect autooxidation and AGE formation: ROS accelerate AGE formation and AGEs in turn supply ROS ("autooxidative glycosylation") (30) (Fig. 1, yellow). AGEs generate ROS through a series of complex biochemical and molecular pathways (31–33). Binding of AGEs to their cell surface receptor (RAGE) is associated with activation and nuclear translocation of the transcription factor NF- $\kappa$ B (34), possibly contributing to endothelial dysfunction (35,36) impaired NBF and ischemia (Fig. 1, black).

Activation of the AR pathway has also been linked to ROS generation (Fig. 1, red). Reduction of glucose to sorbitol by AR oxidizes NADPH, directly impairing antioxidative defense (37). Sorbitol accumulation produces osmotic stress, which may promote oxidative stress through depletion of gluta-

thione and other putative antioxidants such as taurine (38). The novel hypothesis that AR pathway activation produces mitochondrial dysfunction through osmotic stress is discussed in this volume. Mitochondrial dysfunction could impair antioxidative defense by diminishing ATP for the de novo synthesis of glutathione (39). AR pathway activation may also contribute to the activation of PKC reported in some (15,16) but not all tissues prone to diabetic complications (total PKC activity is reduced rather than increased by diabetes in rat sciatic nerve (40,41), but selective activation of specific isoforms in some tissue components has been described in diabetic kidney (42) and has not been excluded in diabetic PNS). Increased AR pathway activity could promote de novo DAG synthesis by diverting dihydroxyacetone phosphate toward formation of  $\alpha$ -glycerophosphate or PKC activation through osmotic stimulation of the JNK-kinase cascade. PKC activation would further exacerbate reciprocal osmolyte depletion promoted by sorbitol accumulation by inhibiting the transport activity of the  $\text{Na}^+$ -myoinositol (43) and the  $\text{Na}^+$ -taurine (44) cotransporters. If activation of endoneurial or perineurial vascular PKC promoted vasoconstriction and nerve ischemia, then this would further exacerbate mitochondrial dysfunction through oxygen deprivation (ischemia). Mitochondrial dysfunction, impaired antioxidative defense, and ischemia would all contribute further to the generation of ROS (which would further exacerbate vasoconstriction (Fig. 1) (19).

ROS may interact with diminished neurotrophic support, impaired energy metabolism, and ischemia in experimental DPN (neurotrophism). Oxidative stress induced by diabetes (45) would be particularly injurious to the PNS, which is particularly vulnerable to oxidative stress (46). Impaired neurotrophic support in diabetes (47) may be mediated by ROS (48). ROS contribute to inschemia-reperfusion injury (49). ROS-induced apoptosis may share similar cell death pathways with neurotrophic withdrawal (50,51), and neurotrophins may protect against ROS damage by inducing antioxidative defense mechanisms (52–54). Recent data suggest that antioxidant therapy may ameliorate some aspects of reduced neurotrophic support in experimental DPN (55).

Thus, oxidative stress and ROS link all of the potential initiators, encompass most of the cellular compartments in the PNS, and relate to virtually all physiological mediators implicated in the progressive nerve fiber dysfunction, damage, and loss in DPN (Table 1). In each of these pathogenetic elements, generation of ROS may initiate a feed-forward cycle, because oxidative stress itself impairs antioxidative defense mechanisms (Fig. 1, double-headed black arrow) (56), resulting in a “viscous” cycle of metabolic damage. The role of ROS and oxidative stress in DPN has only recently begun to emerge, and its

potential as a therapeutic target for DPN holds great but as yet unfulfilled promise.

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# 9

## Experimental Diabetic Neuropathy: Oxidative Stress and Antioxidant Therapy

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There is ample evidence of oxidative stress in both experimental (EDN) and human diabetic neuropathy. Most studies have been done on plasma, with limited study on neural tissues. We briefly review and update our studies.

### **I. MECHANISMS OF OXIDATIVE STRESS**

#### **A. Endoneurial Ischemia/Hypoxia**

There is a perfusion deficit of approximately 50% that affects peripheral nerve endoneurium (1) and the parent cell bodies in relevant dorsal root and sympathetic ganglia (2). The onset of ischemia occurs within the first week (3) and is due to a reduction in nutritive rather than arteriovenous flow. There is attendant hypoxia, seen in both experimental (4) and human diabetes (5).

## B. Hyperglycemic Glycation and Autooxidation

Glucose, by a process of autooxidation in the presence of decompartmentalized trace transitional metals, can cause lipid peroxidation (6). We have evaluated the role of hyperglycemia in lipid peroxidation *in vitro*, using an *in vitro* lipid peroxidation model, with an ascorbate-iron-EDTA system. The addition of 20 mM glucose to the incubation medium increased lipid peroxidation four-fold, confirming rapid and marked glucose-mediated autooxidative lipid peroxidation (7). Glucose autooxidation results in the production of protein reactive ketoaldehydes, hydrogen peroxide highly reactive oxidants, and the fragmentation of proteins (free radical mechanisms). Glycation and oxidation are simultaneous and inextricably linked (8).

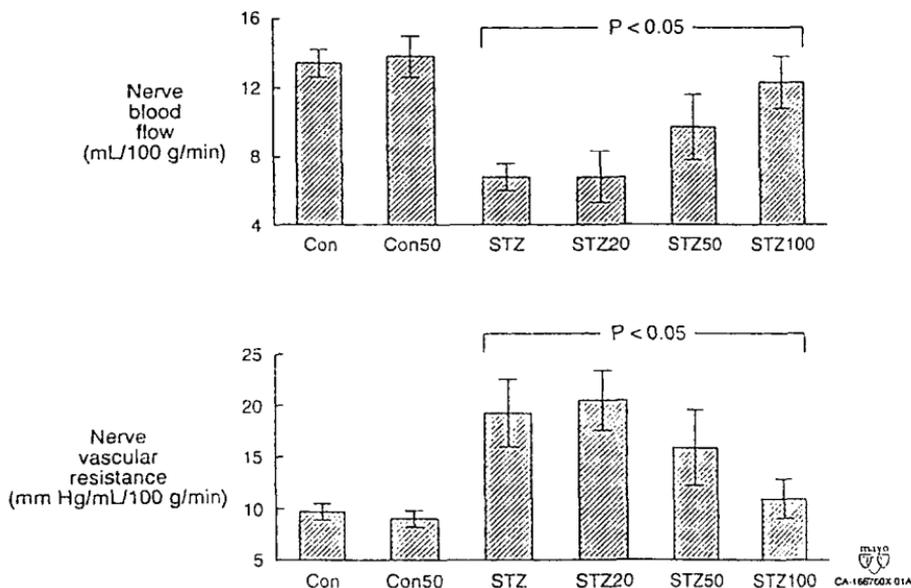
## II. ANTIOXIDANT ENZYMES

Free radical defenses of peripheral nerve are reduced relative to brain and liver, especially involving glutathione (GSH)-containing enzymes (9). Cuprozinc superoxide dismutase (SOD) is reduced in sciatic nerve of experimental diabetic neuropathy, and this reduction is improved by insulin treatment (10). Glutathione peroxidase (GSH-Px) is reported to be further reduced in experimental diabetic neuropathy in alloxan diabetic mice 7–21 days after induction of diabetes, and enzyme activity inversely regresses with glucose level (11). We recently evaluated the gene expression of the antioxidant enzymes, GSH-Px, SOD (cuprozinc [czSOD] and manganese [mnSOD] separately), and catalase (CAT) in L4-L6 dorsal root ganglia (DRG) and superior cervical ganglion (SCG) of rats that had been diabetic for 3 and 12 months (Kishi et al., unpublished data). cDNA fragments for rat GSH-Px, czSOD, mnSOD, CAT, and cyclophilin was obtained by reverse transcriptase polymerase chain reaction of rat DRG RNA using specific primers for each probe and evaluated by Northern blot analysis. We also evaluated GSH-Px activity in sciatic nerve, DRG, and superior cervical ganglion, of these animals. GSH-Px, CAT, czSOD, and mnSOD were not reduced in EDN at either 3 or 12 months. CAT mRNA was significantly increased in EDN more than 12 months. GSH-Px enzyme activity was normal in sciatic nerve. We conclude that gene expression is not reduced in peripheral nerve tissues in EDN. Changes in enzyme activity may be due to posttranslational modifications.

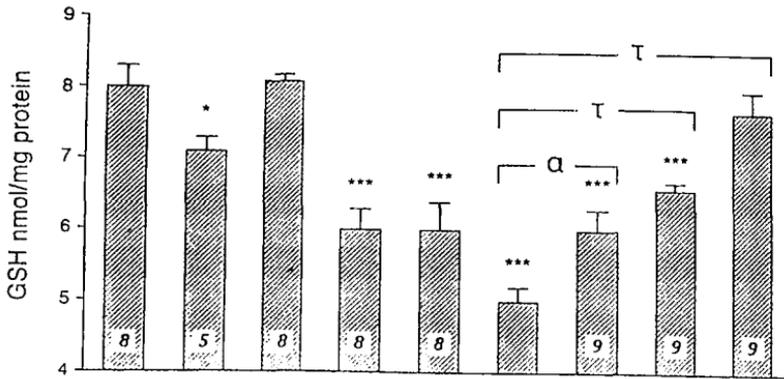
### III. ANTIOXIDANT THERAPY WITH $\alpha$ -LIPOIC ACID IN EDN

Hyperglycemia causes lipid peroxidation of brain and sciatic nerve *in vitro*. We evaluated the effectiveness of the R(+)-, S(-)-enantiomers, and racemate of  $\alpha$ -lipoic acid in reducing thiobarbituric acid reactive substances generation in rat brain and sciatic nerve. Studies were also done in an incubation medium containing 20 mM glucose, which increased lipid peroxidation up to fourfold. A dose-dependent and statistically significant reduction in lipid peroxidation was seen in both tissues with similar potencies for both enantiomers (7).

We also evaluated if lipoic acid will reduce oxidative stress in diabetic peripheral nerve and improve neuropathy *in vivo*, using the model of streptozotocin diabetic neuropathy. End points were nerve blood flow (NBF), electro-



**Figure 1** NBF and nerve vascular resistance of control (Con;  $n = 8$ ), streptozotocin-diabetic neuropathy (STZ;  $n = 5$ ), and animals given lipoic acid supplements at doses of 20 mg/kg (STZ20;  $n = 6$ ), 50 mg/kg (Con50; STZ50;  $n = 5$ ), and 100 mg/kg (STZ100;  $n = 8$ ). Lipoic acid supplementation results in normal flow and nerve vascular resistance. Significance of difference, STZ-supplemented vs. STZ. Bars, SE. (From Ref. 12.)



**Figure 2** Sciatic nerve GSH concentrations in controls (Con) and on restricted caloric intake (Con[R]), streptozotocin diabetic (STZ),  $\alpha$ -tocopherol-depleted (-), and supplemented with lipoic acid at 20, 50, and 100 mg/kg. Lipoic acid supplementation resulted in a dose-dependent prevention of GSH depletion. Significance of difference, vs. control, \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ ; vs. STZ,  $\alpha$ ,  $p < 0.05$ ;  $\tau$ ,  $p < 0.001$ . (From Low et al., *Diabetes* 1997; 46 (suppl 2): S38–S42.

physiology, and indices of oxidative stress in peripheral nerve at 1 month after onset of diabetes and in age-matched control rats (12). Lipoic acid, in doses of 20, 50, and 100 mg/kg, was administered intraperitoneally five times per week after onset of diabetes. NBF in EDN was reduced by 50%; lipoic acid did not affect NBF of normal nerves but improved that of EDN in a dose-dependent fashion. After 1 month of treatment, lipoic acid-supplemented rats (100 mg/kg) had normal NBF (Fig. 1). The most sensitive and reliable indicator of oxidative stress was a reduction in GSH, which was significantly reduced in EDN; it was improved in a dose-dependent manner in lipoic acid-supplemented rats (Fig. 2). The conduction velocity of digital nerve was reduced in EDN and was significantly improved by lipoic acid.

#### IV. ANTIOXIDANT THERAPY WITH $\alpha$ -LIPOIC ACID IN ISCHEMIA-REPERFUSION INJURY

Reperfusion after peripheral nerve ischemia results in reduced reperfusion and a breakdown of the blood–nerve barrier (13). After 1 h of ischemia, the permeability-surface area product (PA) is unaltered but becomes significantly greater with reperfusion. After 3 h of ischemia, PA is increased and becomes further increased with reperfusion (13). Reperfusion results in a significant increase

in endoneurial lipid hydroperoxides (14). Because  $\alpha$ -lipoic acid is a powerful lipophilic antioxidant, we evaluated its efficacy in protecting peripheral nerve from reperfusion injury, using our established model of ischemia-reperfusion injury. We used male Sprague-Dawley rats,  $300 \pm 5$  g. Surgical ligation of the supplying arteries to the sciatic-tibial nerve of the right hindlimb was performed for predetermined periods of ischemia (either 3 or 5 h), followed by the release of the ligatures. Lipoic acid (100 mg/kg/day) was given by intraperitoneal injection daily for 3 days pre- and postsurgery. The same dose of saline was given intraperitoneally to the control rats. A behavioral score of clinical neurological deficits and electrophysiology of motor and sensory nerves was analyzed at 1 week after the surgery. After the electrophysiological examination, the sciatic-tibial nerve was fixed in situ and embedded in epon. One-micron sections with toluidine blue staining were evaluated for ischemic fiber degeneration (IFD) and edema, using previously described methodology (15).

Distal sensory conduction (amplitude of sensory action potential and sensory conduction velocity of digital nerve) was significantly improved in 3-h ischemia treated with lipoic acid ( $p < 0.05$ ). Lipoic acid also improved IFD and edema. The changes after a longer duration of ischemia (5 h) were fewer. These results suggest that the therapeutic window of  $\alpha$ -lipoic acid might be relatively narrow but still has some protective effect on peripheral nerve against mild ischemia and reperfusion insults, especially on distal sensory nerves.

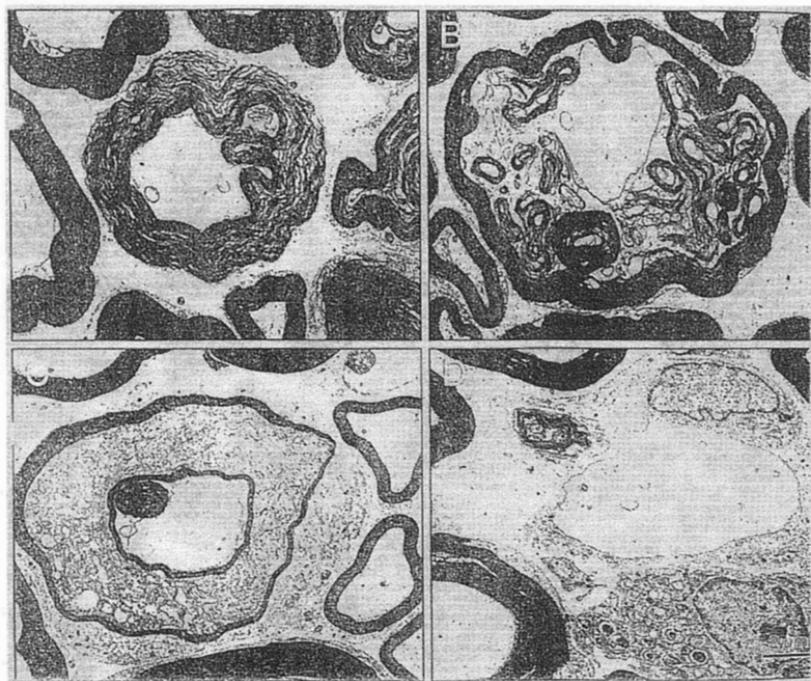
## V. NEUROPATHOLOGY

Neuropathological alterations in sciatic nerve have been modest in EDN. However, with more recent focus on nerve root, marked alterations in the spinal roots have been reported in long-standing streptozotocin-diabetic rats (16). We recently undertook a study addressing the status of vascular perfusion and neuropathology of DRG (17). Vascular perfusion and neuropathologic evaluation of the lumbar spinal roots and DRG were studied in long-standing (duration of 12–18 months) streptozotocin-induced diabetic rats and age- and sex-matched control rats. We also undertook nerve conduction studies, including F wave recordings.

We have undertaken both perfusion-fixed and immersion-fixed ganglia. Light microscopically, changes of the myelin sheath in the dorsal and ventral roots and vacuolated cells in the DRG were the major findings, being significantly higher in diabetic rats than in control rats. The effects of the diabetic state on myelin splitting was greater in the dorsal than ventral roots. Electron

microscopic studies revealed the consecutive changes of myelin from mild separation to severe ballooning of myelin with relative axonal sparing (Fig. 3). DRG cells showed vacuoles of all sizes with cristae-like residua, suggestive of mitochondria. These findings suggest that diabetes mellitus has a dual effect: It accelerates the normal age-related degenerative changes in the spinal roots and DRG and it also has a selective effect on the sensory neuron.

Nerve conduction studies showed markedly reduced conduction velocities in the distal nerve segments and prolonged F wave latency and proximal conduction time despite the shorter conduction pathway in diabetic rats. We suggest that the combination of hyperglycemia and ischemia results in oxidative stress and a predominantly sensory neuropathy.



**Figure 3** Electron micrographs of representative ventral root fibers showing a progression of myelinopathy in experimental diabetes. (A) Myelin decompaction. (B) A rim of intact myelin surrounds degenerating myelin with early myelin balls and a denuded atrophic axon. (C) An atrophic axon is surrounded by myelin, showing residual rims separated by myelin degeneration and assuming a prominent honeycombed appearance. (D) A completely demyelinated axon is seen. (From Ref. 17.)

## VI. GLUCOSE UPTAKE AND ENERGY METABOLISM

$\alpha$ -Lipoic acid has a number of actions in addition to its antioxidant properties. These include its effect on glucose uptake. We therefore evaluated glucose uptake, nerve energy metabolism, and the polyol pathway in EDN induced by streptozotocin. Control and diabetic rats received lipoic acid at various doses (0, 10, 25, 50, and 100 mg/kg). Duration of diabetes was 1 month, and  $\alpha$ -lipoic acid was administered intraperitoneally 5 times during the final week of the experiment. Nerve glucose uptake was reduced to 60%, 37% and 30% of control values in the sciatic nerve, L5 DRG, and superior cervical ganglion, respectively, in EDN.  $\alpha$ -Lipoic acid supplementation had no effect on glucose uptake in normal nerves at any dose but reversed the deficit in EDN, with a threshold between 10 and 25 mg/kg.

Endoneurial glucose, fructose, sorbitol, and myo-inositol were measured in sciatic nerve and L5 DRG. ATP, creatine phosphate, and lactate were measured in sciatic nerve and superior cervical ganglion.  $\alpha$ -Lipoic acid had no significant effect on either energy metabolism or polyol pathway of normal nerves. In contrast, it significantly increased glucose, fructose, and sorbitol but paradoxically increased, rather than reduced, endoneurial myo-inositol.  $\alpha$ -Lipoic acid prevented the reduction in superior cervical ganglion creatine phosphate. We conclude that glucose uptake is reduced in EDN and that this deficit is dose-dependently reversed by  $\alpha$ -lipoic acid, a change associated with an improvement in peripheral nerve function, possibly by improving energy metabolism in ischemic nerve and by increasing endoneurial myo-inositol.

## VII. CONCLUSION

Oxidative stress occurs in EDN due to ischemic and autooxidative lipid peroxidation, with resultant neuropathy. Antioxidant therapy with lipoic acid will improve perfusion, electrophysiology, and indices of oxidative stress. The drug has additional effects, improving glucose uptake and energy metabolism.

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# 10

## Antioxidants in the Treatment of Diabetic Polyneuropathy: Synergy with Essential Fatty Acids

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### I. INTRODUCTION

#### A. Neuropathy and Nerve Blood Flow

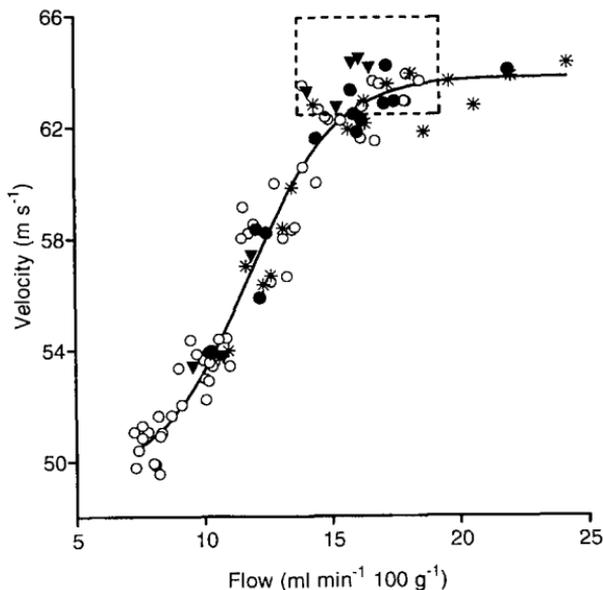
Neuropathy is a common complication of diabetes mellitus. Studies in patients and animal models have shown that endoneurial hypoxia, caused by impaired nerve blood flow, is a major factor in the etiology of diabetic neuropathy (1–4). Changes in vascular function, particularly of the endothelium, occur early after diabetes induction in experimental models, and in some preparations, this may even be partially mimicked by acute exposure to hyperglycemia (5,6). In streptozotocin-induced diabetic rats, sciatic nerve blood flow is reduced by approximately 50% within a week of diabetes induction (7,8), and this precedes changes in nerve conduction velocity (NCV). Large diameter sensory and motor fibers are particularly susceptible to endoneurial hypoxia in experimental diabetes (9,10).

Several treatment strategies have been used to prevent or correct the blood flow deficit in diabetic rats, and, when achieved, this results in improvements of nerve function measures, such as sensory and motor NCV, and the increased resistance to hypoxic conduction failure (3). Powerful evidence for

a direct link between impaired blood flow and nerve dysfunction in diabetes comes from studies using peripheral vasodilators. These do not change the hyperglycemic state or consequent alterations in nerve metabolism such as increased polyol pathway activity; however, they can completely correct reduced NCV and attenuate the development of resistance to hypoxic conduction failure (11–15). Vasodilator treatment has also been used to improve nerve function in diabetic patients (16). Other approaches, such as chronic electrical nerve stimulation (17), and drugs that correct metabolic changes in diabetes, including L-carnitine analogues, *n*-6 essential fatty acids, aldose reductase inhibitors (ARIs), protein kinase C (PKC) inhibitors, antiadvanced glycation agents, and antioxidants, ameliorate NCV defects via their effects on nerve blood flow (3,18). For several of these agents, the vascular endothelium nitric oxide (NO) system appears to be a primary target because their effects on NCV and blood flow are abolished by NO synthase inhibitor cotreatment, whereas many of their other direct biochemical effects on nerve remain unchanged, for example, ARI-mediated suppression of polyol pathway metabolite levels (19–22). Several studies have identified a diminished vasa nervorum NO system in experimental diabetes (23,24). Thus, impaired nerve perfusion lies at the heart of the etiology of diabetic neuropathy. The relationship between sciatic motor NCV and nutritive (capillary) endoneurial blood flow is shown in Figure 1 for groups of diabetic rats pooled from a large number of experiments in which various doses of these drugs were used (reviewed in Refs. 3 and 4). This includes antioxidants, which are the main subject of this review. It is clear that the results of these diverse treatments all fit the same relationship: NCV increases with increasing perfusion and reaches asymptote at blood flow levels within the normal range.

## **B. Sources of Reactive Oxygen Species in Diabetes, NO, and Vasorelaxation**

Reactive oxygen species (ROS) are increased by diabetes. NO is an important vascular target for ROS; superoxide neutralizes NO (25), and the peroxynitrite formed is a source of hydroxyl radicals that can cause endothelial damage (26). Glucose-induced oxidative stress therefore diminishes vessel endothelium-dependent relaxation (27), which contributes to impaired vasa nervorum function (3,4). There are several sources of ROS in diabetes, including those derived from altered metabolism such as autoxidation of glucose and its metabolites, the advanced glycation/glycoxidation process, altered prostanoid production, inefficient mitochondrial function, and upregulation of the vascular NAD(P)H oxidase system (28–30). ROS are also produced as a result of



**Figure 1** Relationship between sciatic nutritive endoneurial blood flow and motor conduction velocity in groups of streptozotocin-diabetic rats ( $n = 6-16$ ) given different drug treatments in our laboratory. Diabetes duration was 1–3 months, and treatment was preventive or corrective. Groups treated with various vasodilators (\*); groups treated with essential fatty acids, miscellaneous metabolically active compounds such as L-carnitine derivatives, aminoguanidine, sorbitol dehydrogenase inhibitors, PKC inhibitors, and myo-inositol (○); groups treated with different antioxidants (●); and data from aldose reductase inhibitor studies (▼). The solid curve is the best-fitting Boltzmann sigmoid curve ( $r^2 = 0.95$  for  $df = 97$ ). The dashed rectangle denotes the nondiabetic range ( $\pm 1$  SD;  $n = 40$ ). All treatment effects appear to follow a similar relationship; conduction velocity is low at low flow rates and reaches an asymptote that approximates the nondiabetic level as perfusion increases.

the blood flow problems they cause during episodes of ischemia-reperfusion by the xanthine oxidase mechanism (31). Another potential source that may be relevant during infection and inflammatory disease is the macrophage respiratory burst. The degree of oxidative stress seen in diabetic patients is inversely proportional to the degree of metabolic regulation (32), and very tight metabolic control is necessary to slow the development of the major diabetic complications, including neuropathy (33). Because strict glycemic control is difficult to achieve and carries with it the risk of hypoglycemic episodes, there is

a strong case for supplementary treatment with antioxidants to further reduce ROS activity.

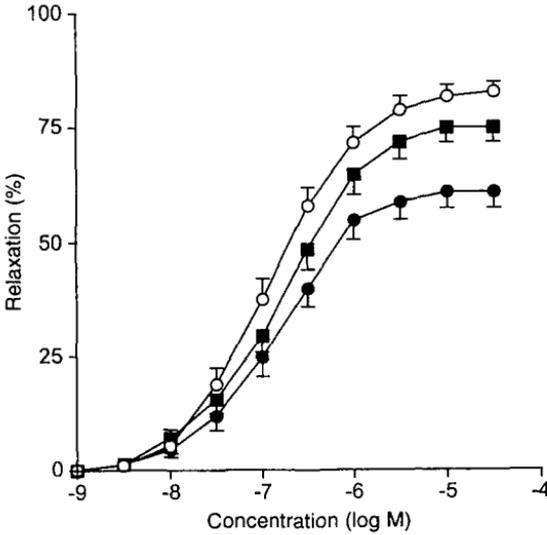
## **II. OXIDATIVE STRESS AND ANTIOXIDANT TREATMENT EFFECTS ON NEUROVASCULAR FUNCTION IN EXPERIMENTAL DIABETES**

Antioxidant protection mechanisms are compromised in nerves of diabetic rats; lipid peroxidation is increased, and the levels of superoxide dismutase and reduced glutathione (GSH) are decreased, although glutathione peroxidase and reductase remain unchanged (34–37). Long-term exposure to elevated ROS, coupled with diminished endogenous antioxidant protection, could lead to cumulative neurodegenerative changes involving axonopathy and demyelination, and damage to dorsal root ganglion cell bodies and their mitochondria has been observed (37,38). However, in the short term, ROS effects on vasa nervorum are more important, being responsible for the earliest defects in nerve function in diabetic rats.

### **A. Antioxidant Treatment, Vascular Endothelium, and Nerve Function**

Defective endothelium-dependent relaxation has been found in diabetic animals and in type 1 and type 2 patients (39–47) and is an important target for antioxidant treatment. An example is shown in Figure 2, where the lipophilic ROS scavenger,  $\alpha$ -tocopherol, protected rat aorta against a diabetic deficit in NO-mediated endothelium-dependent relaxation to acetylcholine (39). A similar protective effect of ROS scavengers has been noted for vasa nervorum blood flow and NCV. The magnitude of effects possible by this approach is illustrated in Figure 3, where a high dose of the probucol analogue, BM 150639, completely corrected motor NCV and blood flow deficits in diabetic rats. These effects were attenuated by cotreatment with a NO synthase inhibitor, emphasizing the importance of the vasa nervorum NO system (19). The effectiveness of a variety of scavengers has been assessed over the last 5 years, including lipophilic drugs like butylated hydroxytoluene,  $\alpha$ -tocopherol (vitamin E),  $\beta$ -carotene, and probucol, and the hydrophilic scavengers, *N*-acetyl-L-cysteine and ascorbic acid (vitamin C) (48–54).

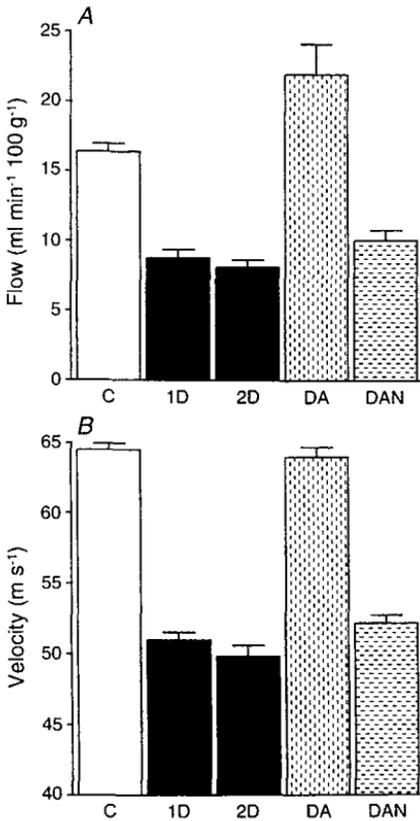
$\alpha$ -Lipoic acid is the subject of considerable current interest and is both lipid and water soluble (55). In experiments on diabetic rats,  $\alpha$ -lipoic acid has been shown to improve nerve antioxidant protection by increasing GSH con-



**Figure 2** Effects of diabetes and antioxidant treatment with  $\alpha$ -tocopherol on endothelium-dependent relaxation of phenylephrine-precontracted aortas to acetylcholine in vitro. Groups ( $n = 14-17$ ); nondiabetic control ( $\circ$ ); 8-week streptozotocin-diabetic ( $\bullet$ );  $\alpha$ -tocopherol (1 g / kg/day) treated from diabetes induction ( $\blacksquare$ ). (From Ref. 39.)

tent and to correct blood flow and motor and sensory NCV deficits (36,56,57). R and S enantiomers of  $\alpha$ -lipoic acid had similar efficacy on impaired nerve function and perfusion (57) and for the inhibition of lipid peroxidation of neural tissues in vitro (56).  $\alpha$ -Lipoic acid was found to be approximately 10 times more potent than  $\alpha$ -tocopherol in correcting motor NCV deficits in diabetic rats (50,57). Clinical trials of symptomatic and cardiac autonomic neuropathy revealed beneficial effects of  $\alpha$ -lipoic acid treatment (58,59).

In rats, the diabetes-induced decrease in sciatic nutritive blood flow was accompanied by a reduction in mean endoneurial oxygen tension, which was prevented by probucol treatment (49). In nondiabetic rats, prooxidant treatment with the antimalarial drug primaquine mimicked the reductions in blood flow, endoneurial oxygen tension, and NCV found in experimental diabetes while having no effect on plasma glucose levels. These effects were blocked by probucol, which stresses the importance of ROS to neurovascular dysfunction. Furthermore, both diabetes and primaquine treatment caused an increase in plasma angiotensin-converting enzyme activity, a marker of endothelial damage, which was attenuated by probucol treatment (49). Effects on plasma



**Figure 3** Reversal of sciatic nutritive endoneurial blood flow (A) and motor conduction velocity (B) deficits in diabetic rats by treatment with the probucol analogue BM 150639. Groups ( $n = 9-10$ ); C, nondiabetic control; 1D, 2D, 1-month, and 2-month streptozotocin-diabetic; DA, 1-month untreated diabetes followed by 1-month BM 150639 (400 mg/kg/day) treatment; DAN, as for DA but cotreated with the nitric oxide synthase inhibitor,  $N^G$ -nitro-L-arginine (10 mg/kg/day) during the second month. Data are mean  $\pm$  SEM. The diabetic deficits in blood flow and motor conduction velocity were well developed after 1 month. BM 150639 treatment was highly effective in reversing these defects, blood flow being approximately 33% supranormal and conduction velocity in the nondiabetic range. The effects of BM 150639 on blood flow and conduction velocity were almost completely blocked by  $N^G$ -nitro-L-arginine, which suggests that blood flow modulates conduction velocity and that antioxidant treatment corrects a diabetic deficit in the vasa nervorum nitric oxide mechanism. (See Ref. 24 for further details.)

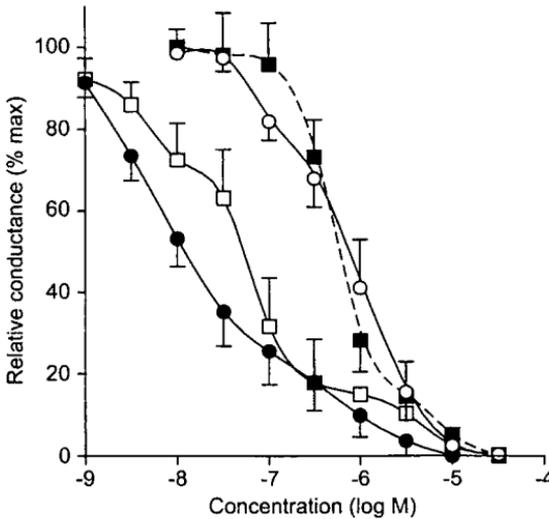
angiotensin-converting enzyme activity suggest increased activation of the vasoconstrictor renin-angiotensin system in diabetes, and studies with angiotensin-converting enzyme inhibitors and angiotensin AT<sub>1</sub> receptor antagonists have shown that this is important for vasa nervorum; these inhibitors correct blood flow, endoneurial hypoxia, and NCV defects in diabetic rats (60). Increased local angiotensin II also causes upregulation of endothelial NADH oxidase, which may exacerbate dysfunction by increasing ROS production (30). Furthermore, oxidative stress also stimulates endothelial endothelin-1 synthesis, and this interacts with the angiotensin II system to increase vasa nervorum vasoconstriction (61). Thus, ROS cause a self-reinforcing cycle that compromises vasodilation by NO and increases local vasoconstrictor mechanisms. This cycle may be interrupted by antioxidant treatment.

## B. Antioxidant Dose Considerations

The normal dietary intake of natural antioxidants could in theory influence NCV and blood flow in diabetic rats. However, high doses that far exceed normal availability are required in practice; for example, 0.62 g/kg/day of  $\alpha$ -tocopherol was necessary to give ~50% protection of sciatic motor NCV in diabetic rats (50). For ascorbic acid, 150 mg/kg/day gave an optimal level of protection that was relatively modest (~35%). At high doses (500 mg/kg/day), protection was less, probably because ascorbic acid is susceptible to autoxidation, acting as a prooxidant (50,62). Under physiological conditions, ascorbic acid may aid the recycling of  $\alpha$ -tocopherol from its tocopheroxyl radical form (63); however, with the pharmacological doses used in diabetic rats, there was no evidence of a synergy between ascorbic acid and  $\alpha$ -tocopherol cotreatment on NCV. Instead, their effects were simply additive, as if they were acting independently in lipid and aqueous phases (50).

## C. Studies Using Transition Metal Chelators

As an alternative to scavenging ROS, it may be therapeutically preferable to prevent their formation by autoxidation, the Fenton reaction, and the advanced glycation process, all of which are catalyzed by free transition metal ions. This can be accomplished using transition metal chelators. Low doses of deferoxamine (relatively specific for iron) and trientine (relatively specific for copper) completely corrected sciatic nerve blood flow and motor and sensory NCV deficits in diabetic rats (64). In vessels such as aorta, chronic trientine and deferoxamine treatment prevented the development of defective NO-mediated, endothelium-dependent relaxation (65,66). For diabetic rat vasa nerv-



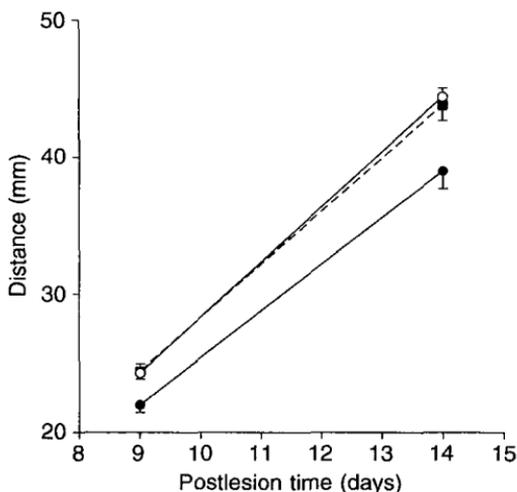
**Figure 4** Cumulative dose-response curves for changes in sciatic vasa nervorum vascular conductance after suffusion of saline containing increasing concentrations of norepinephrine. Blood flow in epi- and perineurial vessels was monitored by laser-Doppler flowmetry, and the results are expressed as a percentage of the maximum vascular conductance. Groups ( $n = 8-12$ ): nondiabetic control (○); 8-week duration diabetic control (●); 8-week diabetic rats treated with deferoxamine (8 mg/kg/day) for the last 2 weeks (■). Data are mean  $\pm$  SEM. Diabetes caused a leftward shift of the dose-response curve, indicating greatly enhanced sensitivity to vasoconstriction by norepinephrine. In nondiabetic rats, cosuffusion with the nitric oxide synthase inhibitor  $N^G$ -nitro-L-arginine (100  $\mu$ M) caused a similar leftward shift (□). Treatment of diabetic rats with deferoxamine completely restored norepinephrine sensitivity, the interpretation being that it corrected a vasa nervorum NO deficit. (See Refs. 52 and 67 for further details.)

orum, the NO deficit markedly increases reactivity to norepinephrine (Fig. 4), and this was corrected by deferoxamine treatment (24,67). The relatively high potency of  $\alpha$ -lipoic acid compared with other natural scavengers such as  $\alpha$ -tocopherol could be due to the additional property of transition metal chelation (55). Thus, transition metal-catalyzed ROS production makes an important contribution to nerve and vascular dysfunction in experimental diabetes.

#### D. Nerve Growth, Regeneration, and Small Fiber Function

In addition to effects on NCV and blood flow, antioxidant treatment improves other aspects of nerve function, including growth and regenerative responses

and the performance of small fibers (which do not normally contribute to NCV measurements). Thus, the hydrophilic scavenger *N*-acetyl-L-cysteine allowed normal nerve maturation in young diabetic rats, preventing a reduction in mean nerve fiber size caused by impaired growth. *N*-acetyl-L-cysteine improved the regenerative response to nerve trauma, which is blunted by diabetes (Fig. 5), inhibited an increase in plasma tumor necrosis factor activity, and prevented red cell lipid peroxidation (51,52). The lipophilic scavengers  $\alpha$ -tocopherol and butylated hydroxytoluene and the metal chelator trientine also prevent blunted nerve growth and regeneration in young diabetic rats (53,54). Recently, improved regeneration, remyelination, and muscle reinnervation have been noted for  $\alpha$ -lipoic acid treatment (Flint H, Cotter MA, and Cameron NE, unpublished observations, 1998). Interestingly, butylated hydroxytoluene and trientine also prevented nerve regeneration and growth deficits in the galactosemic rat model of enhanced polyol pathway and PKC activity (54). Vasodilator treatment had similar effects on these nerve growth parameters in diabetic rats (60); therefore, it is likely that antioxidant-mediated improvements in perfusion and their consequences for the supply of energy and nutrients to nerve fibers and cell



**Figure 5** Effects of 4 weeks of diabetes and *N*-acetyl-L-cysteine treatment on sciatic nerve myelinated fiber regeneration distance 9 and 14 days after a freeze lesion. Groups ( $n = 7-10$ ): nondiabetic control (○); diabetic control (●); diabetic rats treated with 250 mg/kg/day *N*-acetyl-L-cysteine from diabetes induction (■). Data are mean  $\pm$  SEM. At both time points there was a significant regeneration deficit ( $p < 0.01$ ) with untreated diabetes, which was completely prevented by *N*-acetyl-L-cysteine treatment. (See Ref. 52 for further details.)

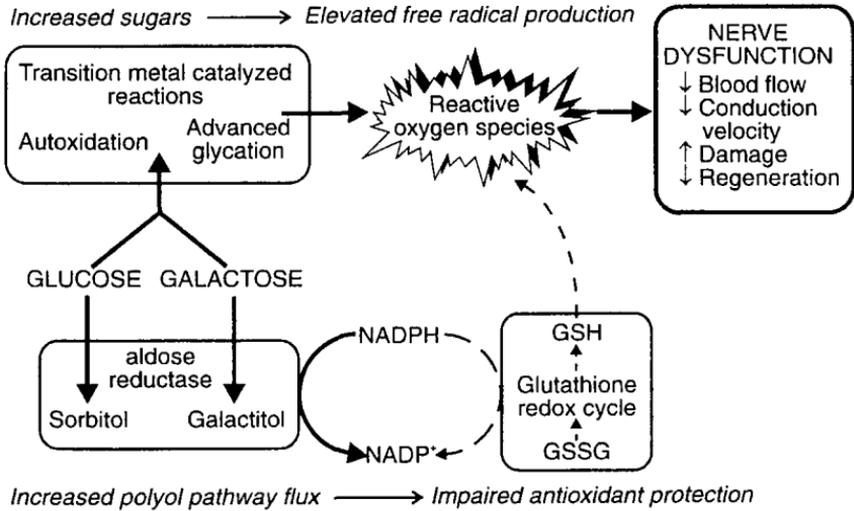
bodies are primarily responsible for their growth and regeneration-promoting actions.

The effects of antioxidant treatment on small fiber function has not been examined in as much detail as large fibers. However, in the isolated corpus cavernosum preparation from diabetic rats, a deficit in vasorelaxation to nitrenergic nerve fiber stimulation was completely prevented by chronic  $\alpha$ -lipoic acid treatment and partially prevented by trientine (68).

### **E. Polyol Pathway, Oxidative Stress, and Neurovascular Dysfunction**

Polyol pathway activity also contributes to oxidative stress; therefore, ARIs have an indirect antioxidant action. The reductions in nerve GSH content found in experimental diabetes and galactosemia were rapidly corrected by ARI treatment (69), which also prevented the elevation of nerve malondialdehyde, a marker of lipid peroxidation, in diabetic rats (70). ARIs correct nerve blood flow and NCV defects in diabetic rats (71,72). They improve defective NO-mediated endothelium-dependent relaxation in vessels from diabetic animals and similar dysfunction resulting from acute hyperglycemic exposure of vessels from nondiabetic animals (3,6). The first half of the polyol pathway, catalyzed by aldose reductase, is much more important than the second half, catalyzed by sorbitol dehydrogenase, because sorbitol dehydrogenase inhibitors did not correct blood flow or NCV in diabetic rats (73) and did not rectify the nerve GSH deficit (Hohman TC, personal communication, 1997).

Aldose reductase requires NADPH as a cofactor, and NADPH is also used by glutathione reductase for maintaining GSH concentrations. Therefore, competition for NADPH in diabetes probably contributes to diminished GSH levels. A further potential polyol pathway action is to increase the formation of advanced glycation end products (AGEs). ARI treatment reduces tissue AGE accumulation, perhaps by inhibiting the synthesis of fructose or by decreasing elevated flux through the pentose phosphate pathway, processes that produce sugars that are considerably more potent glycating agents than glucose (74). Alternatively, the ARI-induced increases in tissue GSH and antioxidant capacity may be sufficient to oppose AGE formation by glycooxidation (75). AGE reactions are an important source of ROS; therefore, their reduction would decrease oxidative stress. Aminoguanidine, although not [directly] an antioxidant, irreversibly binds to reactive carbonyl intermediates, thus blocking AGE formation. Aminoguanidine treatment of diabetic rats has similar functional effects to ARIs and antioxidants in preventing and correcting NCV, nerve blood flow, and NO-mediated endothelium-dependent vasorelax-



**Figure 6** Relation of oxidative stress and nerve dysfunction to elevated polyol pathway activity and transition metal-catalyzed reactions in diabetes or galactosemia. The increased autoxidation and advanced glycation reactions produce ROS, and flux through the first half of the polyol pathway consumes NADPH. This impairs the glutathione redox cycle so that endogenous antioxidant protection is reduced.

ation deficits (3,4,40,76). The putative interrelations between the polyol pathway, advanced glycation, and autoxidation processes in the production of oxidative stress under hyperglycemic conditions are summarized in Figure 6.

**F. Antioxidants, NF-κB, and PKC**

One of the cellular events stimulated by oxidative stress-related biochemical changes in hyperglycemia, either directly or via AGE receptors, oxidized low-density lipoprotein, or cytokine receptors, is the activation of NF-κB, an effect that may be prevented by antioxidant treatment with α-lipoic acid (77). This transcription factor is responsible for changes in gene expression that have important effects on vascular function relevant to diabetes, including elevated endothelin-1 synthesis and upregulation of intercellular and vascular cell adhesion molecules. It has also been linked to increased NADH oxidase activity (30,78).

PKC is another cell-signaling mechanism activated by diabetes, particularly in vascular tissue (79) although not in the nerve itself (80). In the retina

of diabetic rats, there is an early reduction in blood flow paralleling that for nerve. In both retina and nerve, flow was restored by PKC inhibitor treatment (18,81), and nerve NCV deficits were corrected. Diabetes activates PKC via increased de novo synthesis of diacylglycerol from glucose; however, even in the absence of hyperglycemia, PKC is also stimulated by oxidative stress (82). Antioxidants such as  $\alpha$ -tocopherol inhibit PKC both directly and via stimulation of diacylglycerol kinase, which breaks down diacylglycerol (79,82). When activated, PKC can modulate several important vascular systems. For example, it is involved in cell signaling mechanisms for endothelin-1 action and can also stimulate NF- $\kappa$ B, which increases endothelin-1 gene expression in endothelial cells (83). Phosphorylation by PKC controls endothelial constitutive NO synthase, reducing its activity (84). Furthermore, phosphorylation of vascular smooth muscle contractile proteins promotes vasoconstriction (85). Thus, PKC is at the heart of altered vascular responses in experimental diabetes and forms a major component of the dysfunctional mechanisms targeted by antioxidant treatment.

### **G. Therapeutic Implications**

From this brief literature review, it is clear that antioxidant strategies based on the use of ROS scavengers and transition metal chelators can be very effective against experimental models of diabetic neuropathy and vasculopathy. The drawback with the scavenger approach is that very large doses of drug are required, one-two orders of magnitude greater than necessary if using transition metal chelators. However, it is possible that with lower blood glucose concentrations than normally found in the experimental models, more physiological doses, for example, of  $\alpha$ -tocopherol, could be effective as an adjunct to tight metabolic control in patients. The use of agents with both scavenger and chelator properties, such as  $\alpha$ -lipoic acid, or combined therapy with drugs that improve endogenous antioxidant protection mechanisms, such as ARIs, or drugs that target key cell signaling events, such as PKC inhibitors, could provide exciting future strategic approaches to the therapy of diabetic complications including neuropathy.

### **III. INTERACTIONS BETWEEN ANTIOXIDANTS AND ESSENTIAL FATTY ACIDS**

Most of the preceding discussion has dealt with effects of ROS on the NO system and cell-signaling pathways. Another major ROS target is the polyun-

saturated fatty acids, including the essential fatty acids necessary for eicosanoid production and normal membrane structure and fluidity. ROS subject these important molecules to self-propagating destructive chain reactions initiated by lipid peroxidation.

### **A. Essential Fatty Acids, Diabetes, and Neurovascular Dysfunction**

The most common dietary essential fatty acids are *n*-6 linoleic acid and *n*-3  $\alpha$ -linolenic acid. They are metabolized by an alternating series of desaturation and elongation steps to produce *n*-6 arachidonic acid and *n*-3 eicosapentaenoic acid, which are important precursors of prostanoids, leukotrienes, and other mediators. In addition to being subjected to destruction by the elevated ROS in diabetes, metabolism of these essential fatty acids is rate limited by the desaturation steps that are inhibited by diabetes. Depressed hepatic  $\Delta$ -6 desaturation results in lower plasma levels of the *n*-6 metabolites  $\gamma$ -linolenic acid (GLA) and arachidonic acid. This reduces synthesis of the vasodilator, prostacyclin (PGI<sub>2</sub>), by vasa nervorum (86). The desaturation deficit may be bypassed by GLA or arachidonic acid treatments, which improve vasa nervorum prostacyclin production, nerve blood flow, and NCV (3,4,87). In contrast to the *n*-6 essential fatty acids, *n*-3 metabolites, such as eicosapentaenoic and docosahexaenoic acids, have relatively little effect on nerve function in experimental diabetes (88).

### **B. Multiple Endothelial Dysfunction in Diabetes and the Potential for Synergistic Therapies**

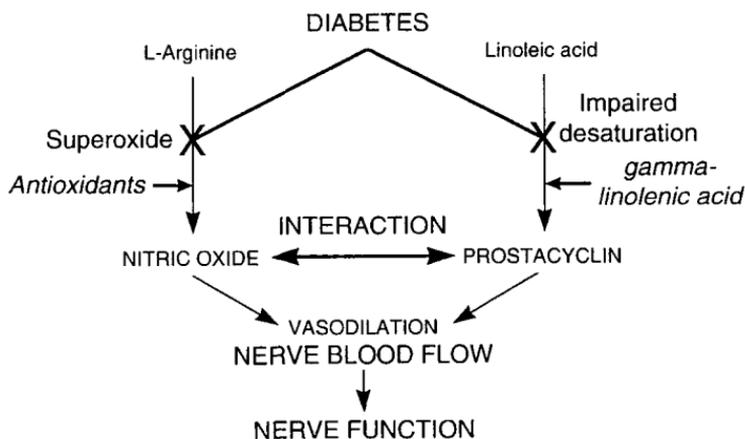
There are multiple defects of vasa nervorum endothelium and possibly smooth muscle in diabetes that cause reduced nerve blood flow and function. The NO deficit is compounded by diminished PGI<sub>2</sub> synthesis and increases in endothelin-1 and angiotensin II. Recently, it was shown that relaxation mediated by endothelium-derived hyperpolarizing factor (EDHF) is also affected by diabetes. In the rat mesenteric vascular bed, EDHF was 76% reduced after 8 weeks of diabetes. In common with the NO defect, the EDHF deficit was attenuated by antioxidant treatment with  $\alpha$ -lipoic acid (89). Vasodilator prostanoid synthesis is also deleteriously affected by oxidative stress; high levels of lipid peroxides inhibit cyclooxygenase (90), and  $\alpha$ -tocopherol treatment corrected the lowering of the PGI<sub>2</sub>/thromboxane A<sub>2</sub> ratio found in diabetic rats (91).

Normally, these different mechanisms provide an integrated local system for nerve blood flow control; however, by disrupting several mechanisms simultaneously, diabetes disintegrates control, and markedly shifts the balance toward vasoconstriction. These individual mechanisms do not exist in isolation but are mutually interactive (92); therefore, changes in one will affect the others. In experiments on nondiabetic rats designed to mimic some of the vasa nervorum changes in diabetes and assess the consequences for nerve function, chronic treatment with a low dose of a cyclooxygenase inhibitor to reduce PGI<sub>2</sub> synthesis or a NO synthase inhibitor caused modest NCV reductions. However, with combined treatment, there was a fivefold amplification of drug effects on NCV compared with that expected for simple summation (93). This demonstrates a marked synergism between blockade of the prostanoid and the NO systems, suggesting that they normally act in a mutually compensatory manner to limit nerve perfusion changes. The converse effect, joint treatment of diabetes with drugs that target the NO and prostanoid mechanisms, as schematized in Figure 7, could potentially offer a marked therapeutic advantage.

### C. Synergy Between Antioxidant and *n*-6 Essential Fatty Acid Treatments

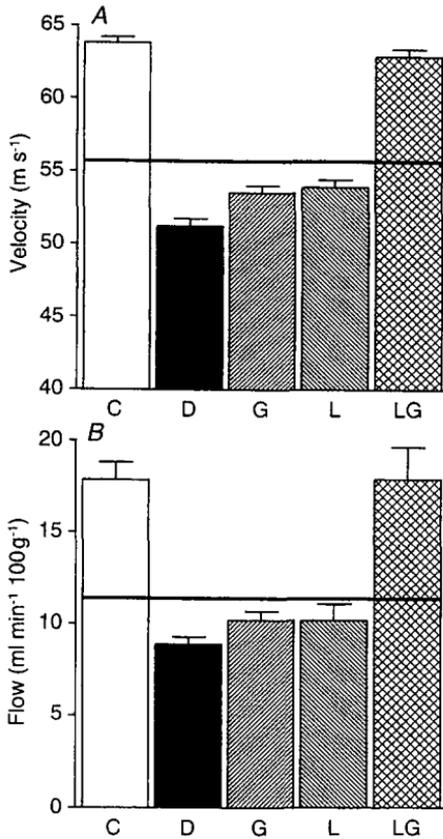
Low doses of an ARI or evening primrose oil (which contains GLA) had modest effects on NCV and blood flow in diabetic rats; however, in combination, improvements matching those obtained by an eightfold increase in the dose of either drug alone were noted (20). A similar magnitude of synergistic interaction was seen for joint treatment with low-dose GLA and the lipophilic scavenger, BM 150639 (94). The notion of antioxidant-*n*-6 essential fatty acid combination therapy has led to the synthesis of hybrid drugs, such as ascorbyl-6-GLA. Although ascorbic acid alone is not particularly effective against NCV deficits in diabetic rats, its combination with GLA increases lipid solubility and therefore the ability to enter cell membranes. It also places the ascorbate moiety in a good physical position to protect the GLA component from ROS damage. Compared with GLA alone, ascorbyl-6-GLA was 4.4-fold more efficacious in correcting NCV and nerve blood flow deficits in diabetic rats (95).

Recent attention has focused on GLA- $\alpha$ -lipoic acid combinations because both drugs have similar dose-response relationships for correcting NCV deficits and because of the greater effective antioxidant power of  $\alpha$ -lipoic acid than ascorbate in experimental diabetic neuropathy. Effects on motor NCV and nerve blood flow are shown in Figure 8. Low doses of GLA or  $\alpha$ -lipoic

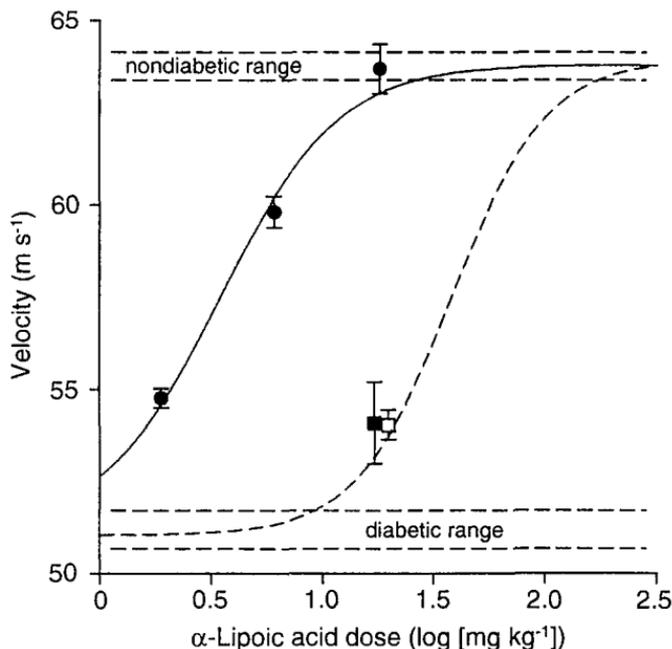
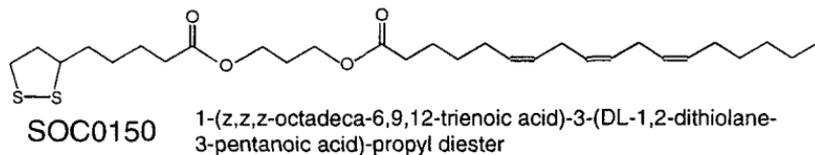


**Figure 7** Schematic for the synergistic interaction between NO and prostacyclin pathways. Increased oxidative stress in diabetes and the generation of superoxide neutralizes endothelial NO production, which impairs vasa nervorum vasodilation. Diabetes also reduces hepatic *n*-6 essential fatty acid metabolism, particularly the rate-limiting desaturation steps in the conversion of dietary linoleic acid to  $\gamma$ -linolenic acid. In turn this reduces vasa nervorum prostacyclin production and vasodilation. Normally, at the level of the endothelial cell, and possibly vascular smooth muscle, there is a mutual facilitatory interaction between NO and prostacyclin systems. This may give a therapeutic advantage for the use of joint treatment with antioxidant, which corrects the NO defect, and  $\gamma$ -linolenic acid, which bypasses the desaturation block to boost prostacyclin production.

acid alone had modest effects on NCV and no statistically significant effects on blood flow. However, when the drugs were combined, NCV and blood flow were in the nondiabetic range, showing a greater than additive effect (57). Synergy was found for GLA- $\alpha$ -lipoic acid mixtures in ratios between 1:3 and 3:1, the greatest amplification of drug action being for ratios near 1:1 or with GLA slightly in excess. Similar interactive effects were noted for the novel orally active drug, SOC0150, which contains equimolar amounts of GLA and  $\alpha$ -lipoic acid (Fig. 9). The dose-response curve for correction of sciatic motor NCV showed that SOC0150 had an  $ED_{50}$  of 9.3 mg/kg/day (giving 3.5 mg/kg/day  $\alpha$ -lipoic acid and 4.5 mg/kg/day GLA). This compares very favorably with the  $ED_{50}$  of  $\alpha$ -lipoic acid alone (38 mg/kg/day). In contrast to SOC0150,  $\alpha$ -lipoic acid-containing compounds in which the GLA was substituted by the *n*-3 essential fatty acids docosahexaenoic acid (57) or eico-



**Figure 8** Effects of low-dose  $\gamma$ -linolenic acid and  $\alpha$ -lipoic acid treatment, alone and in combination, on (A) sciatic motor conduction velocity and (B) sciatic endoneurial blood flow in streptozotocin-diabetic rats. Groups ( $n = 8-12$ ): C, nondiabetic control; D, 8-week diabetic control; G or L, 8-week diabetic treated for the final 2 weeks with  $\gamma$ -linolenic acid (20 mg/kg/day) as the monoester or  $\alpha$ -lipoic acid (20 mg/kg/day); LG, 8-week diabetic given combined  $\gamma$ -linolenic acid and  $\alpha$ -lipoic acid treatment for the final 2 weeks. Data are mean  $\pm$  SEM. The horizontal lines show the predicted conduction velocity and blood flow values for additive drug effects. The LG group greatly exceeded this level for both measures, indicating a marked synergistic drug interaction. (See Ref. 57 for further details.)



**Figure 9** Dose-response relationship for the correction of sciatic motor conduction velocity by the novel drug SOC0150 containing equimolar amounts of  $\gamma$ -linolenic acid and  $\alpha$ -lipoic acid. The chemical name and structure of SOC0150 are shown at the top of the figure. Diabetes duration was 8 weeks, and oral treatment was given for the final 2 weeks. The graph shows data points ( $\pm$  SEM;  $n = 7-10$ ) for SOC0150 (●), plotted in terms of  $\alpha$ -lipoic acid content, and the best-fitting sigmoid dose-response relationship (solid curve) for comparison with the dose-response relationship for  $\alpha$ -lipoic acid (dashed curve). The SOC0150 curve is displaced approximately 1 log unit to the left of that for  $\alpha$ -lipoic acid, indicating an approximately 10-fold increase in efficacy. Also shown are data points for similar drugs in which the  $\gamma$ -linolenic acid moiety was replaced with the  $n-3$  components, eicosapentaenoic acid (■,  $n = 6$ ), or docosahexaenoic acid (□,  $n = 8$ ). Conduction velocity was not significantly different from that predicted from their  $\alpha$ -lipoic acid content alone, showing that synergistic interactions with antioxidants are specific to  $n-6$  essential fatty acids. (From Ref. 57 and Cameron NE, Cotter MA, unpublished observations, 1998).

sapentaenoic acid (Cameron NE, Cotter MA, unpublished observations, 1998) were no more effective than their  $\alpha$ -lipoic acid component alone (Fig. 9). This suggests that synergistic actions are relatively specific to the *n*-6 series.

#### IV. SUMMARY AND CONCLUSIONS

Studies on antioxidant treatment have shown that ROS makes a marked contribution to the etiology of nerve dysfunction in experimental diabetes. Effects on vasa nervorum predominate in the short term; ROS cause dysfunction of vascular endothelium which at the very least reduces NO-mediated vasodilation and increases local vasoconstrictor production and reactivity. The effects of oxidative stress are crucial, complex, and far reaching, causing basic changes in cell signaling such as PKC and NF- $\kappa$ B that affect a plethora of systems involved in the maintenance of vascular control and integrity. ROS effects also impinge on prostanoid and EDHF systems, further exacerbating a diabetic deficit of substrate availability in the former. The result is reduced nerve perfusion, causing endoneurial hypoxia, which in turn is responsible for NCV and other functional deficits. Autooxidation of glucose and its metabolites and other transition metal-catalyzed reactions such as advanced glycation are important sources of ROS. Polyol pathway activity contributes to oxidative stress by compromising the glutathione redox cycle. Antioxidant treatment strategies, in combination with good metabolic control, offer a potential way forward in the prevention or control of diabetic neuropathy and other vascular complications. The powerful synergistic interactions between ROS-NO and *n*-6 essential fatty acid-prostanoid mechanisms on nerve perfusion offer a potential therapeutic advantage for the use of antioxidant-GLA mixtures and novel compounds.

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# 11

## A Thioctic Acid–Gamma-Linolenic Acid Conjugate Protects Neurotrophic Support in Experimental Diabetic Neuropathy

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Defects of the peripheral nervous system are common in patients with diabetes mellitus, and a large fraction of patients will develop a form of diabetic neuropathy within 25 years after diagnosis (1). Although a rigid classification of diabetic neuropathy is difficult, at least three major syndromes are recognized: symmetrical distal polyneuropathy, symmetrical proximal motor neuropathies, and focal asymmetrical neuropathies (2–4). As in most polyneuropathies, sensory, motor, and autonomic nerves are concomitantly involved although sensory dysfunction usually predominates.

Recent attempts to rationalize the etiology of diabetic neuropathy have focused initially on the identification of the biochemical defects that follow directly from hyperglycemia, in this case, the polyol pathway, protein glycation, and oxidative stress (for recent reviews, see Refs. 5 and 6). Second, there is the issue of the importance of reduced nerve blood flow and possible consequent ischemic damage (7–9). Related to this is the proposition that defective metabolism of essential fatty acids, resulting in reduced levels of  $\gamma$ -linolenic acid and subsequently arachidonic acid and vasoactive prostanoids, also has a large part to play in the pathogenesis of diabetic neuropathy (see

Ref. 10 for review). A chain of consequences comprising hyperglycemia, exaggerated polyol pathway flux, oxidative stress, impaired endoneurial nitric oxide production, reduced nerve blood flow, endoneurial hypoxia, and impaired nerve conduction has been suggested (11–13). Impaired neurotrophic support from nerve growth factor (NGF) and neurotrophin 3 (NT-3) (14–17) is also important, because a failure of neurotrophic factors to regulate neuronal phenotype might be expected to result in such a clinical picture as presents in symptomatic diabetic neuropathy.

Recently, the antioxidant thioctic ( $\alpha$ -lipoic) acid has been found to protect against a broad range of defects in diabetic rats, (18–20) including both those related to nerve blood flow and those related to neurotrophins.

## I. NEUROTROPHIC FACTORS

Neurotrophic factors were discovered as agents with the capacity to stimulate neurite outgrowth in culture systems of embryonic sensory or sympathetic neurons. Some of these factors, such as ciliary neurotrophic factor (a member of the interleukin-6 cytokine family) and basic fibroblast growth factor (a member of the fibroblast growth factor family), are quite distinct from NGF, the archetypal neurotrophic factor. It has now been established that NGF belongs to a family of neurotrophic factors known as the neurotrophins that now includes NT-3, brain-derived neurotrophic factor (BDNF), and neurotrophin-4/5 (see Ref. 21 for review).

NGF undergoes retrograde transport in sensory and sympathetic neurons in adult rats (22,23), and about 50% of adult lumbar sensory neurons can bind NGF with high affinity (24). BDNF and NT-3 also undergo retrograde transport from an injection site in the sciatic nerve to the dorsal root ganglia (DRG) and motor neurons of adult rats (25).

The neurotrophins bind to two distinct sites on responsive neurons. Low-affinity binding is associated with the p75 neurotrophin receptor (p75NTR) and shows no selectivity between the neurotrophins. High-affinity binding is provided by a family of trk proto-oncogene receptors that have intrinsic tyrosine kinase activity. trkA is specific for NGF (26,27), trkB for BDNF and NT-4/5 (28,29), and trkC for NT-3 (30). However, NT-3 is promiscuous and will also bind to trkB and trkA at increasing concentrations.

There is a large body of evidence suggesting that NGF and the other neurotrophins are involved, not just in the survival of neurons during embryonic development but also in the regulation of neuronal phenotype in the adult. Deprivation of trophic support by nerve transection provokes a pattern of

change in the nerve cell body that is prevented by the administration of exogenous neurotrophin. Direct evidence for modulation of adult phenotype was provided when it was demonstrated that NGF can regulate the expression of substance P (SP) and calcitonin gene-related peptide (CGRP) in primary cultures of adult rat DRG neurons (31). Additionally, deprivation of NGF has been found to cause a proportional reduction in the expression of these neuropeptides (32,33).

## II. NEUROTROPHINS IN DIABETIC NEUROPATHY

Decreased capture and retrograde transport of iodinated NGF in the sciatic nerve was observed in diabetic rats many years ago (34). Reduced retrograde transport of iodinated NGF in ileal mesenteric nerves has also been demonstrated (35). These observations imply that even in the absence of any deficit in production of NGF in diabetes, a deficit in the amount delivered to the cell body might be expected. In diabetic rats, there are reduced levels of NGF in the submandibular gland, superior cervical ganglion, and sciatic nerve (36–38). NGF levels have also been shown to be decreased in the serum of diabetic patients with symptomatic peripheral neuropathy (39).

Work in our laboratory has shown that with increasing duration of diabetes, progressive reductions in NGF mRNA appear in leg muscle and sciatic nerve followed by reductions in skin. There is a profound reduction in the retrograde transport of NGF in the sciatic nerve, which can be reversed by intensive insulin treatment, and dose-related increases in sciatic nerve NGF retrograde transport were seen with recombinant human NGF (rhNGF) treatment (33). Additionally, it is clear that there are also deficits in the production of NGF target genes, and deficits in expression of SP and CGRP are easily demonstrable in experimental diabetic neuropathy (32).

It is quite apparent that there are deficits in NGF exaptation in experimental diabetes, but this does not explain the earlier observation of reduced capture and transport of exogenous NGF. Recent work in our laboratory has revealed a marked decrease in the retrograde transport of the p75NTR, which closely follows the changes seen with NGF transport in diabetes (40). No changes in transport of trkA were observed, but it is not yet possible to suggest which are the precedent changes because NGF availability is known to affect the expression of the p75NTR (41).

NT-3 mRNA levels are reduced in leg muscle from diabetic rats, but an assessment of NT-3 neurotrophic support is difficult because gene targets of NT-3 have yet to be identified. Work in our laboratory showed that treat-

ment of diabetic rats with rhNT-3 for the last 4 weeks of a 12-week period of diabetes could completely normalize the reduced sensory nerve conduction velocity, which is characteristic of diabetic rats (16). This implies that NT-3 may be even more instrumental than NGF in the development of important functional deficits in diabetic neuropathy. However, although treatment of animals with NGF or NT-3 prevents some of the deficits characteristic of experimental neuropathy (14,16), these agents do not influence reduced nerve blood flow or motor nerve conduction velocity (42), other classic hallmarks of experimental neuropathy.

### III. NEUROTROPHINS AND OXIDATIVE STRESS

Shifts in cellular redox balance due to increased levels of free radicals may cause or result from neuronal injury (43,44). Oxygen free radicals are generated as a consequence of ischemia-reperfusion, inflammation, traumatic, and oxidative injury and are associated with neuronal cell death (45,46).

Peripheral nerves, including the sciatic nerve, have inherent low antioxidant defenses compared with the central nervous system, because total reduced glutathione (GSH) content and activities of GSH utilizing enzymes like glutathione peroxidase (GSH-Px) are about 10-fold lower than they are in brain (47).

NGF stimulates cellular resistance to oxidative stress in PC12 cells (48). In particular, NGF protects from oxidative injury induced by hydrogen peroxide and 6-hydroxydopamine, (48–50) both of which generate hydroxyl radicals. From these observations, it might be expected that NGF regulates cellular oxidant-antioxidant equilibrium.

NGF has been found to regulate the expression of the antioxidant enzymes, catalase (Cat), and GSH-Px. Application of NGF to PC12 cells in culture results in an increase in the transcription of the mRNA for Cat and GSH-Px and, in addition, appears to stabilize the transcript for Cat (51). This is associated with an increase in the activities of both enzymes (52). Furthermore, newborn rat astrocytes in culture synthesise NGF in a dose-dependent fashion in response to superoxide anion, as generated by xanthine/xanthine oxidase and to hydrogen peroxide (53).

### IV. OXIDATIVE STRESS IN DIABETES

Diabetes is associated with increases in oxidative stress in humans and in experimental animal models. Chronic hyperglycemia *per se* results in autoxi-

dative glycation/oxidation and lipid peroxidation (54–56), and hyperglycemia alone will cause lipid peroxidation of peripheral nerve *in vitro* (57).

Diabetic peripheral nerve has increased levels of conjugated dienes (end products from peroxidation of polyunsaturated fatty acids) (57,58), decreased levels of GSH (19), and reduced activity of copper/zinc-superoxide dismutase that is reversible with reinstatement of moderate glycemic control (58).

Further support for the role of oxidative stress in the pathogenesis of diabetic neuropathy come from the effectiveness of antioxidant treatment in reversing some of the functional neurological deficits observed in experimental animal models.

Probucol, a powerful free radical scavenger, normalizes both decreases in endoneurial nerve blood flow and motor nerve conduction velocity (59,60). Dietary treatment with 1% butylated hydroxytoluene or  $\alpha$ -tocopherol also has similar effects (61,62). Intravenous administration of GSH can also partially prevent motor nerve conduction velocity slowing in diabetic rats (63). Beneficial effects on conduction velocity have also been reported using the metal chelator deferoxamine (64).

## V. THIOCTIC ACID

Thioctic acid (TA) was first isolated in 1951 (65) and is now known by a variety of different names, including  $\alpha$ -lipoic acid, 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane valenic acid, and 6,8-thioctic acid. As lipoamide, it functions as a cofactor in the multienzyme complexes that catalyze the oxidative decarboxylations of  $\alpha$ -keto acids such as pyruvate,  $\alpha$ -ketoglutarate, and branched chain  $\alpha$ -keto acids (66). In addition, TA is a powerful antioxidant (67) and a potent free radical scavenger in peripheral nerve (57,68) that would appear to act by substituting for  $\alpha$ -tocopherol (57). Interestingly, endogenous TA is depleted in diabetes (69,70).

A study carried out by Kahler et al. (71) confirmed the association of long-term diabetic complications with increased lipid peroxidation and enhanced lipid peroxidation. Treatment with TA decreased peroxidation rate, improved thermal and vibration sensibilities, and improved patellar and Achilles tendon reflexes. Furthermore, results from the  $\alpha$ -Lipoic Acid in Diabetic Neuropathy study (72) revealed that TA at a dose of 600 or 1200 mg administered intravenously significantly reduced the incidence of burning sensation, parasthesiae, and numbness in patients with symptomatic diabetic neuropathy.

TA has effects beyond that of a simple antioxidant. TA stimulates glucose utilization by tissues (73,74) and may also act as a chelator of transition

metal ions, which are thought to contribute to oxidative stress (75–77). Furthermore, TA also inhibits nonenzymatic glycation/glycosylation, which is now thought to play a role in the pathogenesis of diabetic late complications (see Ref. 78 for review).

Unrelated to this, TA is also able to induce neurite outgrowth (79) and promote nerve regeneration (80). TA can also stimulate NGF synthesis and secretion (81).

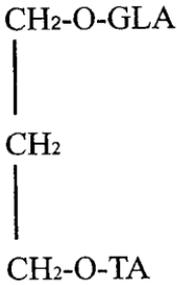
## VI. IN VIVO STUDIES WITH THIOCTYL- $\gamma$ -LINOLENIC ACID

$\gamma$ -Linolenic acid (GLA) is an  $\Omega$ -6 essential fatty acid that is thought to be the active ingredient of evening primrose oil (EPO). Previous studies using EPO as a dietary supplement have shown complete reversal of motor nerve conduction deficits in diabetic rats (82,83). EPO has also been found to increase prostacyclin release in diabetic sciatic nerve (84), increase nerve blood flow, reduce subsequent nerve trunk ischemia (85), and reduce resistance to ischemic conduction failure (86,87).

Given the known effects of TA in experimental diabetes (19,20) and the ability of EPO to reverse some of the deficits seen in experimental diabetes, the rationale for use of a TA- $\gamma$ -linolenic acid conjugate (thioctyl  $\gamma$ -linolenic acid; GLA<sup>TA</sup>) are not difficult to realize. However, in the search for compounds that are active against a broad spectrum of defects encountered in diabetes, no such compound has fit that bill. Therefore, *in vivo* studies with the conjugate were designed to reflect two classes of defect, with motor and sensory nerve conduction velocities indicating the polyol-nerve blood flow pathway and NGF and neuropeptides as markers of neurotrophic support.

*In vivo* studies evaluated the efficacy of dietary supplementation with 2.5% GLA<sup>TA</sup> (see Fig. 1 for structural representation), 100 mg/kg TA IP five times per week, and dietary supplementation with 1.5% butylated hydroxytoluene (BHT) in 8-week streptozotocin-induced diabetic rats.

Untreated diabetic animals showed both motor and sensory nerve conduction velocity deficits (Fig. 2A) and reduced levels of NGF, neuropeptide Y (NPY), and SP (Fig. 2B) in their sciatic nerves. Treatment with BHT was without effect on any of these variables. Treatment with TA increased the NGF content of sciatic nerve and produced small arithmetic increases in both SP and NPY, which did not attain significance. There was no effect on either sensory or motor nerve conduction velocity. Treatment with the GLA<sup>TA</sup> conjugate increased sciatic nerve levels of both SP and NPY, so that they were significantly higher than those measured in nerves from untreated diabetic rats ( $p < 0.05$ ), although they remained significantly lower than those of controls

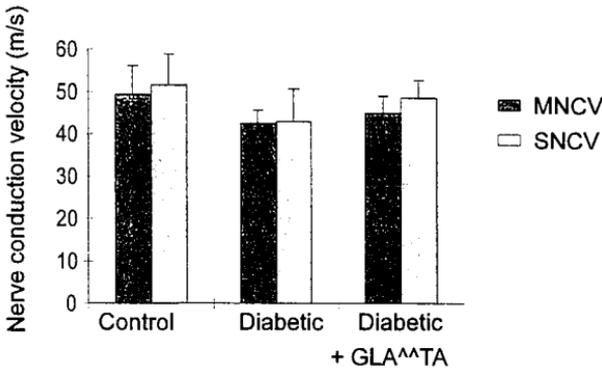


**Figure 1** Structure of thioctyl- $\gamma$ -linolenic acid (GLA<sup>^^</sup>TA). The molecule is a 1,2,3-propanediol diester with one molecule of GLA and one molecule of TA joined to the propanediol backbone by ester linkages.

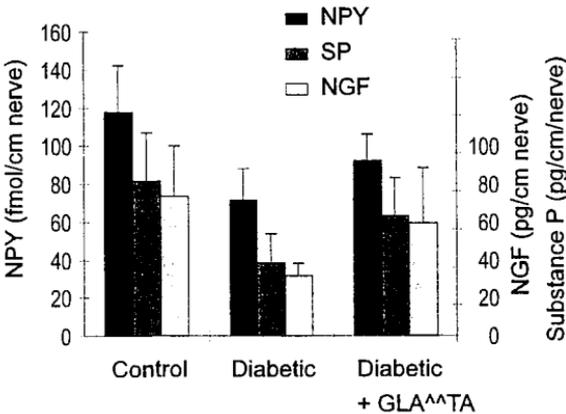
( $p < 0.05$  and  $0.01$  respectively) (Fig. 2B). GLA<sup>^^</sup>TA also increased NGF levels (Fig. 2B) and both motor and sensuous nerve conduction velocities (Fig. 2A), so that these values were not significantly different from those of control rats. The potential dependence of the neuropeptide changes on NGF levels was examined by regression analyses. For the dependence of SP on NGF,  $r^2$  was  $0.257$  ( $p < 0.005$ ), but the levels of NPY were less closely related to those of NGF, where  $r^2$  was  $0.145$  and the regression was barely significant ( $p < 0.05$ ).

NPY in the sciatic nerve may derive from mixed fiber populations—some must be in sympathetic postganglionic fibers (88,89), but there may also be some in somatic sensory fibers. However, the level of expression in somatic afferents is low unless they are damaged, when it increases (90); after axotomy, this increased expression may be reduced by either NT-3 or NGF (91,92). Expression of NPY by the sympathetic phenotype is clearly stimulated by NGF (93), and NGF-responsive elements have been identified on the NPY promoter (94). Thus, the findings reported here might be most easily explained by the proposition that the NPY deficit in sciatic nerves of diabetic rats is also derived from reduced NGF neurotrophic support. However, our previous study showed that treatment of diabetic rats with NGF, although normalizing the SP levels in sciatic nerve, did not affect the NPY deficit (20). Thus, there may be control of NPY expression in these fibers by another neurotrophin, and the NGF response elements become functional only when other influences are removed. This might explain the NGF effects on NPY in vitro (93,95) and increases in NPY after axotomy in vivo (90,96). Thus, the evolution of the decrease in NPY expression in our diabetic rats cannot be explained as yet, though the deficit clearly responds to treatment with the GLA<sup>^^</sup>TA conjugate and, in our previous study, also responded to TA (20).

A



B



**Figure 2** (A) Bar chart showing nerve conduction velocities in control, diabetic, and 2.5% GLA<sup>TA</sup>-treated diabetic rats. Control versus diabetic  $p < 0.01$  for both motor and sensory nerve conduction velocity; 2.5% GLA<sup>TA</sup> treated animals were not statistically different from controls or untreated diabetic rats by ANOVA with Duncan's multiple range tests. (B) Bar chart showing sciatic nerve levels of SP, NPY, and NGF. Control versus diabetic  $p < 0.01$  for SP, NPY, and NGF; 2.5% GLA<sup>TA</sup> increased sciatic nerve levels of SP and NPY ( $p < 0.05$ ), although they remained significantly different from controls,  $p < 0.05$  and  $p < 0.01$ , respectively. GLA<sup>TA</sup> 2.5% also increased NGF levels, so that these values were not different from controls.

## EFFECT OF THIOCTYL- $\gamma$ -LINOLENIC ACID ON THE ACTIVATION OF STRESS-ACTIVATED PROTEIN KINASES

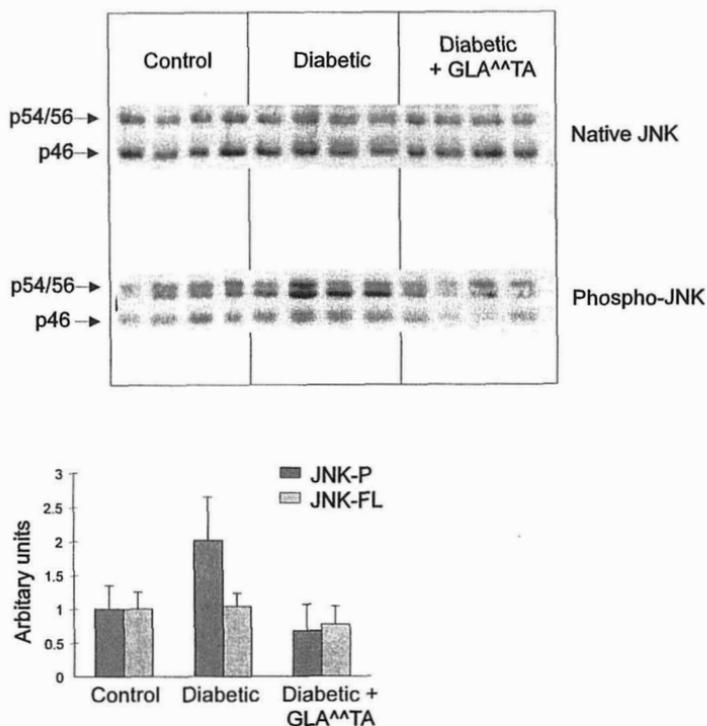
Mitogen-activated protein (MAP) kinases are proline-directed serine/threonine kinases that are activated by dual phosphorylation in response to a wide variety of extracellular stimuli. Three distinct groups of MAP kinases have been identified in mammalian cells; extracellular signal-regulated kinase (ERK), *c-jun* N-terminal kinase (JNK), and p38MAP kinase (p38) (see Refs. 97 and 98 for review). MAP kinase activation is achieved through kinase cascades, which serve as information relays connecting cell surface receptors to specific transcription factors and other regulatory proteins, thus allowing extracellular signals to regulate the expression of specific genes (99).

Recently, among this large family of MAP kinases, a family of stress-activated protein kinases (SAPKs), including JNK and p38, have been delineated and characterized (see Ref. 100 for review). JNK exists in three forms in mammalian cells: JNK1, JNK2, and JNK3 of 46, 54, and 56 kDa molecular weight, respectively. JNK has been found to be activated by a variety of different stimuli, including inflammatory cytokines such as interleukin-1 and tumor necrosis factor  $\alpha$ ,  $\gamma$ -irradiation, ultraviolet irradiation, and oxidative stress (101). Once activated, JNK phosphorylates a number of different substrates, including *c-jun* and ATF2. Both of these transcription factors can form part of the AP-1 transcription factor complex, which is known to regulate the transcription of many different genes (reviewed in Ref. 98). Because oxidative stress, as part of the dysmetabolism of diabetes mellitus, seemed an excellent candidate for activation of SAPKs, we included measurement of activation of these molecules in the present study.

The expression of the subtypes of JNK was investigated using antibodies raised against either the native protein (JNK-FL) of the phosphorylated (activated) form (pJNK) in the DRG from 8-week diabetic rats and diabetic rats treated with 2.5% GLA<sup>^^</sup>TA. Western blots were analyzed by densitometry and levels of protein compared by normalizing to controls.

Eight weeks of diabetes resulted in a significant increase in the level of the p54 form of activated JNK ( $p < 0.05$  compared with controls). This increase in activation of p54 JNK as measured by Western blotting with phospho-specific antibodies was reversed by 2.5% GLA<sup>^^</sup>TA (Fig. 3) Total levels of the full-length JNK protein were unchanged by diabetes or GLA<sup>^^</sup>TA treatment.

Levels of the phosphorylated transcription factors, ATF2, and *c-jun* were investigated using antibodies specific to the phosphorylated forms of these transcription factors and were also unchanged by diabetes or GLA<sup>^^</sup>TA treatment.



**Figure 3** Western blots from lumbar DRG exposed to antibodies against a nonphosphorylated epitope of JNK (JNK-FL) or a phosphorylated epitope (pJNK). Bar chart shows diabetes-induced increase in p54/p56 JNK phosphorylation ( $p < 0.05$  controls) can be reversed by GLA<sup>TA</sup>. There were no changes in the levels of the native protein, JNK-FL.

The reduction in the activation of JNK by GLA<sup>TA</sup> may reflect a reduction in neuronal stress. TA treatment is known to increase levels of GSH in diabetic sciatic nerve (19), and GSH is a critical regulator for the induction of SAPKs, including JNK (102). However, it is not yet known whether the conjugate has similar effects on glutathione metabolism, but this warrants closer investigation.

### VIII. ROLE FOR GLA- $\beta$ -TA IN THE TREATMENT OF DIABETIC NEUROPATHY

This is the first instance of a treatment that is capable of attenuating both electrophysiological and neurochemical deficits in the nerves of diabetic rats. The effect of the GLA- $\beta$ -TA conjugate is also remarkable when related to the current effects of TA and previously reported effects of EPO. Approximate calculations suggest that the dose of GLA- $\beta$ -TA used here could deliver about 14 mg/kg/day TA and 18 mg/kg/day GLA. TA must be given at doses of at least 50 mg/kg/day to influence nerve conduction (19), and in the present and in previous (20) studies had little effect on sciatic nerve NGF levels at 100 mg/kg/day. Assuming an approximate content of 10% GLA in EPO (83), a daily consumption of about 180 mg oil would be required to match the current dose of the conjugate. A previous study demonstrated no effect of EPO at about 3.5 g/day per rat (equivalent to about 10 g/kg/day) on the deficit in sciatic nerve SP in diabetic rats (82). It is therefore clear that the properties of the conjugate are significantly greater than those of its constituent molecules. We suspected that a membrane localization might be important and that was why BHT, a lipid-soluble antioxidant, was included in this study, but it showed no efficacy, indicating that simple membrane sequestration of TA does not provide an explanation for the efficacy of the conjugate. Furthermore, although NGF treatment of diabetic rats does not affect nerve conduction deficits, the conjugate had multiple protective effects on different fiber groups—*viz*, increasing nerve NGF levels, stimulating NPY expression by a different mechanism, and boosting deficient conduction velocities. Clearly, this molecule shows clinical potential.

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# 12

## Clinical Trials of $\alpha$ -Lipoic Acid in Diabetic Polyneuropathy and Cardiac Autonomic Neuropathy

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Polyneuropathy involving the somatic and autonomic nervous system is responsible for substantial morbidity and increased mortality among diabetic patients. Near normoglycemia is now generally accepted as the primary approach to prevention of diabetic neuropathy (1,2). However, in diabetic patients with advanced stages of peripheral neuropathy, relatively long periods of near-normal glycemic control for several months or even years may be needed to retard the progression of nerve dysfunction (3). Because normoglycemia is not achievable in most diabetic patients, the effects of several medical treatments derived from the pathogenetic concepts of diabetic neuropathy have been evaluated in numerous randomized clinical trials during the past two decades. However, due to various reasons, none of these compounds has been marketed as yet in the major European countries or in the United States. Nonetheless, in symptomatic diabetic neuropathy, additional pharmacological treatment of painful neuropathic symptoms is frequently required to maintain the patients' quality of life. Although treatment of pain with antidepressants is effective, it may be of limited value because of frequent adverse reactions (4). Other symptomatic approaches including anticonvulsants, mexiletine, and topical capsaicin either have not been unequivocally effective, have shown only partial effects, or caution has been expressed as to potential neurotoxic

side effects in view of longer term treatment (4). Furthermore, these medications are designed to modulate symptoms without influencing the underlying neuropathy.

Cardiovascular autonomic neuropathy (CAN) is a serious complication of diabetes that is associated with a poor prognosis and may result in severe clinical symptoms (5). Although CAN is appreciated as a clinical entity since 1945, in the past it has received less attention than peripheral sensorimotor neuropathy, one reason being that noninvasive quantitative and reliable methods for assessment of cardiovascular autonomic function are available since only the last two decades. After the introduction of cardiovascular reflex tests based on changes in heart rate variability (HRV) and blood pressure regulation into clinical routine, it became evident that CAN may be frequently detected at early stages in asymptomatic diabetic patients (5).

The question of long-term primary prevention of CAN has been previously addressed only in trials that studied the role of near normoglycemia in Type 1 diabetic patients. These studies have shown that the development of abnormalities in HRV can be prevented or retarded by intensive insulin therapy (1,6). Secondary intervention trials in patients with advanced CAN have demonstrated that its progression can be delayed during long-term near normoglycemia, but periods of more than 2 years are needed (3,5).

## **I. ROLE OF OXIDATIVE STRESS IN DIABETIC NEUROPATHY: CLINICAL AND EXPERIMENTAL EVIDENCE**

A growing body of evidence suggests that oxidative stress resulting from enhanced free radical formation and/or defects in antioxidant defense is implicated in the development of various disorders, including neurodegenerative diseases (7) and diabetic complications (8,9). Increased free radical formation and changes in hemostatic variables related to endothelial damage have been found in Type 2 diabetic patients with microalbuminuria (10). Impaired endothelium-dependent vasodilation is improved by administration of vitamin C, suggesting that nitric oxide inactivation by increased oxygen free radical activity contributes to abnormal vascular reactivity in diabetes (11). Furthermore, impaired cellular scavenging activity against oxidative stress (12) and elevated levels of plasma hydroperoxides in conjunction with a trend to lower vitamin E levels (13) have recently been demonstrated in patients with Type 2 diabetes.

In experimental diabetic neuropathy, oxygen free radical activity in the sciatic nerve is increased (9). Treatment with  $\alpha$ -lipoic acid, a potent lipophilic

free radical scavenger (14), results in prevention of neurovascular abnormalities associated with experimental diabetic neuropathy (15). It has been demonstrated that reduced digital nerve conduction velocity (NCV), nerve blood flow, and glutathione levels in diabetic rats are normalized and in vitro lipid peroxidation of neural tissue reduced by  $\alpha$ -lipoic acid in a dose-dependent manner (15,16), suggesting that the improvement in neurovascular changes were induced by improving oxygen free radical scavenging activity. One mechanism of reduced nerve blood flow is the inhibitory effect of superoxide anion on nitric oxide synthase. Because nitric oxide synthase is reduced in experimental diabetic neuropathy (17),  $\alpha$ -lipoic acid might prevent this inhibition by reducing oxidative stress (15). A recent study has also demonstrated that treatment with  $\alpha$ -lipoic acid may correct neuropeptide deficits in diabetic rats, indicating that the compound may boost neurotrophic support (18). Administration of low doses of an  $\alpha$ -lipoic acid–gamma-linolenic acid conjugate corrects the nerve conduction and nerve blood flow deficits (19) and sciatic nerve contents of nerve growth factor, substance P, and neuropeptide Y (20) in diabetic rats, suggesting a marked synergistic action of these compounds. These experimental findings provide the rationale for a potential therapeutic value of  $\alpha$ -lipoic acid in diabetic patients with neuropathy.

## **II. MULTICENTER CONTROLLED CLINICAL TRIALS**

Randomized clinical trials (RCTs) are a widely accepted means of applying experimental methods to a clinical setting and have been advocated as the gold standard for comparing and evaluating different treatments (21). However, the quality of the RCTs that evaluated the effects of medical treatment in diabetic polyneuropathy was poor. Cavaliere et al. (22) assessed the quality of scientific evidence for the efficacy of various pathogenetically oriented treatment approaches for diabetic polyneuropathy examined in RCTs published between 1981 and 1992. They used a quality system covering the internal (scientific) validity (the ability to demonstrate a treatment effect if it really exists) and external validity (the possibility of generalizing the study results to patients seen in clinical practice). The analysis based on 38 RCTs in total revealed a devastating picture: The methods of randomization were unspecified and a detailed a priori estimate of the sample size needed to detect a treatment difference was not reported in 95% of the RCTs, respectively. Only 11% of the RCTs had sufficient statistical power to detect a clinically meaningful difference (22). More generally spoken, a recent analysis by Freiman et al. (23) revealed that only 4 of 71 controlled clinical trials (5.6%) that reported nega-

**Table 1** Multicenter, Randomized, Double-Blind, Placebo-Controlled Trials Using  $\alpha$ -Lipoic Acid in Diabetic Peripheral and Cardiac Autonomic Neuropathy

	ALADIN Study <sup>a</sup>	DEKAN Study <sup>b</sup>	ALADIN 3 Study <sup>c</sup>
Number	328	73	509
Design	Four parallel groups	Two parallel groups	Three parallel groups
Dose	1,200/600/100 mg $\alpha$ /P I.V.	800 mg $\alpha$ /P PO	600 IV/1800 mg PO( $\alpha$ - $\alpha$ / $\alpha$ -P/P-P)
Duration of treatment	3 wk	4 mo	3 wk + 6 mo
Effect	Symptoms+, NDS+	HRV+, TSS	TSS, NIS+

$\alpha$ ,  $\alpha$ -Lipoic acid; P, placebo; +, improvement; IV, intravenous; PO, orally.

<sup>a</sup>Diabetologia, 38, 1995.

<sup>b</sup>Diabetes Care, 20, 1997.

<sup>c</sup>Diabetes Care, 22, 1999.

tive results ( $p > 0.1$ ) in major medical journals between 1960 and 1977 included a sample size large enough to have a 90% chance of detecting a 25% difference in treatment effects. In another analysis, the situation did not improve as the number was 4 of 65 trials (6.2%) (23). Thus, as recently stated by Altman and Bland (24), absence of evidence is not evidence of absence. In other words, to interpret most published RCTs as providing evidence of an ineffectiveness of the respective treatment in diabetic neuropathy would be clearly misleading, because the likelihood to detect differences in the parameters of nerve function between the treatment groups was too low in view of the small sample sizes.

Adequate designs for RCTs in diabetic neuropathy have to consider the following aspects: type and stage of neuropathy, homogeneity of the study population, outcome measures (neurophysiological markers, intermediate clinical end points, ultimate clinical outcomes, quality of life), natural history, sample size, study duration, reproducibility of neurophysiological and intermediate end points, regression to the mean, true and perceived placebo effects, measures of treatment effect, and the generalizability of the overall trial result to individual patients (25). The results of three multicenter RCTs that evaluated the effects of  $\alpha$ -lipoic acid in Type 2 diabetic patients with polyneuropathy are summarized and discussed below (Table 1).

## A. ALADIN Study

The improvement in neuropathic symptoms during a 3-week period of intravenous treatment with 600 mg  $\alpha$ -lipoic acid/day as compared with vitamin B<sub>1</sub>

(26) in conjunction with the recent experimental evidence (15) formed the rationale for a large-scale, 3-week, multicenter, randomized, double-blind, placebo-controlled trial (*Alpha-Lipoic Acid in Diabetic Neuropathy: ALADIN*) (27). Assuming from previous studies a placebo response of about 30% and a drug response of about 60%, the a priori estimate of the required sample size yielded  $n = 67$  patients per group, with  $\alpha = 0.05$  and  $\beta = 0.1$  for the two-tailed log-rank test.

The efficacy and safety of intravenous infusion of  $\alpha$ -lipoic acid were evaluated in 328 NIDDM outpatients with symptomatic peripheral neuropathy who were randomly assigned to treatment using three doses ( $\alpha$ -lipoic acid 1200 mg/day, 600 mg/day, 100 mg/day) or placebo. Neuropathic symptoms (pain, burning, paresthesias, and numbness) were assessed by the total symptom score (TSS) at baseline and each visit (days 2–5, 8–12, and 15–19) before infusion. In addition, the Hamburg Pain Adjective List (HPAL), a multidimensional specific pain questionnaire, and the Neuropathy Symptom and Disability Scores (NDS) were assessed at baseline and at day 19 (27).

According to the protocol, 260 patients ( $n = 65$ , 1200 mg/day;  $n = 63$ , 600 mg/day;  $n = 66$ , 100 mg/day;  $n = 66$ , placebo) completed the study. No significant differences were noted for the mean changes in HbA<sub>1c</sub> and blood glucose levels between the four groups studied. TSS in the feet decreased from baseline to day 19 (mean  $\pm$  SD) by  $-4.5 \pm 3.7$  ( $-58.6\%$ ) points in  $\alpha$ -lipoic acid 1200,  $-5.0 \pm 4.1$  ( $-63.5\%$ ) points in  $\alpha$ -lipoic acid 600,  $-3.3 \pm 2.8$  ( $-43.2\%$ ) points in  $\alpha$ -lipoic acid 100, and  $-2.6 \pm 3.2$  ( $-38.4\%$ ) points in placebo ( $\alpha$ -lipoic acid 1200 vs. placebo,  $p = 0.003$ ;  $\alpha$ -lipoic acid 600 vs. placebo,  $p < 0.001$ ). The response rates after 19 days, defined as an improvement in the TSS of at least 30%, were 70.8% in  $\alpha$ -lipoic acid 1200, 82.5% in  $\alpha$ -lipoic acid 600, 65.2% in  $\alpha$ -lipoic acid 100, and 57.6% in placebo ( $\alpha$ -lipoic acid 600 vs. placebo,  $p = 0.002$ ). The total scale of the HPAL was significantly reduced in  $\alpha$ -lipoic acid 1200 and  $\alpha$ -lipoic acid 600 as compared with placebo after 19 days (both  $p < 0.01$ ). Detailed analysis of pain revealed that both the affective and sensory components of pain, representing pain experience and pain perception, could be improved. NDS decreased by  $-1.8 \pm 0.3$  points in  $\alpha$ -lipoic acid 1200, by  $-1.5 \pm 0.3$  points in  $\alpha$ -lipoic acid 600, by  $-0.9 \pm 0.3$  in  $\alpha$ -lipoic acid 100, and by  $-1.0 \pm 0.2$  in placebo after 19 days ( $p = 0.03$  for  $\alpha$ -lipoic acid 1200 vs. placebo). The rates of adverse events were 32.6% in  $\alpha$ -lipoic acid 1200, 18.2% in  $\alpha$ -lipoic acid 600, 13.6% in  $\alpha$ -lipoic acid 100, and 20.7% in placebo. These findings demonstrate that parenteral treatment with  $\alpha$ -lipoic acid over 3 weeks using a dose of 600 mg/day in NIDDM patients is associated with a significant reduction of various symptoms of peripheral neuropathy including pain, paresthesias, and numbness as

compared with placebo. Furthermore, it is evident that a dose of 100 mg/day does not exert an effect superior to that seen with placebo. An increase in the dosage to 1200 mg/day is associated with an enhanced rate of adverse events rather than with maximized efficacy. The increased risk of gastrointestinal side effects associated with 1200 mg/day precludes from using this dose (27).

It may be argued that nerve conduction studies have not been used in this study as objective measures of neuropathy. However, electrophysiological changes are not relevant in patients with pain. In addition, in a short-term study of this kind, a significant difference between the groups treated with  $\alpha$ -lipoic acid and the placebo group regarding NCV would not appear likely to occur. Previous studies using drugs such as the aldose reductase inhibitors have shown that NCV was either unchanged (28) or only a minimal increase was seen within several weeks of treatment (29) that was subject to substantial criticism as to whether it represented a clinically meaningful degree of change or merely a physiological variation (30). By contrast, neuropathic symptoms have been shown susceptible to intervention within a few weeks. Painful symptoms but not motor and sensory NCV were improved after 4 weeks of treatment with sorbinil as compared with placebo (28), and withdrawal of tolrestat resulted in a rapid worsening of pain scores (31).

It may also be argued that the exclusion of 51 patients due to failures to adhere to the protocol potentially could have introduced bias. However, an additional analysis of the results of the ALADIN Study based on the intention to treat revealed no appreciable differences in the outcome of the parameters studied when compared with the per-protocol analysis, indicating that the adherence to the study protocol did not introduce bias. In summary, it has been demonstrated that in diabetic subjects a relief of neuropathic symptoms can be achieved by short-term intravenous with  $\alpha$ -lipoic acid.

## B. DEKAN Study

An effective treatment aimed at improving or retarding the progression of reduced HRV, the hallmark and earliest sign of CAN, might potentially favorably influence the poor prognosis among these patients.

The efficacy and safety of  $\alpha$ -lipoic acid were studied in a randomized, double-blind, placebo-controlled, multicenter trial (*Deutsche Kardiale Autonome Neuropathie: DEKAN Study*) in NIDDM patients with CAN (32). Inclusion criteria were age  $\geq 18$  and  $\leq 70$  years, NIDDM treated with diet, oral antidiabetic agents and/or insulin, spectral power of HRV in the low-frequency band (0.05–0.15 Hz) and/or high-frequency band (0.15–0.5 Hz) below

the 2.5 centile of age-related normal ranges (33). Eligible patients were randomly assigned to treatment with an oral dose of 800 mg/day (200 mg four times daily)  $\alpha$ -lipoic acid ( $n = 39$ ) or placebo ( $n = 34$ ) for 4 months. Parameters of HRV at rest, including the coefficient of variation (CV), root mean squared successive difference (RMSSD), and spectral power in the low-frequency (0.05–0.15 Hz) and high-frequency (0.15–0.5 Hz) bands, and the QTc interval were assessed at baseline, 2 weeks, and at the end of each month of study using a validated computer system (33). Seventeen patients ( $\alpha$ -lipoic acid,  $n = 10$ ; placebo,  $n = 7$ ) dropped out of the study, but only 3 ( $\alpha$ -lipoic acid,  $n = 1$ ; placebo,  $n = 2$ ) of these dropouts were due to adverse reactions.

Mean blood pressure, heart rate, and HbA<sub>1c</sub> levels did not differ between the groups during the study. RMSSD increased from baseline to 4 months by 1.5 (–37.6–77.1) ms (median [min–max]) in the group treated with  $\alpha$ -lipoic acid and decreased by –0.1 (–19.2–32.8) ms in the placebo group ( $p < 0.05$  for  $\alpha$ -lipoic acid vs. placebo). Power spectrum in the low-frequency band increased by 0.06 (–0.09–0.62) bpm<sup>2</sup> (beats per minute squared) in  $\alpha$ -lipoic acid, whereas it declined by –0.01 (–0.48–1.86) bpm<sup>2</sup> in placebo ( $p < 0.05$  for  $\alpha$ -lipoic acid vs. placebo). Furthermore, there was a trend toward a favorable effect of  $\alpha$ -lipoic acid versus placebo for the CV and for the high-frequency band power spectrum. QTc interval was shortened insignificantly by –8.8 (–87.9–46.5) ms in  $\alpha$ -lipoic acid and remained unchanged in placebo (0.0 [–118–77.9]). No differences between the groups were noted regarding the rates of adverse events. These findings suggest that oral treatment with  $\alpha$ -lipoic acid for 4 months using a well-tolerated dose of 800 mg/day in NIDDM patients is associated with a significant (two indices) or borderline (two indices) improvement in HRV but not QTc interval as compared with placebo (32).

Apart from the DEKAN study, the effects of antioxidants on CAN have not been previously examined in a controlled clinical trial. However, a recent study has demonstrated that glutathione, a physiological antioxidant, normalizes reduced HRV during the squatting test (baroreflex changes) induced by acute hyperglycemia in healthy nondiabetic subjects (34). Presumably, such an effect is mediated by a reduction in free radical activity. In the isolated perfused diabetic rat heart, treatment with vitamin E completely prevented a progressive loss of histofluorescent nerve fibers in the myocardium and intraneural catecholamines (35). There are no studies using  $\alpha$ -lipoic acid in experimental diabetic cardiac neuropathy, but the compound normalizes reduced glucose uptake and glucose utilization and consequently oxygen uptake, myocardial ATP levels, and cardiac output in the isolated diabetic rat heart model

(36). However, it is not known whether and to which degree these experimental findings relate to the chronic process that characterizes human diabetic autonomic neuropathy.

### C. ALADIN 3 Study

This was a randomized, double-blind, placebo-controlled, multicenter trial including type 2 diabetic outpatients with symptomatic distal symmetric polyneuropathy. Patients were allocated to three parallel groups receiving  $\alpha$ -lipoic acid or placebo: group 1 ( $n = 167$ ),  $\alpha$ -lipoic acid 600 mg intravenously for 3 weeks, followed by  $\alpha$ -lipoic acid 600 mg three times a day orally for 6 months; group 2 ( $n = 174$ ),  $\alpha$ -lipoic acid 600 mg intravenously for 3 weeks, followed by placebo orally for 6 months; group 3 ( $n = 168$ ), placebo intravenously for 3 weeks, followed by placebo orally for 6 months. At baseline, there were no significant differences between the groups regarding the demographic variables such as age, sex, body mass index, duration of diabetes, and HbA<sub>1c</sub>. After 3 weeks, the Neuropathy Impairment Score (NIS) decreased significantly from baseline by  $-4.34 \pm 0.35$  ( $\pm$  SEM) in the patients treated with  $\alpha$ -lipoic acid and by  $-3.49 \pm 0.58$  points in the placebo group ( $p = 0.016$  for  $\alpha$ -lipoic acid vs. placebo). The NIS of the lower limbs (NIS[LL]) declined by  $-3.32 \pm 0.26$  on  $\alpha$ -lipoic acid and  $-2.79 \pm 0.42$  points on placebo ( $p = 0.055$ ). After completion of the 6-month oral phase, the reduction in the NIS was  $-5.82 \pm 0.73$  in group 1,  $-5.76 \pm 0.69$  in group 2, and  $-4.37 \pm 0.83$  points in group 3 ( $p = 0.095$  for group 1 vs. 3), whereas the reduction in NIS[LL] was  $-4.39 \pm 0.51$  in group 1,  $-4.20 \pm 0.52$  in group 2, and  $-3.37 \pm 0.54$  points in group 3 ( $p = 0.086$  for group 1 vs. 3). Regarding the TSS, no significant differences were noted between the groups at baseline and after 6 months. These results indicate a trend toward an improvement of neuropathic deficits but not symptoms after 6 months of oral treatment with  $\alpha$ -lipoic acid (600 mg three times a day) after a 3-week intravenous phase in type 2 diabetic patients with polyneuropathy. The percentages of adverse events were comparable in the three groups throughout the study.

### III. FUTURE ASPECTS

Dyck et al. (37) recently calculated the sample size needed in a clinical trial based on the 2-year follow-up of the Rochester Diabetic Neuropathy Study. The estimates were derived from the changes of a composite score including NIS of the lower limbs plus seven tests (NIS[LL]+7: VPT great toe; R-R

variation to DB; peroneal CMAP, MNCV, and MNDL; tibial MNDL; and sural SNAP) that among other measures performed best at showing monotone worsening over time. Assuming that a treatment effect of two NIS points is clinically meaningful, a 2-year study would need 68 patients in each treatment arm to have a power of 0.90 at the two-sided 0.05 level. If the effect of treatment is to halt the progression of neuropathy without improving it, a study of 3.7 years would be required. A 4-year study would require 45 patients per arm to achieve power of 0.90 to detect a treatment effect that inhibits progression of neuropathy, and a clinically meaningful effect could be expected after approximately 2.4 years. Thus, a conservative estimate would yield 70–100 patients per arm for a period of at least 3 years to achieve a high probability of detecting a clinically meaningful effect (37). A 4-year RCT evaluating the effects of  $\alpha$ -lipoic acid in diabetic polyneuropathy (Neurological Assessment of Thioctic Acid in Diabetic Neuropathy 1 Study) has been designed on the basis of these estimates. The design of this trial is summarized in Table 2.

In conclusion, it is conceivable that the initial diabetes-related changes in the nerve are mediated by oxidative stress that, on a long-term basis, could result in progressive neuronal damage and therefore would be of pathogenetic relevance. Studies in proof of the promising results reported herein are needed. An ongoing pivotal long-term trial of oral treatment with  $\alpha$ -lipoic acid designed along the recent guidelines of the Peripheral Nerve Society (38) aimed at slowing the progression of clinical neuropathy using a reliable clinical end

**Table 2** Outline of the NATHAN 1 (Neurological Assessment of Thioctic Acid in Diabetic Neuropathy) Study

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Design: Randomized, double-blind, placebo-controlled, multicenter trial
Subjects: Two parallel groups of type 1 or type 2 diabetic patients ( $n = 500$ enrolled)
Medication: Thioctic acid 600 mg or placebo tablets once daily orally
Duration: Screening, 2 wk; placebo run-in, 6 wk; treatment, 192 wk; follow-up, 4 wk (interim analysis, at 96 wk)
Inclusion criteria: Stage 1 or 2a polyneuropathy [NIS(LL) + 7 $\geq$ 97.5 centile; TSS $\leq$ 5]
Primary outcome measure: NIS(LL) + 7 tests score (VDT; HBDB; peroneal CMAP, MNCV, and MNDL; tibial MNDL; sural SNAP)
Secondary outcome measures: NSC, TSS, CDT, HP, other NIS and NC

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VDT: vibration detection threshold; HBDB: heart beat to deep breathing; CMAP: compound muscle action potential; MNCV: motor nerve conduction velocity; MNDL: motor nerve distal latency; SNAP: sensory nerve action potential; NSC: neuropathy symptoms and changes; CDT: coding detection threshold; HP: heat as pain; NC: nerve conduction.

point addresses the question as to whether the observed improvement in neuro-pathic symptoms and autonomic dysfunction can be translated into long-term effects on objective neurophysiological parameters and neuropathic deficits.

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# 13

## Oxidative Stress, NF- $\kappa$ B Activation, and Late Diabetic Complications

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Oxidative stress is widely believed to play a central role in the pathogenesis of late diabetic complications. Recently, the understanding of oxidative stress in diabetes has been improved by the availability of assays exactly determining defined products of reactive oxygen species. These studies have revealed oxidative stress to occur before diabetic complications are present, further supporting the concept that oxidative stress is pivotal for the development of diabetic complications. Studies looking at the oxidative stress-activated transcription factor NF- $\kappa$ B help to understand the cellular consequences of oxidative stress at the molecular level. However, the occurrence of oxidative stress and oxygen species-mediated secondary end products are not sufficient proof for the hypothesis of oxidative stress-dependent diabetic complications. It has to be demonstrated that antioxidant therapy does not only reduce plasmatic markers of oxidative stress and subsequent NF- $\kappa$ B activation but also late diabetic complications.

## I. OXIDATIVE STRESS IN PATIENTS WITH DIABETES MELLITUS

Several studies have shown that increased production of reactive oxygen species and antioxidant depletion occurs in patients with diabetes mellitus (1–11). Oxidative stress may lead to endothelial cell damage and vascular dysfunction through various mechanisms (12–32).

Lately, considerable effort has been devoted to gain insights into the role of oxidative stress in the development and progression of late micro- and macrovascular complications in diabetes (17,23,27,28,31–34). Although hyperglycemia is an acknowledged pathogenic factor in diabetic complications, it is not known through which mechanism an excess of glucose results in tissue damage. Accumulating data support the hypothesis that oxidative stress might play an important role in the pathogenesis of late diabetic complications. Several pathways are leading to oxidative stress associated with acute or chronic hyperglycemia, such as the polyol pathway, prostanoid synthesis, glucose autooxidation, and protein glycation by increasing the production of free radicals (35–39). A close relationship of oxidative stress with glycemic control has been described, showing a significant positive correlation between malondialdehyde (MDA) and both fasting blood sugar and glycosylated hemoglobin (40). Moreover, many injurious effects of hyperglycemia on endothelial functions, such as delayed cell replication, impaired endothelial cell-dependent relaxation, and the activation of NF- $\kappa$ B, are reversed by antioxidants (38,41).

Several studies indicate that not only increased production of free radicals but also the depletion of antioxidative capacities may play an important role in the pathogenesis of late diabetic complications (42,43). In this regard, a prospective study has described an association between low lipid-standardized  $\alpha$ -tocopherol levels and the incidence of type 2 diabetes, suggesting a link between hyperglycemia-induced depletion of antioxidants and the pathology of diabetes (44). Recently, it has been shown that oxidative stress appears to be primarily related to the underlying metabolic disorder, occurring before manifestation of late diabetic complications, consistent with the idea that oxidative stress is an early event in the pathology of diabetes and its complications (45). The imbalance between lipid peroxidation products and antioxidant capacities in diabetic patients has been demonstrated using a precise technique for measurement of plasma lipid hydroperoxides. A significantly higher ratio of hydroperoxides to cholesterol-standardized  $\alpha$ -tocopherol has been found in diabetics compared with control subjects (45). Further, it has been described that endothelial dysfunction is associated with oxidant injury and tubular dam-

age and may precede microalbuminuria in development of diabetic nephropathy (46). Free radicals produced by the system myeloperoxidase/hydrogen peroxide/halogen derivatives activate proteinases, which break down collagen and other components of the extracellular matrix present in the basal membrane of the glomeruli and in the mesangium. It has been shown that hydroxyl radicals may depolarize glomerular heparan sulfate *in vitro* and in experimental nephrotic syndrome, leading to loss of glomerular basement membrane integrity and albuminuria (47). Thus, oxygen radicals and proteinases can cause and amplify glomerular damage.

## **II. HYPOTHESIS OF ADVANCED GLYCATION END PRODUCTS AND ITS RECEPTOR**

### **A. Advanced Glycation End Products**

Advanced glycation end products (AGEs) are a heterogeneous group of irreversible adducts resulting from nonenzymatic glycation and oxidation of proteins, lipids, and nucleic acids. Glucose and other reducing sugars react in a nonenzymatic reaction (Maillard reaction) with the N-terminal residues and/or  $\epsilon$ -amino groups of proteins initially forming a Schiff base. Rearrangement of this aldimine leads after a short time to the formation of more stable but still reversible Amadori adducts. The open chain of the resulting ketoamin can react with other amino groups. Oxidation, dehydration, and condensation reactions finally lead to the production of irreversible crosslinks, which are proteinase resistant.

The formation of AGEs *in vitro* and *in vivo* depends on the turnover rate of the modified substrate, sugar concentration, and time. Recent studies have shown that AGEs can be formed not only at long-living proteins but occur also on short-living proteins (48), peptides (48), lipids (49), and nucleic acids (50–52).

AGE formation and protein crosslinking alter the structural and functional properties of proteins, lipid components, and nucleic acids. AGEs have also been shown to induce cellular signaling, activation of transcription factors, and consequently gene expression *in vitro* and *in vivo* (32). They have been suggested to represent general markers of oxidative stress and long-term damage to proteins and to induce pathogenic changes in endothelial cells. Thus, AGEs are not only markers but also mediators of chronic vascular diseases and late diabetic complications.

## B. Formation of AGEs in Diabetes

AGE formation proceeds slowly under normal glycemic conditions but is enhanced in the presence of hyperglycemia, oxidative stress, and/or conditions in which protein and lipid turnover are prolonged. For example, *N*-epsilon-(carboxymethyl)lysine (CML), one of the various AGE structures postulated to date, has been found to be a product of both glycooxidation (combined non-enzymatic glycation and oxidation) and lipid peroxidation reactions (53). CML and pentosidine have been shown to accumulate in diabetic kidneys in colocalization with a marker of lipid peroxidation (MDA), suggesting an association of local oxidative stress with the etiology of diabetic glomerular lesions (54). Evidence for an age-dependent increase in CML accumulation in distinct localizations and acceleration of this process in diabetes has been provided by immunolocalization of CML in skin, lung, heart, kidney, intestine, intervertebral discs, and particularly in arteries (55). In diabetic kidneys, AGEs were preferentially localized in vascular lesions (56), renal cortex (57), expanded mesangial areas (58), and the glomerular basement membrane (56–59). An increased CML content in serum proteins of diabetic patients (55) and a correlation of serum AGE levels with the progressive loss of kidney function was found (60). The increased formation of tissue AGEs has been described to precede and to correlate with early manifestations of renal and retinal complications in patients with diabetes (61).

Increased levels of AGE-modified low-density lipoprotein (LDL) with a markedly impaired clearance have been found in the plasma of diabetic patients, suggesting a pathway for pathogenic modification of LDL (62). The mediating role of AGEs in development of late diabetic complications (Table 1) (49,63–70) has been studied in animal models by short- and long-term administration of AGEs. Short-term administration of AGEs led to increased vascular permeability and leakage, impaired endothelial relaxation, subendothelial mononuclear recruitment, activation of NF- $\kappa$ B, and subsequent VCAM-1 gene expression (71–73). Long-term administration of AGEs resulted in arteriolar basement thickening and complex vascular dysfunction (74) and in glomerular basement thickening, mesangial expansion, glomerulosclerosis, and proteinuria (68).

## C. AGE–RAGE Interactions

The principal means through which AGEs exert their cellular effects is via specific cellular receptors (Table 2). One of them, the receptor for AGE (RAGE), a 35-kDa protein, is also expressed by endothelial cells (23,75,76).

**Table 1** The Role of AGEs in Diabetic Complications

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Formation of HbA <sub>1c</sub> as a marker of production of AGEs: poor glycemic control increases formation of AGEs and AGE-dependent cell activation (37,41,63)
Toxic effects of AGEs on retinal endothelial cells (64) and positive correlation between accumulation of AGEs, expression of vascular endothelial growth factor, and nonproliferative and proliferative diabetic retinopathy (65)
Inhibition of development of experimental diabetic retinopathy by aminoguanidine treatment (49).
Excessive deposition of intra- and extracellular AGEs in human diabetic peripheral nerve (66)
Inhibition of AGE formation prevents diabetic peripheral nerve dysfunction (67)
Accumulation of AGEs in the kidney of diabetic patients (56,60)
Injection of AGE-albumin in normal rats induces symptoms of diabetic nephropathy (68)
Blocking of AGE binding to RAGE reduces albuminuria (69)
Inhibitor AGEs reduces urinary albumin excretion, mesangial expansion, and glomerular basement membrane thickening (70)

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**Table 2** AGE Binding Proteins and Their Localization

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AGE binding proteins	Localization
AGE-R <sub>1</sub> (OST-48)	Monocytes/macrophages, endothelial cells, T lymphocytes, mesangial cells, neurons
AGE-R <sub>2</sub> (80KH)	Monocytes/macrophages, endothelial cells, T lymphocytes, fibroblasts, mesangial cells, neurons
AGE-R <sub>3</sub> (Galectin-3 or GBP-35)	Monocytes/macrophages, endothelial cells, T lymphocytes,
RAGE	Endothelial cells, monocytes/ macrophages, smooth muscle cells, mesangial cells, neurons, T lymphocytes, erythrocytes
Lactoferrin, lysozyme	Endothelial cells
Fructosylline-specific binding protein	Monocytes
Macrophage scavenger receptor	Macrophages

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An induction of endothelial RAGE expression has been shown on vessels from patients with arteriosclerosis, diabetes, uremia, and vasculitis (77–79). Binding of AGEs to their cellular binding sites results in generation of oxygen free radicals and depletion of antioxidants such as glutathione and ascorbate (32,80). The consequently enhanced cellular oxidative stress leads to activation of the redox-sensitive transcription factor NF- $\kappa$ B in endothelial cells, smooth muscle cells, mesangial cells, and monocytes/macrophages (23,25,32,78–81).

### III. ACTIVATION OF NF- $\kappa$ B

The multiprotein complex NF- $\kappa$ B resides as an inactive form in the cytoplasm associated with its inhibitor, I $\kappa$ B (Table 3). NF- $\kappa$ B translocates to the nucleus after phosphorylation and proteolytic degradation of I $\kappa$ B. NF- $\kappa$ B activation is modulated by redox reactions that increase the cytosolic phosphorylation, and degradation of I $\kappa$ B and requires a thioredoxin-dependent status in the nucleus (82–85). NF- $\kappa$ B-dependent genes and their products [include] I $\kappa$ B $\alpha$ , RAGE, cytokines (tumor necrosis factor- $\alpha$ , interleukin-6 and -8), adhesion molecules (VCAM-1, ICAM-1, ELAM), receptors for coagulation factors such as the procoagulant tissue factor endothelin-1, inducible nitric oxide synthase, inducible cyclooxygenase, heme oxygenase type 1, and 5-lipoxygenase (23,77,86). Because transcription of I $\kappa$ B $\alpha$  is autoregulated by NF- $\kappa$ B (87), activation of NF- $\kappa$ B terminates itself (86,88), leading to a short-living acute cellular response. Recent studies showed that I $\kappa$ B $\beta$  mediates a more sustained activation of NF- $\kappa$ B that lasts up to 48 h (89,90).

Activation of NF- $\kappa$ B and induction of increased binding activity of NF- $\kappa$ B are believed to have a pivotal role in the pathogenesis and progression of chronic diseases, such as diabetes and atherosclerosis (39,86,91,92). Accumu-

**Table 3** Proteins of the NF- $\kappa$ B and I $\kappa$ B Families

Proteins of the NF- $\kappa$ B family	Proteins of the I $\kappa$ B family
P50 (p150)	I $\kappa$ B $\alpha$
P52/p49 (p100)	I $\kappa$ B $\beta$
P65 (relA)	I $\kappa$ N $\gamma$
c-rel	I $\kappa$ B $\epsilon$
relB	I $\kappa$ B-R

lating data indicate a close link between hyperglycemia, oxidative stress, formation of AGEs, and induction of NF- $\kappa$ B to the etiology of late diabetic complications. Increased glucose concentration has been shown to induce NF- $\kappa$ B activation in endothelial cells (38) and to increase NF- $\kappa$ B binding activity in peripheral blood mononuclear cells isolated from diabetic patients with poor glycemic control (37), suggesting that NF- $\kappa$ B activation is an early event in response to elevations in glucose contributing to diabetes-induced endothelial cell injury.

AGEs interacting with endothelial cell RAGE have been identified as relevant mediators of NF- $\kappa$ B activation by generating intracellular oxidative stress (39,41,76,80). Recently, it has been shown that the binding of AGEs or amyloid- $\beta$  peptides to RAGE leads to perpetuated NF- $\kappa$ B activation in vitro and in vivo resulting in a 1-week translocation of NF- $\kappa$ B (p50/p65) from the cytoplasm into the nucleus (39). The AGE-RAGE-mediated NF- $\kappa$ B activation was initiated by the degradation of both I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ . The key event in maintaining the activation of NF- $\kappa$ B is the induction of de novo synthesis of p65-mRNA, leading to a constantly growing pool of free NF- $\kappa$ Bp65. Thus, AGEs are capable of activating NF- $\kappa$ B in vitro and in vivo, pointing to a central role of AGE-mediated NF- $\kappa$ B activation in late diabetic complications.

#### **IV. INHIBITION OF DIABETIC COMPLICATIONS BY ANTIOXIDANT TREATMENT**

In patients with diabetes mellitus, oxidative stress is increased by enhanced production of free radicals and by antioxidant depletion, resulting in an increased susceptibility to oxidative damage and possibly development of late diabetic complications. Endogenous antioxidant proteins such as superoxide dismutase, glutathione peroxidase, and metal-binding proteins may protect the body against the effect of prooxidant reactions. Multiple antioxidants, including  $\alpha$ -lipoic acid, vitamins C and E, urate, carotenoids, flavonoids, the amino acid methionine, and protein-bound zinc and selenium, are interacting additively in these biological systems. In vitro and in vivo studies using antioxidants support the concept of radical-mediated diabetic complications.

##### **A. Vitamin E**

Vitamin E is the most abundant antioxidant in LDL. In vitro it scavenges peroxy radicals 10,000-fold avidly, and then these react with fatty acids. But

in LDL, vitamin E is located in the more rigid outer layer of the particle, whereas the free radicals seem to accumulate in the more fluid core and might therefore not be scavenged effectively by the antioxidant. However, vitamin E has been shown to inhibit lipid peroxidation and to reduce protein glycation in diabetic patients (93–95).

Intervention studies have shown that vitamin E treatment could prevent early changes of diabetic glomerular dysfunction in diabetic rats through activation of diacylglycerol kinase, decreasing diacylglycerol and protein kinase C levels (96,97). Further, vitamin E has been demonstrated to improve significantly diabetes-induced abnormal contractility and endothelial dysfunction (98) and to exhibit cardiovascular protection in diabetes (99). Thus, these studies support the concept of oxidative stress-mediated diabetic complications.

## B. Vitamin C

Vitamin C is the most effective water-soluble antioxidant in the organism. It acts as a potent electron donor, which is then reduced back to ascorbic acid primarily by glutathione. Ascorbate is the first antioxidant consumed in plasma exposed *in vitro* to aqueous peroxy radicals, followed by sulfhydryl groups, urate, and vitamin E. Vitamin C supplementation has been shown to regenerate vitamin E and to increase glutathione levels (94).

Treatment with vitamin C improved endothelium-dependent vasodilatation in patients with insulin-dependent diabetes mellitus (100) and decreased albuminuria, glomerular transforming growth factor- $\beta$ , and glomerular size in diabetic rats (101). These data are in support for a role of water-soluble antioxidants in diabetic complications.

## C. $\alpha$ -Lipoic Acid

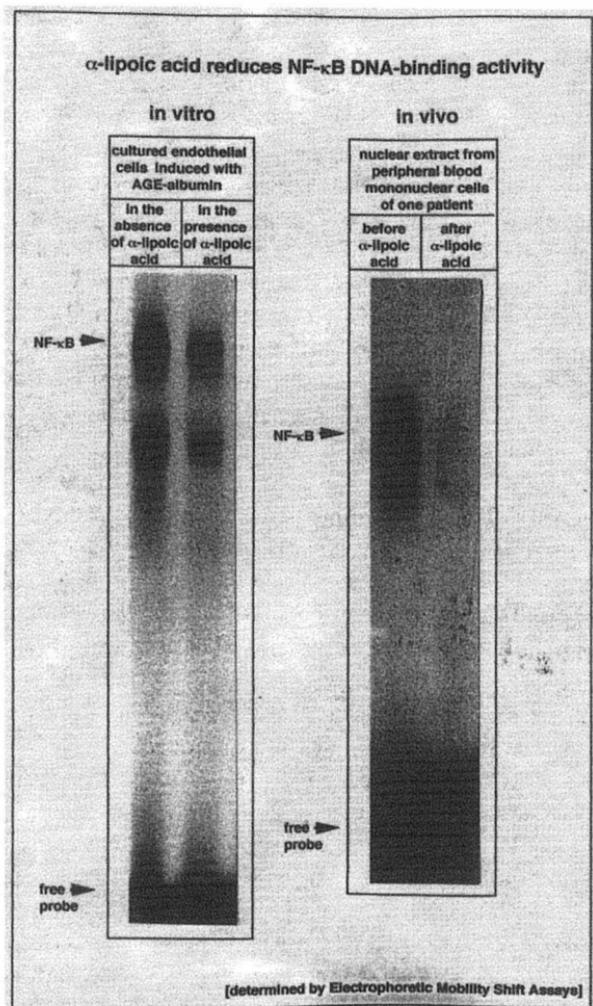
$\alpha$ -Lipoic acid occurs naturally in physiological systems as a cofactor for enzymatically catalyzed acyl transfer reactions. It has powerful antioxidant actions *in vitro* and *in vivo* (102,103).  $\alpha$ -Lipoic acid exists in oxidized and reduced forms and regenerates  $\text{NAD}^+$  from  $\text{NADH}$  (104–106). It acts as a universal antioxidant both in the membrane and the aqueous phase by reducing peroxy, ascorbyl, and chromanoxyl radicals (107) and by decreasing microsomal lipid peroxidation (108).  $\alpha$ -Lipoic acid participates in establishing a cellular antioxidant network, raising intracellular glutathione levels, reducing the oxidized amino acid cysteine to cystine and regenerating other important antioxidants such as the vitamins C and E. On the cellular level,  $\alpha$ -lipoic acid has also been shown to prevent single oxygen-induced DNA damage (104,109). Also

of interest, and related to diabetic complications, are the findings regarding the effects of  $\alpha$ -lipoic acid with respect to the glucose homeostasis and the production of AGEs (110).  $\alpha$ -Lipoic acid has been found to reduce glycemia and to stimulate glucose uptake and transport activity in skeletal muscle both human and experimental diabetes (111,112).

One of the earliest events in atherogenesis is the adhesion of monocytes to the endothelium and its migration into the arterial intima. Endothelin-1, which is increased in diabetes and is believed to be relevant for the progression of nephropathy (113), has been shown to increase monocyte chemotaxis in a dose-dependent manner (97).  $\alpha$ -Lipoic acid inhibits migration (114).  $\alpha$ -Lipoic acid has also been shown to be an effective inhibitor of aldose reductase (115). Aldose reductase inhibitors have been suggested to prevent or reduce the different components of vascular dysfunction, cataract, neuropathy, and nephropathy in animal models of diabetes.

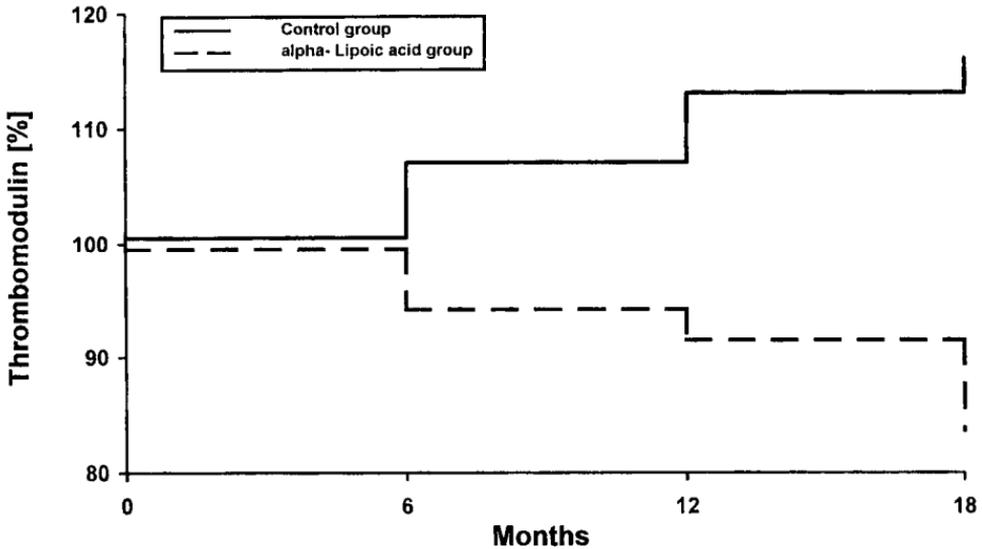
Several intervention studies have been performed to establish the role of  $\alpha$ -lipoic acid as a powerful antioxidant in diabetes. Therapeutic effects of  $\alpha$ -lipoic acid in the prevention of diabetic retinopathy and cataract have been described (116). The neuroprotective effect of  $\alpha$ -lipoic acid in the treatment of symptomatic diabetic peripheral neuropathy by reducing oxidative stress and improving nerve blood flow and distal nerve conduction is well documented (117–119). The increased blood flow is consistent with the vascular protective effect of  $\alpha$ -lipoic acid.

Recently, it has been reported that  $\alpha$ -lipoic acid completely prevented the AGE-dependent depletion of glutathione and ascorbate in vitro and reduced in a time- and dose-dependent manner the AGE albumin-mediated activation of NF- $\kappa$ B in endothelial cells as long as  $\alpha$ -lipoic acid was added at least 30 min before AGE albumin stimulation (Fig. 1) (41). It was shown that the inhibition of NF- $\kappa$ B activation was not due to physical interactions with protein DNA binding, because  $\alpha$ -lipoic acid did not prevent binding activity of recombinant NF- $\kappa$ B when it was included directly into the binding reaction. Furthermore, it was demonstrated by Western blots that  $\alpha$ -lipoic acid inhibited the release and translocation of NF- $\kappa$ B from the cytoplasm into the nucleus. In addition,  $\alpha$ -lipoic acid reduced the NF- $\kappa$ B-mediated transcription of tissue factor and endothelin-1, both of them being relevant for endothelial cell dysfunction in diabetes (41). Ongoing studies demonstrated that  $\alpha$ -lipoic acid-dependent downregulation of NF- $\kappa$ B is also evident in monocytes of diabetic patients under  $\alpha$ -lipoic acid therapy (Fig. 1) (37,41). Thus,  $\alpha$ -lipoic acid reduces oxidative stress-dependent NF- $\kappa$ B activation in vitro and in vivo. It is of interest that these effects are present even in patients with poor glycemic control.



**Figure 1** Inhibition of NF- $\kappa$ B DNA binding activity by  $\alpha$ -lipoic acid in vitro (left) and in vivo (right).

The effects of  $\alpha$ -lipoic acid on the development of late diabetic complications have been studied with respect to the progressive endothelial cell damage and albuminuria in patients with diabetes mellitus (120). The progression of endothelial cell damage has been evaluated in a pilot study over 18 months that assessed the course of plasma thrombomodulin as a marker of endothelial

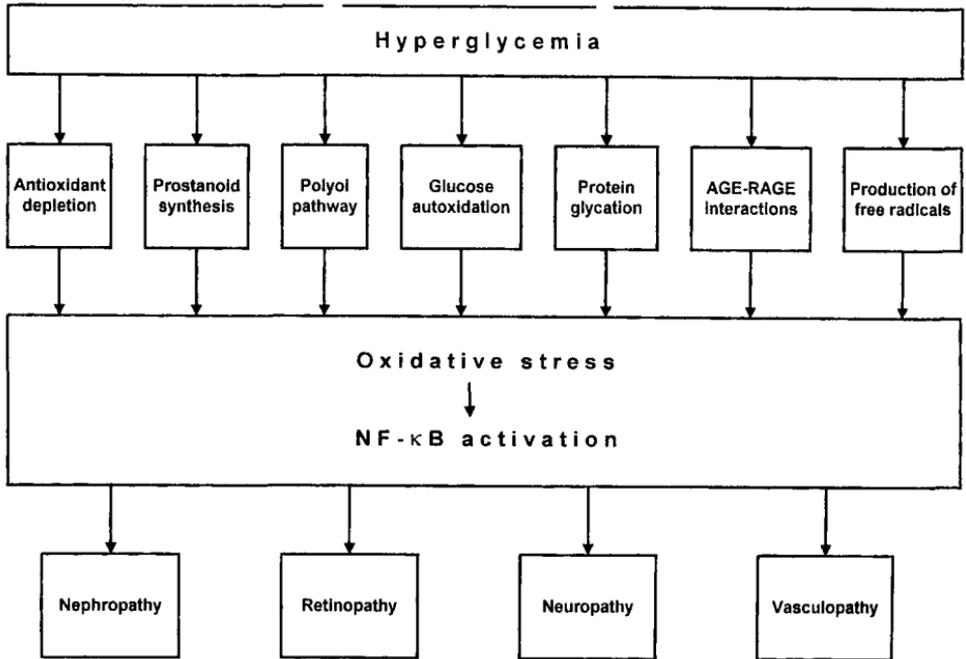


**Figure 2** Progression of thrombomodulin in 205 patients with diabetes mellitus studied over 18 months according to the use of  $\alpha$ -lipoic acid expressed as relative values (% of study entry values). —,  $n = 151$  patients without  $\alpha$ -lipoic acid treatment; ---,  $n = 54$  patients treated with 600 mg/day  $\alpha$ -lipoic acid.

injury. It has been shown that  $\alpha$ -lipoic acid significantly reduced the time-dependent increase of plasma thrombomodulin that was seen in the control patients (Fig. 2). Treatment with  $\alpha$ -lipoic acid was found to be the only factor significantly predicting a decrease of the urinary albumin concentration and a decrease of plasma thrombomodulin in multiple regression analysis (120).

## V. SUMMARY

There is increasing evidence that oxidative stress plays a major role in the development of late diabetic complications. Oxidative stress generated by hyperglycemia, AGEs, or other factors of cellular activation results in activation of NF- $\kappa$ B activation correlates in diabetic patients with glucose control and can be reduced by treatment with  $\alpha$ -lipoic acid. The vasculoprotective action of  $\alpha$ -lipoic acid supports the hypothesis shown in Figure 3.



**Figure 3** Possible linkage between hyperglycemia-induced oxidative stress, subsequent activation of NF- $\kappa$ B, and development of secondary complications in patients with diabetes mellitus.

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# 14

## Role of Oxidative Stress and Antioxidants on Adhesion Molecules and Diabetic Microangiopathy

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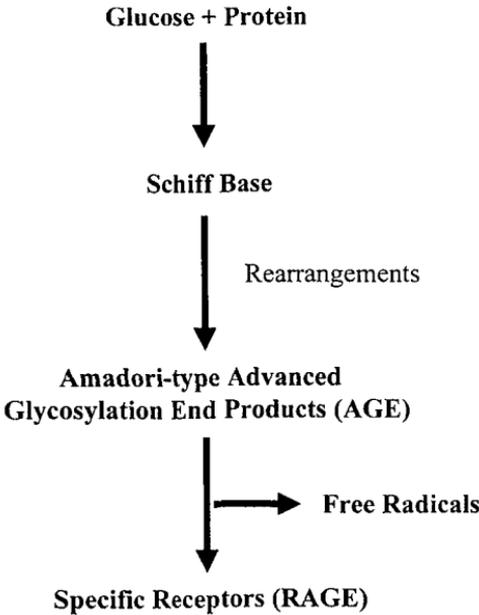
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Each cell can mobilize an armory of antioxidant defense systems. Under normal metabolic conditions, the production of free radicals and the antioxidant capacity are balanced. Hyperglycemia in diabetes mellitus is associated with an increased production of free radicals. Furthermore, observational studies indicate lower levels of antioxidants like vitamin E, vitamin C, carotene, ascorbate, and thiols in patients with diabetes mellitus (1,2). Imbalance between free radical production and the antioxidant defense system leads to oxidative stress. In diabetic patients, oxidative stress can be demonstrated by increased levels of lipid peroxidation products (3–8). There is a body of evidence that vascular and neurological complications in patients with diabetes mellitus are a consequence of oxidative stress (9–12).

### I. FREE RADICALS AND DIABETES MELLITUS

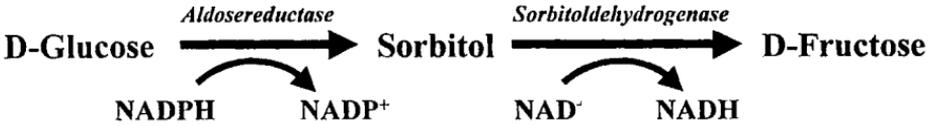
Various sources of free radicals are considered in patients with diabetes mellitus. Free radicals are produced during autooxidation (10). Furthermore, glu-



**Figure 1** The production of AGEs. The interaction of AGEs with their specific receptors (RAGEs) generates free radicals.

Glucose is known to form glycosylation products with protein (Fig. 1). These Schiff bases rearrange and form more stable Amadori-type glycosylation end products (13). Some of these early glycosylation end products on collagen or proteins of the vessel wall undergo a complex series of chemical rearrangements to form irreversible advanced glycosylation end products (AGE). Receptors specific for AGEs (RAGE) have been identified on endothelial cells, monocytes, neurons, and smooth muscle cells (14–20). AGE–RAGE interaction induces free radicals (18).

The sorbitol pathway is another mechanism involved in glucotoxicity (21) (Fig. 2). Glucose is reduced to sorbitol by aldose reductase. Then sorbitol is oxidized by sorbitol dehydrogenase to fructose. The second reaction is coupled with the reduction of  $\text{NAD}^+$  to  $\text{NADH}$  (Fig. 1). The pathophysiological consequences are similar to changes during ischemia, and the increased  $\text{NADH}/\text{NAD}^+$  ratio has been termed pseudohypoxia (11). During hyperglycemia, the sorbitol pathway activity is increased. The increased ratio of  $\text{NADH}/$



**Figure 2** Sorbitol oxidation increases cytosolic NADH/NAD<sup>+</sup>, which is linked to hyperglycemic pseudohypoxia (11).

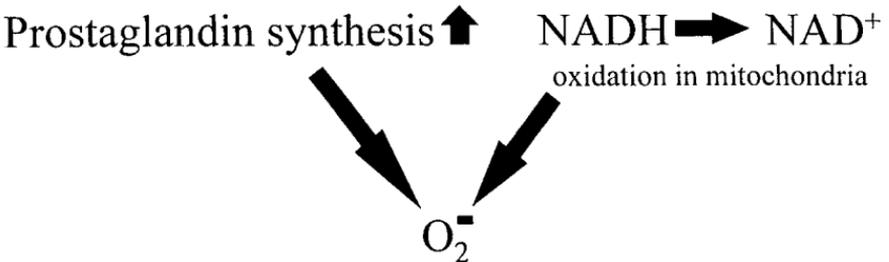
NAD<sup>+</sup> enhances the synthesis of prostaglandins, which leads to free radical production. Further, the oxidation of NADH to NAD<sup>+</sup> in the electron transport chain in the mitochondria involves increased production of superoxide radical (Fig. 3).

The superoxide radical is normally catalyzed by superoxide dismutase to hydrogen peroxide. The iron-dependent Fenton reaction leads to the aggressive hydrogen peroxide radical that further attacks the side chains of lipids,

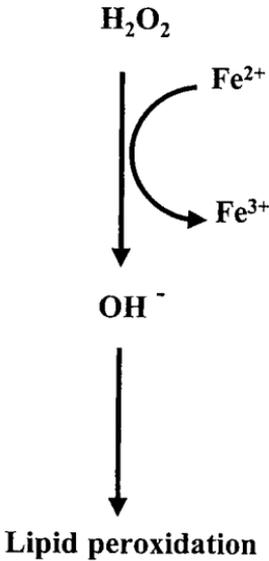
## Hyperglycemia

Sorbitol pathway activity ●

NADH/NAD<sup>+</sup> ↑



**Figure 3** Hyperglycemia activates the sorbitol pathway. Sorbitol oxidation increases cytosolic NADH/NAD<sup>+</sup>. Increased NADH/NAD<sup>+</sup> ratios increase prostaglandin synthesis, leading to free radical production. In the mitochondria, the superoxide radical is generated by oxidation of NADH in the electron transport chain.

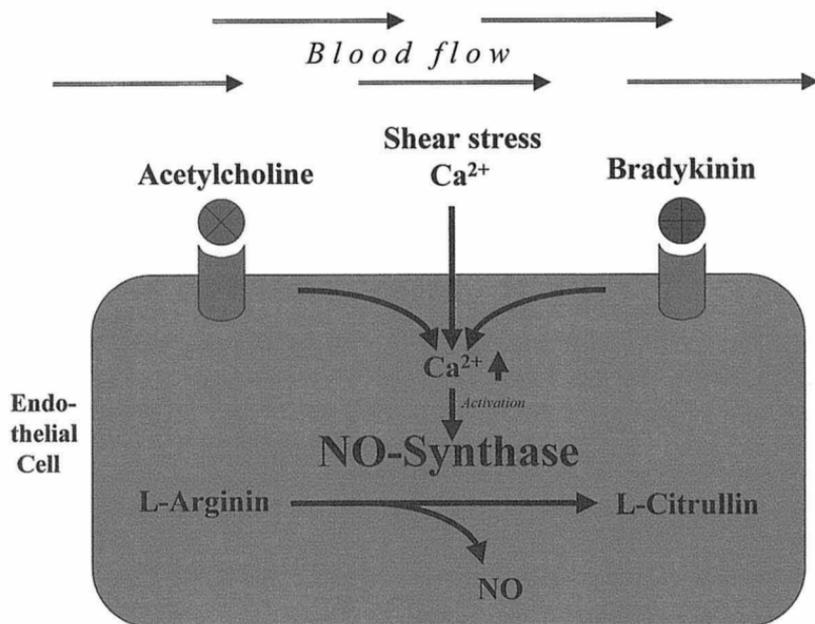


**Figure 4** The superoxide radical is rapidly dismutized to  $\text{H}_2\text{O}_2$  by superoxide dismutase. The iron-dependent Fenton reaction produces a hydrogen peroxide radical, which induces lipid peroxidation.

resulting in lipid peroxidation products (22) (Fig. 4). Patients with diabetes mellitus have increased lipid peroxidation products (3–8).

## II. ENDOTHELIUM-DEPENDENT VASODILATION

In physiological terms, hyperglycemia increases blood pressure and leads to endothelial dysfunction with impaired vascular reactivity (23). Hypoxia is accompanied with an influx of calcium, which might activate nitric oxide (NO) synthase followed by vasodilation and hyperemia (11) (Fig. 5). In diabetes, increased free radicals might quench NO, leading to ischemia (11,24). In vitro acetylcholine-induced vasodilation of vasculature from diabetic animals is impaired (25,26). The rate of NO synthesis in vivo compared with the rate of NO quenching is unclear. We measured the blood flow of the arteria iliaca in diabetic and nondiabetic rats. The NO-mediated stimulation by acetylcholine was impaired in diabetic rats, but in contrast to in vitro experiments, treatment



**Figure 5** The increase of cytosolic calcium activates nitric oxide synthase.

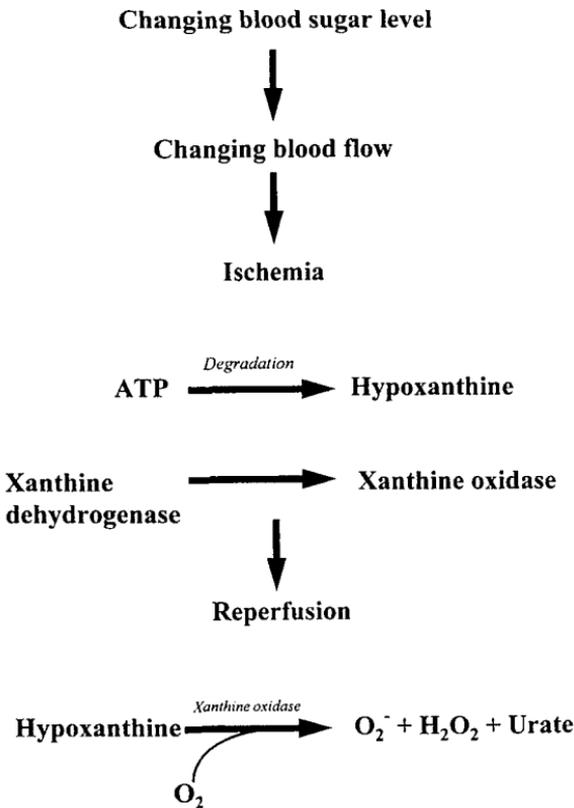
with antioxidants did not restore the impaired endothelium-dependent vasodilation (unpublished results).

### III. ISCHEMIA-REPERFUSION IN DIABETES MELLITUS

Microvascular dysfunction has been studied extensively in animal models. One of the most widely used models is streptozotocin-induced diabetes in the rat. In this model, rats are treated with a single toxic dose of streptozotocin, which destroys the islets of Langerhans (27). The effect of hyperglycemia on the microvasculature is then evaluated after 4–12 weeks (21,28–37). Glycemic control by insulin treatment can prevent the microvascular dysfunction. In diabetic humans, metabolic status is not stable, and episodes of normoglycemia are followed by phases of hyper- and hypoglycemia. Thus, blood sugar levels vary considerably, and in consequence to the pathophysiological changes presented above, the status of oxidative stress changes and blood flow will go up and down. Therefore, it is reasonable to assume that under realistic conditions, a diabetic patient undergoes episodes of ischemia and reperfusion

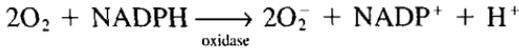
during the time until manifestation of microangiopathy. The time intervals of the increased blood sugar levels are sufficient to induce leukocyte adherence to the vascular endothelium.

We therefore propose an additional pathophysiological approach to the understanding of microangiopathy (Fig. 6). A patient with diabetes mellitus has frequent episodes of ischemia followed by reperfusion. During ischemia, there is a loss of energy-rich phosphates leading to an accumulation of hypoxanthine (38). In the endothelial cell, xanthine dehydrogenase is converted to xanthine oxidase. During reperfusion, superoxide radical and hydrogen perox-



**Figure 6** Changes in blood sugar are associated with changes in blood flow. Frequent episodes of hyperglycemia in a diabetic patient will induce frequent episodes of ischemia–reperfusion. Ischemia–reperfusion generates free radicals and induces leukocyte adherence to vascular endothelium followed by tissue injury.

ide are produced by the oxidation of hypoxanthine. In addition, ischemia–reperfusion induces the adherence of leukocytes to the endothelium. The adherent leukocytes migrate to the tissue of the vessel wall and release their inflammatory mediators, including free radicals. Free radicals are produced by leukocytes by the following reaction:

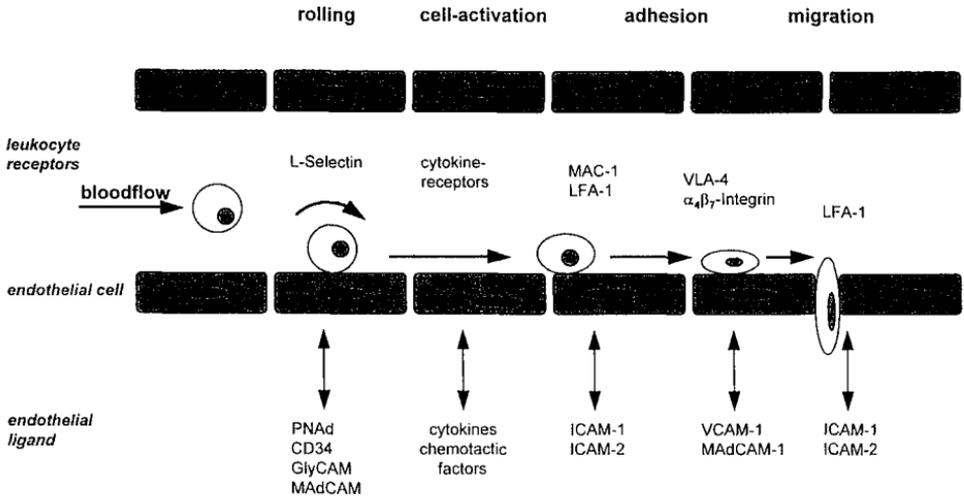


To test this hypothesis, we first evaluated the effect of lipoic acid on leukocyte adherence induced by ischemia–reperfusion. Lipoic acid was chosen because its beneficial effect in diabetic neuropathy has been demonstrated in animals and multicenter trials of clinically manifested polyneuropathy. Lipoic acid is an effective radical scavenger (39). Nerve conduction and blood flow of the nerves improved by the treatment with lipoic acid (37). In multicenter clinical trials, symptoms in diabetic patients with polyneuropathy improved (40). Ischemia–reperfusion experiments were performed in groups of rats receiving either solvent propylenglycol or 25, 50, or 100 mg/kg intravenous (IV) lipoic acid, respectively, 30 min before the beginning of the ischemia. Ischemia of 30 min was produced by means of a plastic ring in mesentery vessels. With *in vivo* microscopy, leukocyte adherence was measured during 10, 20, and 30 min of reperfusion. Leukocyte adherence was reduced dose dependently by lipoic acid. Diabetic animals were treated either with propylenglycol or lipoic acid at 100 mg/kg intraperitoneally (IP) 5 days a week for 1 month. After 1 month of treatment with propylenglycol IP, the acute pretreatment with lipoic acid 100 mg/kg IV did not prevent the increased leukocyte adhesion in diabetic rats. Treatment with lipoic acid (100 mg/kg/day IP) from the induction of diabetes combined with an IV bolus 10 min before the experiment reduced ischemia–reperfusion-induced leukocyte adhesion from  $7550 \pm 1073/\text{mm}^2$  vein cross-section to  $1774 \pm 840$  ( $p < 0.001$ ).

In conclusion, the therapeutic agent for polyneuropathy, lipoic acid, reduces reperfusion injury. Thus, ischemia–reperfusion caused by changing blood sugar levels might contribute to the pathogenesis of diabetic polyneuropathy, and the beneficial effects of lipoic acid in diabetic polyneuropathy might be partially explained by the inhibition of leukocyte adherence.

#### IV. ADHESION MOLECULES

The migration of leukocytes from the bloodstream into inflamed tissue requires a cascade of events in the microcirculation (41). The sequence of bind-



**Figure 7** The migration of leukocytes from the bloodstream to the tissue starts with rolling of the leukocytes, which is mediated by selectins. Chemoattractants or cytokines increase the expression of adhesion molecules on the endothelium or increase the avidity of the integrins on the leukocytes, leading to adhesion of leukocytes to the endothelium and finally to emigration from the bloodstream.

ing events starts with leukocytes rolling along the endothelium (Fig. 7). This first step is mediated by selectins (42–47). Upon activation by inflammatory signals such as chemoattractants, rolling progresses to firmer adhesion, due to interaction of the integrins on leukocytes with adhesion molecules of the immunoglobulin family, such as ICAM-1 or VCAM-1, on the endothelium. Monoclonal antibodies that interfere with different steps of the cell-binding cascade have shown a beneficial effect on ischemia–reperfusion injury (48).

Enhanced expression of ICAM-1 and P-selectin has been demonstrated in the diabetic human retina and choroid (49). This increased expression of cell adhesion molecules may contribute to the retinal and choroidal microangiopathy in diabetic patients. A potential mechanism for the accelerated vasculopathy has been proposed recently (14). The interaction of AGEs with their endothelial receptor induced the expression of VCAM-1 in cultured human endothelial cells and in mice (14). AGE–RAGE interaction generates free radicals that activate NF- $\kappa$ B. NF- $\kappa$ B regulates in addition to other genes the expression of VCAM-1. The incubation of human umbilical vein endothelial cells with AGE increased the endothelial expression of VCAM-1. Preincubation of the cells with lipoic acid suppressed VCAM-1 expression to baseline

levels (50). The AGE-induced endothelial binding of monocytes was also reduced by lipoic acid (50).

Soluble adhesion molecules are detectable only in small quantities in the serum of healthy individuals (51,52). However, increased serum levels of soluble adhesion molecules have been described in different pathologic situations, including ischemia–reperfusion injuries (53,54), insulin-dependent (IDDM) (55), and non–insulin-dependent diabetes mellitus (NIDDM) (56). In IDDM, circulating ICAM-1 and VCAM-1 but not ELAM-1 was elevated. The increased levels were found in IDDM patients with and without microangiopathy. In first-degree relatives of NIDDM, the levels of E-selectin relates to vascular risk in contrast to soluble VCAM-1, which showed no difference compared with control subjects (56). Thus, elevated levels of circulating adhesion molecules can be used as a risk marker for developing microangiopathy. The pathophysiological role of the circulating adhesion molecules is unclear. It might be that they just indicate endothelial cell stimulation by oxidative stress or that they are a reaction to protect the endothelium from further leukocyte attack. The second hypothesis might be reasonable because we could recently demonstrate that leukocyte adhesion during ischemia–reperfusion can be reduced by the application of naturally occurring soluble recombinant ICAM-1 (57).

## **V. CONCLUSION**

In line with many other investigators, oxidative stress is an important pathogenic factor for the development of microangiopathy in diabetic patients. In addition to the well-characterized sources of free radicals, we propose a new concept for free radical production: Changes of metabolic control are coupled to episodes of ischemia–reperfusion with subsequent free radical production and leukocytes adherence. Lipoic acid prevents increased leukocyte adherence in diabetic and nondiabetic rats, which might contribute to its beneficial effect in diabetic neuropathy.

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## Molecular Basis of $\alpha$ -Tocopherol Action and Its Protective Role Against Diabetic Complications

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Atherosclerosis is a pathology frequently associated with diabetes (1–4). Migration and proliferation of smooth muscle cells (smc) from the media to the intima of the arterial wall take place early at the beginning of the atherosclerotic process (5).

Proliferation of smc is accompanied by an increased expression of extracellular matrix protein, which contributes to the development of diabetic vascular complications (6–8). Several studies have shown beneficial effects of antioxidant vitamins, in particular vitamin E, in protection against the vascular complications seen in diabetic patients (9–12).

In recent years, the mechanism of action of vitamin E has been discussed (13,14) because its simple role as an antioxidant and protective component of the cell membrane (15) is becoming insufficient to explain the action of this compound.

Vitamin E deficiency leads to premature cell aging and to the binding of immunoglobulin G to erythrocytes (16), and it has been shown to play an important role in porphyrin metabolism (17). Moreover, reports suggest an effect of vitamin E on the arachidonic acid cascade, related to the biosynthesis of prostaglandins (18). A specific neuropathology ataxia with vitamin E defi-

ciency (AVED) has also been found to be due to familiar vitamin E deficiency (19–21).

In our laboratory we found that  $\alpha$ -tocopherol (the most active form of vitamin E) inhibits vascular smooth muscle cell proliferation (22–25). smc are under the control of several growth factors that activate a cascade of kinases and phosphatases ending with the activation of transcription factors (such as AP-1 and NF- $\kappa$ B) and those proteins involved in the cell cycle progression, in particular protein kinase C (PKC) (26).

First described 20 years ago as a proteolytically activated serine/threonine kinase, PKC still justifies the attention of researchers, providing new surprises. The enormous number of weekly publications requires a continuous effort to summarize and simplify the knowledge on the field. Since its original discovery, PKC has expanded into a family of closely related proteins, which can be subdivided on the basis of certain structural and biochemical properties. The original members of the PKC family are the  $\text{Ca}^{2+}$ -dependent or conventional (c)  $\alpha$ ,  $\beta_1$ ,  $\beta_{II}$ , and  $\gamma$  isoforms. Later, with the discovery of  $\text{Ca}^{2+}$ -independent PKC isoenzymes, the group of a new or novel (n) isoforms has been classified, including  $\epsilon$ ,  $\eta$ , and  $\theta$  PKCs. Finally, there are the so-called atypical (a)  $\zeta$ ,  $\tau$ ,  $\lambda$ , and  $\mu$  isoforms, which take an intermediate position between the nPKC and aPKC isoforms. For a complete description of the structures, there are several recent reviews (27–29).

PKC has played an important role in the field of cancer research since the discovery that the tumor-promoting phorbol ester class of compounds caused PKC activation. For the first time, a connection between the process of signal transduction and tumor promotion could be made. Since then, PKC isoforms have been implicated in several pathways not always related to carcinogenesis. Today, the observation that individual isoenzymes are located in different subcellular compartments and undergo different regulation strongly suggests that each isoform has a unique individual role. For example, PKC $\mu$  has been found to be associated with the B-cell antigen receptor complex (30), PKC $\eta$  is thought to mediate transcriptional activation of the human transglutaminase 1 gene (31), and PKC $\theta$  has been described to stimulate the transcription factor complex AP-1 in T lymphocytes (32). Implication of specific PKC isoenzymes has also been shown in pathological conditions such as Alzheimer's (33–36), atherosclerosis (37–39), and diabetes (40–42).

In our laboratory we observed the inhibition of PKC activity and smc proliferation by physiological concentrations of  $\alpha$ -tocopherol. By using isoform-specific inhibitors and activators, we found that PKC $\alpha$  is the selective target of  $\alpha$ -tocopherol action.  $\beta$ -Tocopherol, an antioxidant almost as potent as  $\alpha$ -tocopherol, did not show any effect on cell proliferation of PKC

activity, suggesting that the mechanism of action of  $\alpha$ -tocopherol is not related to its antioxidant properties. Our results could offer a model to explain the beneficial effects of vitamin E on diabetic vascular complication.

## I. METHODS

Rat A7r5 aortic vascular smc were obtained from American Type Culture Collection. Purified PKC $\alpha$  and protein phosphatase type 2A were from UBI (New York). Tissue culture media and polyclonal antibodies to PKC $\alpha$ ,  $\delta$ , and  $\epsilon$  isoforms were from Life Technologies, Inc. (Grand Island, NY). Fetal calf serum (FCS) was from PAA (Linz, Austria). Anti-rat PKC  $\zeta$  polyclonal antibody, purified PKC $\alpha$ , and trimeric PP2A were from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-PKC $\mu$  antibody, caliculin A, okadaic acid, phorbol 12-myristate 13-acetate, and Gö 6976 were from LC Laboratories (Woburn, MA). Ly379196 was a gift of Eli Lilly (Indianapolis, IN). ( $\gamma$ - $^{32}\text{P}$ ) ATP (3000 Ci/mmol),  $^{32}\text{P}_i$ , monoclonal anti-PKC $\alpha$  (clone MC5), ECL detection system, and ECL Hyperfilm were from Amersham International (Buckinghamshire, UK). Anti-protein kinase C $\alpha$  rabbit polyclonal used for the kinase reactions of the immunoprecipitated protein was from Oxford Biomedical Research, Inc. (Oxford, MI). Phosphorylase- $\beta$ , phosphorylase kinase, streptolysin-O, Histone III-SS, and phorbol dibutyrate were from Sigma (St. Louis, MO). C $_2$ -ceramide was from BioMol (Hamburg, Germany). Phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, and aprotinin were from Böhringer (Mannheim, Germany). Protein A-Sepharose 4B was from Pharmacia Biotech Inc. PKC $\zeta$  antisense oligodeoxynucleotide was from MWG Biotech (Ebersberg, Germany). The peptide PLSRTLVAACK used as substrate for assay PKC activity was synthesized by Dr. Servis (Epalinges, Switzerland). Myelin basic protein (4-14 fragment) was produced by Bachem (Switzerland). RRR- $\alpha$ -tocopherol and RRR- $\beta$ -tocopherol are obtained from Henkel (LaGrange, IL). Tocopherols are adsorbed to FCS before the addition to the cells, as described (43). Protein concentration was determined using a Pierce kit according to the manufacturer's procedures.

### A. Cell Cultures

Rat A7r5 cells are maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1.0 g/L glucose, 60 U/mL penicillin, 60 mg/mL streptomycin, and supplemented with 10% (v/v) FCS. Cells in a subconfluent state were made quiescent by incubation in DMEM containing 0.2% FCS for 48

h. Cells were then washed with phosphate-buffered saline (PBS) and treated as indicated in the figure legends. Cell viability, determined by Trypan blue exclusion method, was 90–95% in all experiments. Cells are used between passages 7 and 15.

## **B. Animals**

Thirty male albino rabbits aged 2–4 months were assigned randomly to one of the following four groups. All rabbits were fed 100 g/day vitamin E-free diet as described (44,45). One group of rabbits was only fed the diet without addition of treatments. The second group received daily injections of 50 mg/kg vitamin E. The diet of the third group contained 2% cholesterol, and the fourth received the same diet with daily injections of 50 mg/kg vitamin E. After 4 weeks, the thoracic aortas were removed, and media strips were minced, homogenized, and cytosolic and membrane extracts were prepared for analysis of PKC activity (46).

## **C. Cell Proliferation**

Quiescent A7r5 cells were restimulated to grow by addition of 10% FCS.  $\alpha$ -Tocopherol was added to cells at the indicated concentrations. Cell number was determined 30 h later by using a hemocytometer. To measure DNA synthesis, cells were pulsed for 1 h with [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci}/\text{well}$ ) during the S phase (11 h after entry into the cycle). After labeling, cells were processed as described (22), and radioactivity was determined.

## **D. PKC Activity in Permeabilized Cells**

Quiescent A7r5 cells were subjected to different treatments as indicated in the figure legends. During the last hour of the preincubation period, cells were treated with 100 nM phorbol 12-myristate 13-acetate. Aliquots of cells were resuspended in a reaction buffer containing 5.2 mM  $\text{MgCl}_2$ , 94 mM  $\text{KCl}$ , 12.5 mM HEPES pH 7.4, 12.5 mM EGTA, and 8.2 mM  $\text{CaCl}_2$ , and assays were started by adding [ $\gamma$ - $^{32}\text{P}$ ]ATP (9 cpm/pmol, final concentration 250  $\mu\text{M}$ ), peptide substrate (final concentration 70  $\mu\text{M}$ ), and streptolysin-O (0.3 IU). Samples were incubated at 37 °C for 10 min, quenched, and analyzed as described previously (22,47).

### E. Immunoprecipitation of PKC Isoforms

After treatment, cells were harvested in 1 mL lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% [v/v] Triton X-100, 1 mM EGTA, 2 mM EDTA, chymostatin, leupeptin, antipain, pepstatin [5 mg/L each], 1 mg/L E64, and 1 mM PMSF). Cell lysates were forced through a 25-gauge syringe (15 times) and cleared by centrifugation at  $15,800 \times g$  for 10 min. Immunoprecipitation was carried out on equal amounts of protein with the indicated anti-PKC antibody (3  $\mu$ g) incubated for 1–3 h at 4 °C followed by adsorption to protein A-Sepharose beads (10 mg) for 1 h at 4 °C. Precipitated samples were recovered by centrifugation and proteins were either resolved by SDS-PAGE or used in autophosphorylation and kinase reactions.

### F. Western Blot Analyses

Immunocomplexes were dissolved in Laemmli's sample buffer and separated by electrophoresis on a 10% polyacrylamide gel followed by electrotransfer to poly (vinylidene difluoride) (PVDF) membranes (DuPont, NEN Research Products). Membranes were incubated at room temperature with 0.1 mg/mL anti-PKC isoforms. Proteins were detected with the ECL system (Amersham).

### G. Autophosphorylation of PKC Isoforms

Immunoprecipitated PKCs bound to protein A-Sepharose beads were washed three times with lysis buffer and once with the same buffer containing 0.4 M NaCl and without EDTA/EGTA. Samples were incubated in 40 mL of a mixture containing 5  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP, 10 mM ATP, 400  $\mu$ M MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, 400  $\mu$ M phosphatidylserine, 100 nM phorbol 12,13-dibutyrate, 1 mM sodium orthovanadate, and 20 mM Tris, pH 7.4 at 37 °C for 10 min. The reactions were terminated by addition of 10  $\mu$ L of boiling SDS sample buffer and electrophoresed on a 10% polyacrylamide gel. Gels were stained using the SYPRO protein gel stain kit (Molecular Probes), blotted, and radioactivity in the membranes was detected by using a BioRad GS-250 Molecular Imager. Alternatively, gels were dried down for autoradiography on Kodak X-Omat S films. Quantification was done by using a BioRad GS-700 imaging densitometer.

### H. Activity of Immunoprecipitated PKC $\alpha$

Confluent A7r5 cells, after the treatment described in the figure legends, were lysed in a buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1%

Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, protease inhibitor cocktail (Böhringer), 1 mM  $\text{Na}_3\text{VO}_4$ , and 1 mM PMSF. Extracts were prepared by passing the lysates through a 25-gauge needle (15 times) and cleared by centrifugation at  $15,800 \times g$  for 10 min. Anti-protein kinase C $\alpha$  antibody (3  $\mu\text{g}$ ) was added to the supernatants for 1 h at 4 °C, and afterward protein A-Sepharose was added for an additional hour. The resulting immunocomplexes were collected by centrifugation, washed in lysis buffer, and finally in kinase buffer (50 mM Tris-HCl pH 7.4, 10 mM NaF, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM  $\text{MgCl}_2$ , 1 mM PMSF, and protease inhibitor cocktail). Kinase reactions with the immunocomplexes were carried out in a 40- $\mu\text{L}$  final volume of an activation buffer containing 20 mM Tris-HCl pH 7.4, 10 mM  $\text{MgCl}_2$ , 10 mM ATP, 2.5  $\mu\text{Ci}$  ( $\gamma$ - $^{32}\text{P}$ ) ATP (600 Ci/mmol), 0.4 mg/mL histone III-S, 1.2 mM  $\text{CaCl}_2$ , 40 mg/mL phosphatidylserine, and 3.3 mM diolelylglycerol. Reactions were terminated by adding 20  $\mu\text{L}$  boiling SDS sample buffer and frozen until use. Samples were loaded in a 10% SDS-PAGE and blotted on a PVDF membrane for 1 h at 100 mA. Histone phosphorylation was detected by using a phosphorimager, and the signals were quantified by densitometric scanning and normalized in respect to the amount of immunoprecipitated PKC $\alpha$ , which was detected by immunoblots using the MC5 anti-PKC antibody.

### I. In Vivo Labeling of Cells

Quiescent A7r5 cells ( $6 \times 10^5$ ) were incubated in phosphate-free DMEM (Amimed) with 0.25 mCi/mL  $^{32}\text{P}_i$  during 14 h at 37 °C. Then cells were stimulated with 10% dialyzed FCS (Sigma) in the presence of the indicated agents and further incubated for 7 h. During the last hour, cells received 100 nM phorbol 12-myristate 13-acetate (PMA). Cells were then washed exhaustively with PBS, lysed in SDS buffer, and subjected to immunoprecipitation for PKC $\alpha$ . PKC $\alpha$  was resolved on SDS-PAGE, and its phosphorylation was analyzed on a BioRad Molecular Imager GS-250. Staining with the SYPRO kit has been used to control protein loading of the gels.

### J. Protein Phosphatase Activity Assay

Purified  $\text{PP}_2\text{A}$  (25 ng) resuspended in 10  $\mu\text{L}$  assay buffer (Tris 50 mM, EDTA 1 mM pH 7.6) was preincubated with a solution of either  $\alpha$ -tocopherol (50  $\mu\text{M}$ ) or  $\beta$ -tocopherol (50  $\mu\text{M}$ ) for 10 min at 30 °C. Control reactions contained vehicle (ethanol 0.1%) alone. Assays were started by addition to the mixtures of [ $^{32}\text{P}$ ]phosphorylase- $\alpha$  solution (8.5 mg protein,  $9 \times 10^4$  dpm) and further incubated for 10 min at 30 °C. Reactions were stopped by adding 120  $\mu\text{L}$  ice-cold trichloroacetic acid 10% and 150 mg albumin in 20  $\mu\text{L}$   $\text{H}_2\text{O}$ . Samples

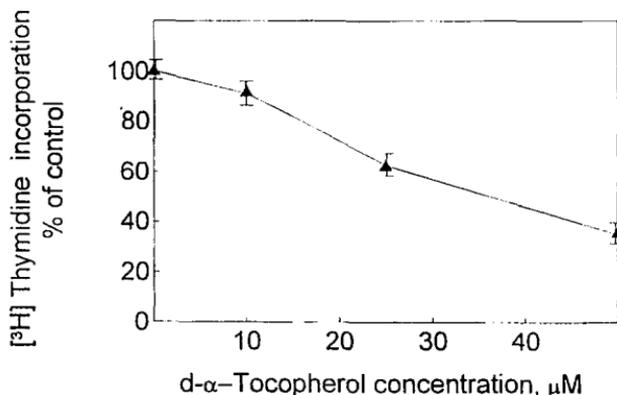
were left on ice and centrifuged 2 min at  $12,000 \times g$ . The clear supernatant was counted in a liquid scintillation counter.

### K. PKC $\alpha$ /Protein Phosphatase Assay

PKC $\alpha$  (16 nM) was incubated for 10 min at 30°C with PP<sub>2</sub>A or PP<sub>1</sub> at the indicated concentrations, in 40  $\mu$ L activation buffer containing 10 mM MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.2, 0.5 mM DTT (1,4-dithio-DL-threitol), 100  $\mu$ M MBP<sub>4-14</sub> (myelin basic protein peptide fragment 4-14), 0.25 mM ATP, 20 mM MgCl<sub>2</sub>, 5  $\mu$ g phosphatidylserine, 5  $\mu$ g diacylglycerides, and 5  $\mu$ Ci [<sup>32</sup>P]-ATP. Reaction was stopped with 20  $\mu$ L 25% TCA (trichloroacetic acid). Aliquots of 50  $\mu$ L were spotted onto 3  $\times$  3 cm P81 Whatman filters, washed twice with 0.75% phosphoric acid, and then washed once with acetone. Radioactivity was counted in a liquid scintillation analyzer.

## II. $\alpha$ -TOCOPHEROL IS A SPECIFIC INHIBITOR OF CELL PROLIFERATION

$\alpha$ -Tocopherol, at concentrations between 10 and 50  $\mu$ M, was shown to inhibit rat A7r5 smc proliferation (Fig. 1), whereas  $\beta$ -tocopherol appeared ineffective (48). When  $\alpha$ -tocopherol and  $\beta$ -tocopherol were added together, no inhibition of cell proliferation was seen. Both compounds were transported equally in



**Figure 1** Inhibition of SMC proliferation. Quiescent cells were restimulated with FCS in the presence of the indicated concentrations of  $\alpha$ -tocopherol. For DNA synthesis determination, [<sup>3</sup>H]thymidine was given to the cells in the S-phase. The control represents  $84,332 \pm 5150$  cpm.

cells and did not compete with each other for uptake (48). The oxidized product of  $\alpha$ -tocopherol,  $\alpha$ -tocopheryl quinone, and several other water- and lipid-soluble antioxidants were not inhibitory, indicating that the effects of  $\alpha$ -tocopherol were not related to its antioxidant properties (48).

Inhibitory effects of  $\alpha$ -tocopherol were also observed in primary human aortic smc, Balb/3T3 mouse fibroblasts, and NB-2a mouse neuroblastoma but not in Chinese hamster ovary (CHO), Saos-2 human osteosarcoma cells, or P388 mouse macrophages.

### III. $\alpha$ -TOCOPHEROL IS A SPECIFIC INDIRECT INHIBITOR OF PKC

#### A. $\alpha$ -Tocopherol Inhibits PKC Activity

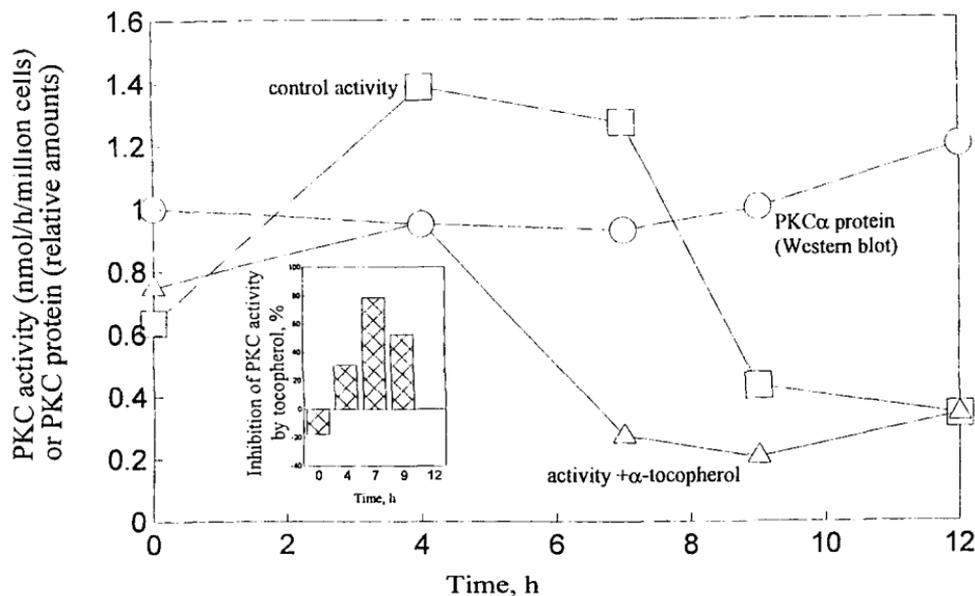
During the transition  $G_0 \rightarrow G_1$  phase of the cell cycle, an  $\alpha$ -tocopherol-sensitive increase in PKC activity not paralleled by changes in the mRNA levels of the PKC $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  isoforms was observed (Fig. 2) (Ricciarelli R., et al., 1997, unpublished results). Similarly, no changes in the protein levels of the major isoform (PKC $\alpha$ ), expressed during the transition were observed in the presence or absence of  $\alpha$ -tocopherol (Fig. 2).

Maximal PKC inhibition by  $\alpha$ -tocopherol was found 6–7 h after the entry of cells into the  $G_1$  phase (Fig. 2, inset), and this inhibition was only observed when  $\alpha$ -tocopherol was added at the time of restimulation (22).

PKC activity has been also measured in homogenates of aortas from rabbits fed different dietary supplements. As shown in Table 1, an approximate 50% reduction in PKC activity is observed in vitamin E-treated animals compared with controls (vitamin E-poor diet). With cholesterol supplementation, PKC activity increases to 10.2  $\Delta$ -absorbency units/min/mg protein, which is significantly reduced by vitamin E treatment.

#### B. PKC Isoforms in A7r5 smc

The finding that PKC is involved in the  $\alpha$ -tocopherol inhibition of smc proliferation (22) prompted the question of which isoform(s) is(are) affected by  $\alpha$ -tocopherol. In A7r5 cells the presence of PKC $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ , and  $\mu$  was documented (Fig. 3, lane A). To determine the specificity of the reaction, the corresponding competitor peptides were used (lane B). The PKC $\beta$  isoform was determined in a separate experiment as well and shown to be present in smc. It was also



**Figure 2** Inhibition of PKC activity by  $\alpha$ -tocopherol is a function of the cell cycle. Quiescent A7r5 cells were stimulated for different times with FCS in the absence or presence of  $50 \mu\text{M}$   $\alpha$ -tocopherol. At the indicated points, PMA-stimulated PKC activity in permeabilized cells was measured. PKC $\alpha$  levels were analyzed by Western analysis and the signals scanned by densitometry. Data are expressed as arbitrary units of absorbency. The inset shows the percentage of PKC inhibition by  $\alpha$ -tocopherol at different incubation times. Data are representative of four independent experiments.

found that long PMA treatment downregulated the PKC $\alpha$ ,  $\delta$ ,  $\epsilon$  (lane C) and  $\beta$  and  $\mu$  isoforms (not shown).

### C. $\alpha$ -Tocopherol Selectively Inhibits PKC $\alpha$

Nanomolar concentrations of Gö 6976 have been shown to inhibit PKC $\alpha$  and  $\beta$ , whereas even micromolar concentrations have no effect on the activity of PKC $\delta$ ,  $\epsilon$ , or  $\zeta$  (49).

As can be seen in Figure 4, when PKC $\alpha$  and  $\beta$  isoforms were inhibited by Gö 6976, the residual activity was not sensitive to  $\alpha$ -tocopherol, indicating that the PKC $\delta$ ,  $\epsilon$ ,  $\zeta$ , and  $\mu$  isoforms were not involved in the  $\alpha$ -tocopherol-induced PKC inhibition. Differently, after PKC $\beta$  activity was inhibited by the specific inhibitor Ly379196 (42), the effect of  $\alpha$ -tocopherol was still present,

**Table 1** PKC Activity from smc Homogenates Obtained from Differently Treated Rabbits

Treatment	PKC activity
Control	8.4 ± 1.1
Vitamin E	4.5 ± 2.5*
Cholesterol	10.2 ± 2.4
Cholesterol + vitamin E	4.5 ± 1.0†

\*  $p < 0.01$  compared with control group.

†  $p < 0.02$  with respect to cholesterol group.

Aortic media are minced and homogenized, and nuclei are sedimented by centrifugation. Supernatants are centrifuged again at  $100,000 \times g$  to obtain cytosolic fractions. Pellets are used for preparation of membrane fractions. Protein kinase C activity is measured in both fractions. Since the homogenates did not show significant membrane/cytosol distribution changes, only the values of total PKC activity are reported. Results are expressed as mean  $\pm$  SD ( $n = 5$ ). Statistical analysis was performed by one-way analysis of variance (ANOVA).

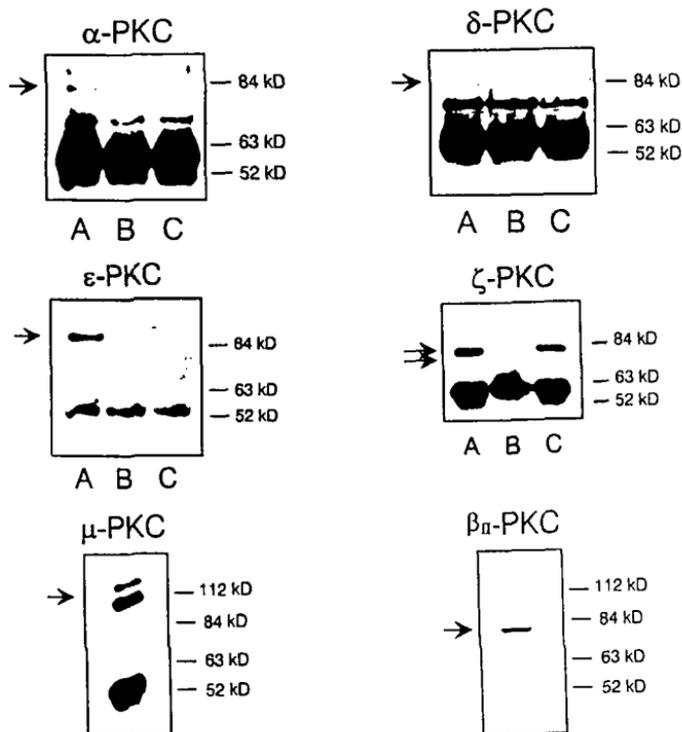
indicating that this isoform of PKC is not involved in  $\alpha$ -tocopherol inhibition (Table 2).

Further evidence was obtained by the experiments reported in Figures 5 and 6. Figure 5 shows that after the inhibition of PKC $\alpha$  and  $\beta$  by Gö 6976 and  $\zeta$  by PKC $\zeta$  specific antisense oligonucleotide, the remaining PKC isoforms ( $\delta$ ,  $\epsilon$ , and  $\mu$ ) were not affected by  $\alpha$ -tocopherol. Finally, when PKC $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$ , and  $\mu$  isoforms were downregulated, the residual PKC $\zeta$  was not sensitive to  $\alpha$ -tocopherol either in the absence or presence of the activator C<sub>2</sub>-ceramide (50) (Fig. 6).

Taken together, the above experiments suggested that PKC $\alpha$  is the specific target for  $\alpha$ -tocopherol. To further substantiate this finding, immunoprecipitation of the different PKC isoforms and the determination of the kinase and autophosphorylating activity were carried out.

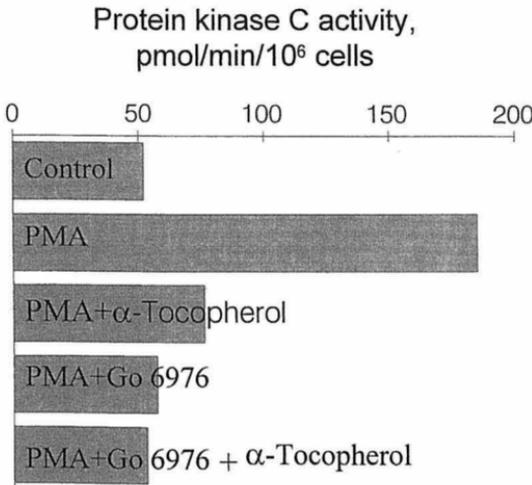
#### **D. Effect of $\alpha$ -Tocopherol on PKC Isoforms Autophosphorylation**

PKC autophosphorylation in immunoprecipitates has been found to correlate with its enzymatic activity and has been taken as a reliable indication of PKC activity (28).



**Figure 3** Characterization of PKC isoforms in A7r5 cells. Cells ( $1 \times 10^6$ ) at 90% confluence were harvested. PKC isoforms were immunoprecipitated with the corresponding antibodies and subjected to SDS-PAGE and immunoblotting as described in Methods. A, control; B, plus competitor peptide; C, cells treated for 24 h with 1  $\mu$ M PMA.

Cells were incubated in the absence or presence of  $\alpha$ -tocopherol or  $\beta$ -tocopherol for 7 h during the  $G_1$  phase. Then extracts were prepared, and immunoprecipitation of the individual PKC isoforms was performed. Auto-phosphorylation activity and protein amounts were determined for each isoform. In Figure 7, the effects on PKC $\alpha$  and PKC $\zeta$  are shown. The bar graphs correspond to the PKC activity, and values are normalized with respect to the protein content. As can be seen, only PKC $\alpha$  from  $\alpha$ -tocopherol-treated cells was less active relative to its control. The activity of all other PKC isoforms was not affected by the treatment of cells with  $\alpha$ -tocopherol (data not shown).



**Figure 4** Selective inhibition of PKC $\alpha$  by  $\alpha$ -tocopherol. Quiescent A7r5 cells were restimulated for 7 h with FCS in the absence or presence of 50  $\mu$ M  $\alpha$ -tocopherol. Gö 6976 (20 nM) was added to the permeabilized cells where indicated, and PMA-stimulated PKC activity was measured as described in Methods.

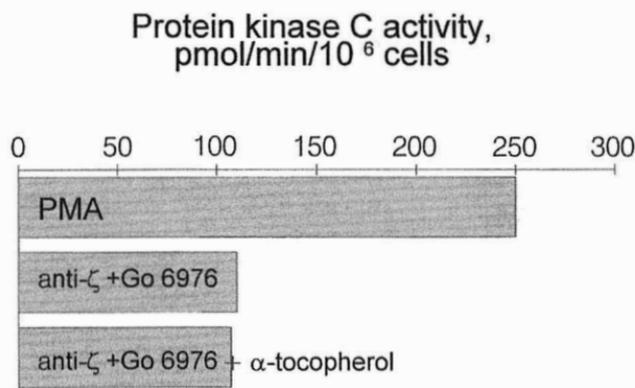
### E. $\alpha$ -Tocopherol Selectively Inhibits PKC $\alpha$ Activity and Phosphorylation State

To establish whether the incubation of cells with  $\alpha$ -tocopherol resulted in a change in the phosphorylation state of PKC $\alpha$ , an *in vivo* labeling reaction was carried out. Cells were labeled with <sup>32</sup>P<sub>i</sub> overnight, and after stimulation with

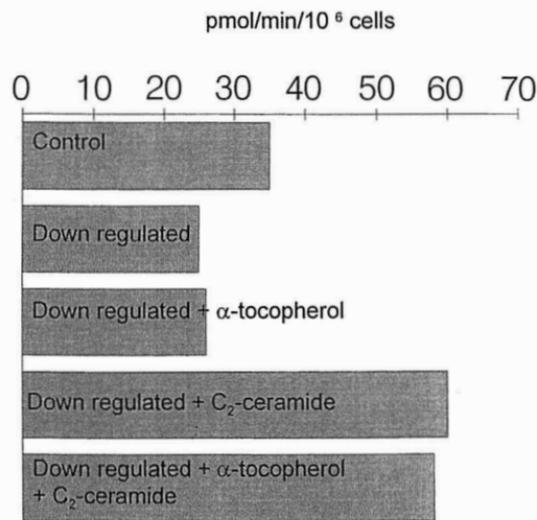
**Table 2** Effect of the PKC  $\beta$ -Specific Inhibitor Ly379196 on the  $\alpha$ -Tocopherol-Induced Inhibition of PKC

Treatment	cpm	% Change	Inhibition
PMA	13,428	100	0
$\alpha$ -tocopherol	8,928	67	33%
Ly379196	15,280	113	-13
Ly379196 + $\alpha$ -tocopherol	9,228	69	31%

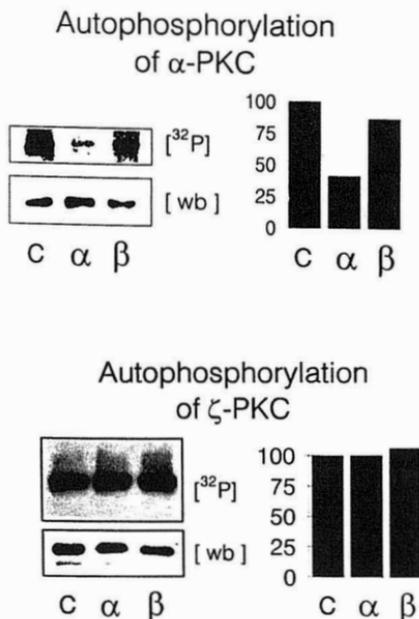
Quiescent A7r5 cells were restimulated for 7 h with FCS in the absence or presence of 50  $\mu$ M  $\alpha$ -tocopherol. Ly379196 (20 nM) was added to the permeabilized cells where indicated, and PMA-stimulated PKC activity was measured as described in Method. PMA, phorbol 12-myristate 13-acetate.



**Figure 5** Selective inhibition of PKC $\alpha$  by  $\alpha$ -tocopherol. Quiescent cells were treated for 24 h with 1 mM phosphorothioate antisense oligodeoxynucleotide designed to hybridize PKC $\zeta$  mRNA (anti- $\zeta$ ). Then cells were restimulated with FCS for 7 h in the absence or presence of 50  $\mu$ M  $\alpha$ -tocopherol. Gö 6976 was added to the PKC assay as described above.

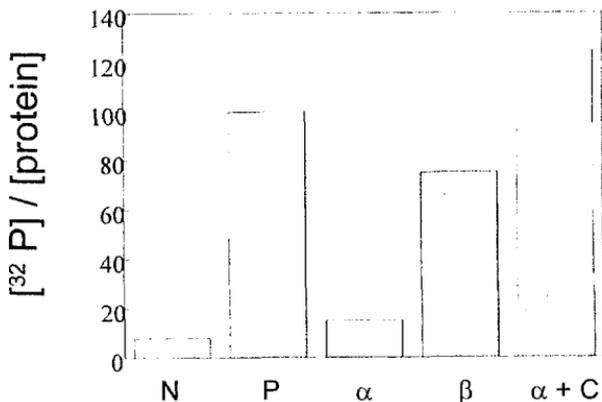


**Figure 6** Effect of  $\alpha$ -tocopherol on PKC $\zeta$ . Quiescent cells were treated for 24 h with 1  $\mu$ M PMA (downregulation, DR) and afterward treated for 7 h with 50  $\mu$ M  $\alpha$ -tocopherol. C<sub>2</sub>-ceramide (20  $\mu$ M) was added to the PKC assay in permeabilized cells as described.



**Figure 7** Autophosphorylation activity of different PKC isoforms. Quiescent cells were restimulated for 7 h with FCS in the absence (C) or presence of either 50  $\mu\text{M}$   $\alpha$ -tocopherol ( $\alpha$ ) or  $\beta$ -tocopherol ( $\beta$ ). PMA (100 nM) was added for the last hour of the preincubation period. Then, cell extracts were prepared, and PKC isoforms were immunoprecipitated with the indicated antibodies. Autophosphorylation reaction of the individual isoforms was performed as described in Methods. Samples were electrophoresed and blotted, and the calculated ratio between incorporated radioactivity [ $^{32}\text{P}$ ] and the protein levels (Western blot) is represented in the bar graphs for each condition. Data are representative of three independent experiments.

FCS for 7 h in the presence or absence of  $\alpha$ -tocopherol, PKC $\alpha$  was immunoprecipitated, blotted, and the  $^{32}\text{P}$  incorporation measured. Figure 8 shows a bar graph presentation of the radioactivity intensities integrated and normalized with respect to the protein levels of each sample. Relative to the control (N), the PMA-treated cells (P) showed a significant increase in  $^{32}\text{P}$  incorporation into PKC $\alpha$ . Cells pretreated with  $\alpha$ -tocopherol (column  $\alpha$ ) showed a large inhibition of PKC $\alpha$  phosphorylation, whereas cells preincubated with  $\beta$ -tocopherol (column  $\beta$ ) showed much less inhibition. The inhibitory effect of  $\alpha$ -tocopherol was reversed by two potent protein phosphatase inhibitors, okadaic acid 2 nM (not shown) or calyculin A 2 nM (column  $\alpha + \text{C}$ ). Figure 9 shows the PKC $\alpha$  activity measured after immunoprecipitation of the enzyme



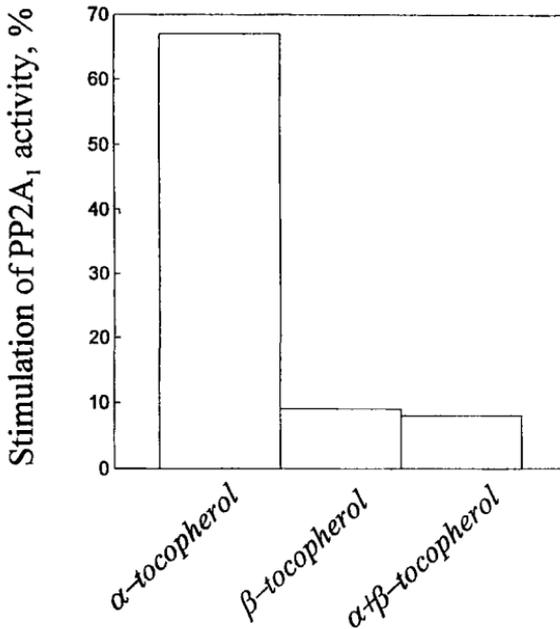
**Figure 8** Effect of  $\alpha$ -tocopherol and  $\beta$ -tocopherol on PKC $\alpha$  phosphorylation state. Quiescent A7r5 cells were incubated in phosphate-free DMEM medium (ICN) for 48 h. They received 0.25 mCi/ml P<sub>i</sub> for the last 14 h. Cells were restimulated for 7 h with FCS in the absence (P) or presence of either 50  $\mu$ M  $\alpha$ -tocopherol ( $\alpha$ ) or  $\beta$ -tocopherol ( $\beta$ ). PMA (100 nM) was added for the last hour to all samples except N. Caliculin A (2 nM) was added to cells for 1 h where indicated ( $\alpha + C$ ). Cell extracts were prepared and immunoprecipitated with anti-PKC $\alpha$ . Proteins were resolved by SDS-PAGE and radioactivity [<sup>32</sup>P] and protein levels were quantified as described in Methods. The bar graph represents the ratio between cpm incorporated to PKC $\alpha$  and the protein levels. Data are representative of three independent experiments.

from cells preincubated with  $\alpha$ -tocopherol or  $\beta$ -tocopherol at the late G<sub>1</sub> phase of the cell cycle. As can be seen,  $\alpha$ -tocopherol inhibited PKC $\alpha$  activity more strongly compared with  $\beta$ -tocopherol.

In the case of PKC $\beta$ , no significant PKC activity changes were observed if the cells were preincubated with either  $\alpha$ -tocopherol or  $\beta$ -tocopherol.

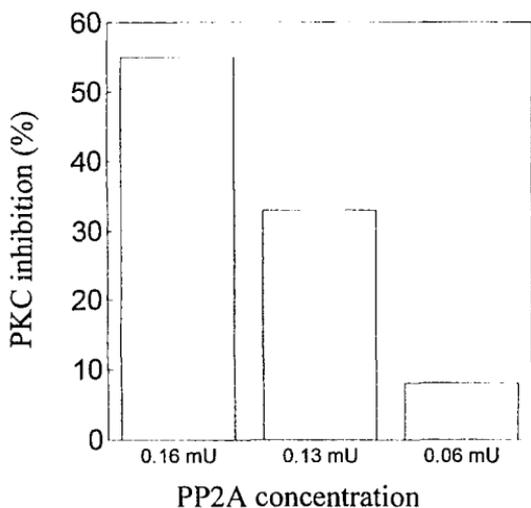
#### **F. Protein Phosphatase PP2A Is Activated by $\alpha$ -Tocopherol and Can Dephosphorylate PKC $\alpha$**

The role of a phosphatase PP2A on the deactivation of PKC $\alpha$  has been postulated on the basis of previous experiments (22). To establish if a direct effect of PP2A on PKC took place, the following experiment was carried out (Fig. 10). The two enzymes were preincubated together and then the activity of PKC $\alpha$  was measured. It was observed that PP2A produced a deactivation of PKC $\alpha$ . The inhibition of approximately 50% in PKC $\alpha$  activity obtained at a ratio 1 PP2A/16 PKC molecules indicates a catalytic role of PP2A on PKC inactivation.

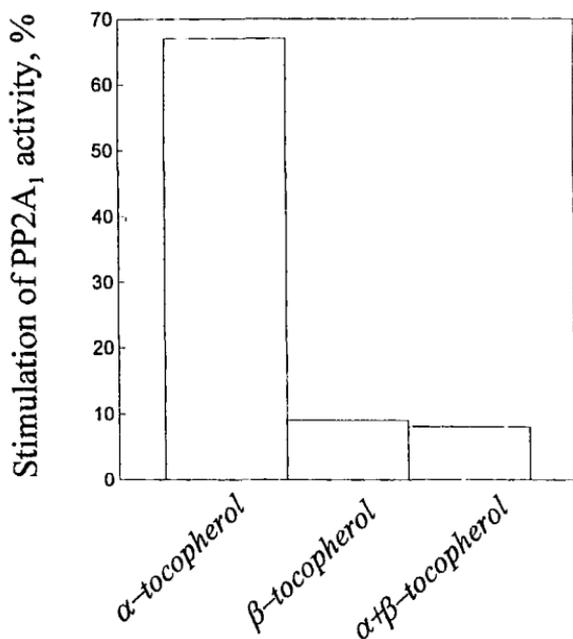


**Figure 9** Determination of PKC $\alpha$  activity after its immunoprecipitation from cells treated with  $\alpha$ -tocopherol or  $\beta$ -tocopherol. Cells were stimulated for 7 h with FCS in the absence (Control) or presence of either 50  $\mu$ M  $\alpha$ -tocopherol or  $\beta$ -tocopherol as indicated. During the last hour of preincubation, they received 100 nM PMA. Then extracts were prepared, PKC $\alpha$  was immunoprecipitated, and a kinase reaction using Histone III-SS was performed as described in Methods. Proteins were resolved by electrophoresis, and radioactive bands were quantified with a BioRad Molecular Analyst software. Protein levels were estimated by staining the gel with the SYPRO kit or by immunoblots with the MC5 monoclonal antibody. The ratio between radioactivity incorporated into the substrate and the amount of PKC $\alpha$  precipitated was expressed as arbitrary units of the densitometric scanning of the bands. Data are representative of three independent experiments.

The inhibition by  $\alpha$ -tocopherol of PKC $\alpha$  at a cellular level may thus be related to a possible activation of PP2A by  $\alpha$ -tocopherol. This hypothesis was investigated in the experiment outlined in Figure 11. Purified PP2A was incubated with [ $^{32}$ P]phosphorylase- $\alpha$  as a substrate; its activation by  $\alpha$ -tocopherol,  $\beta$ -tocopherol, and a mixture of the two was analyzed.  $\alpha$ -Tocopherol produced almost a twofold activation.  $\beta$ -Tocopherol was slightly inhibitory, and the mixture of both tocopherols was without significant effect. It thus appears that one of the cellular targets of  $\alpha$ -tocopherol may be PP2A.



**Figure 10** Inhibition of PKC $\alpha$  activity by PP2A. Purified PKC $\alpha$  (16 nM) was incubated for 10 min at 30°C with PP2A in 40  $\mu$ L activation buffer as described in Methods. PKC $\alpha$  activity was determined using a peptide substrate as described previously. Data are representative of three independent experiments.



**Figure 11** Effect of  $\alpha$ -tocopherol and  $\beta$ -tocopherol on PP2A activity. Phosphatase activity, using pure PP2A, was assayed in the presence of different  $\alpha$ -tocopherol or  $\beta$ -tocopherol concentrations as indicated. PP2A activity stimulation was calculated with respect to control samples. The background in the absence of the enzyme represented less than 3.5% of the initial total counts. Data are representative of three separate experiments.

#### IV. CONCLUSIONS

Cell proliferation, especially of smc and mesangial cells, plays an important role in the pathogenesis of diabetic complications. Increase in smc proliferation rate is of primary importance in the progression of atherosclerosis. Increased growth of mesangial cells, a cell type similar to smc, takes place during nephrosclerosis. PKC has been shown to be involved in both diseases. Smooth muscle cells from aortas of rabbits fed a high cholesterol diet show increased PKC activity.  $\alpha$ -Tocopherol treatment of the animals has been shown to diminish such activity.

In vitro smooth muscle cells are subject to a fine control by  $\alpha$ -tocopherol. Their proliferation is strongly diminished by  $\alpha$ -tocopherol in a specific way. Many other cell types are, in fact, insensitive to  $\alpha$ -tocopherol.  $\alpha$ -Tocopherol is also unique in its action in smooth muscle cells, because other tocopherols, tocotrienols, and general antioxidants are not as potent as  $\alpha$ -tocopherol. Some of them, such as probucol, are not effective at all. It is thus conceivable that the action of  $\alpha$ -tocopherol is not mediated by its radical scavenging function.

Data on the competition of  $\alpha$ -tocopherol and  $\beta$ -tocopherol suggest the existence of a receptor with the capacity of recognizing  $\alpha$ -tocopherol as an agonist and  $\beta$ -tocopherol as an antagonist. The finding that a phosphatase PP2A is modulated in an opposite way to proliferation by several tocopherols may indicate a role of this enzyme in the cascade of events at the basis of  $\alpha$ -tocopherol inhibition of cell proliferation. In the studies reported above, it has also been clarified that PKC, in smooth muscle cells, is inhibited by  $\alpha$ -tocopherol. Also, such an event is  $\alpha$ -tocopherol specific, with a specificity pattern similar to that of cell proliferation. PKC inhibition takes place only at cellular level and is associated with a diminution of PKC phosphorylation. This finding parallels the observed activation pattern of a phosphatase. Consequently, the entire picture relative to the understanding of smc inhibition may be understood in the following way. In the hypothesis that a receptor for  $\alpha$ -tocopherol exists, this may produce PP2A increase expression. Alternatively, the latter enzyme could be by itself the receptor, distinguishing between  $\alpha$ -tocopherol and  $\beta$ -tocopherol. In either case, the increased PP2A activity results in a dephosphorylation of PKC and in a reversible diminution of its activity. The role of  $\alpha$ -tocopherol has also been described in terms of inhibition, in mesangial cells, of PKC activity via diminution of diacylglycerol, the physiological activator of PKC. Such a decrease would be the consequence of the specific activation by  $\alpha$ -tocopherol of the enzyme diacylglycerol kinase.

The two described mechanisms of cell proliferation control by  $\alpha$ -tocopherol are both centered on PKC regulation. In one (22,48), emphasis is given to the posttranslational modifications of PKC mediated by an  $\alpha$ -tocopherol-sensitive protein phosphatase. In the other (42), the role of  $\alpha$ -tocopherol would be to subtract PKC major activator by biochemical conversion. Both mechanisms may coexist or acquire a major role in one or the other cell type.

## ACKNOWLEDGMENTS

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## **Protein Kinase C Activation, Development of Diabetic Vascular Complications, and Role of Vitamin E in Preventing These Abnormalities**

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Hyperglycemia induces multiple changes in vasculature or in neuronal cells in animal models of diabetes or in diabetic patients. The multifactorial nature of the changes is not surprising because the flux of glucose and its metabolites are known to affect many cellular pathways. The main challenge in this area has been to identify hyperglycemia-induced biochemical changes that can have a significant impact on vascular dysfunction and subsequent development of pathologies. Multiple theories have been proposed to explain the pathogenesis of the various complications involving retina, glomeruli, peripheral nerves, cardiovascular tissues, wound healing, and pregnancy. No one single theory, however, has emerged to account for all these changes.

Extracellularly, glucose can react nonenzymatically with the primary amines of proteins, forming glycated compounds or oxidants (1). These prod-

ucts can secondarily act on inflammatory cells or vascular cells directly via receptor- or nonreceptor-mediated processes to cause vascular dysfunction (2,3). Excessive glucose can also be transported intracellularly, mainly by the glucose transporter GLUT-1, and the resulting metabolism can cause changes in the redox potential, increase sorbitol production via aldose reductase, or alter signal transduction pathways such as the activation of diacylglycerol (DAG) and protein kinase C (PKC) pathway activation (4–10). It is probable that all hyperglycemia-induced intra- and extracellular changes and their adverse effects are being mediated through the alteration of some of these signal transduction pathways.

The effect of hyperglycemia on signal transduction pathways has not been extensively studied except for the activation of the DAG–PKC pathway. This pathway is known to be important in vascular cells because it is associated with the regulation of vascular permeability, contractility, extracellular matrix, cell growth, angiogenesis, cytokine actions, and leukocyte adhesions, all of which are abnormal in diabetes (11,12).

## I. PROTEIN KINASE C

PKC includes at least 11 isoforms ( $\alpha$ ,  $\beta 1$ ,  $\beta 2$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ,  $\theta$ ,  $\lambda$ ,  $\mu$ ), representing the major downstream targets for lipid second messengers or phorbol esters (11–13). The conventional PKC isoforms ( $\alpha$ ,  $\beta 1$ ,  $\beta 2$ ,  $\gamma$ ) are  $\text{Ca}^{2+}$ -dependent, containing two cysteine-rich, zinc finger-like motifs (C1 region), which are the binding sites of DAG or phorbol ester, and a  $\text{Ca}^{2+}$ /phospholipid (C2 region). New PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ,  $\mu$ ) are DAG sensitive but  $\text{Ca}^{2+}$  independent due to the absence of the C2 region. The two atypical PKCs,  $\zeta$ , and  $\lambda$ , are insensitive to DAG and lack one of the cysteine-rich motifs in the C1 region, but they can be activated by phosphatidylserine.

The source of DAG resulting in PKC activation can be derived from the hydrolysis of phosphatidylinositides (PI) or from the metabolism of phosphatidylcholine (PC) by phospholipase C (PLC) or D (PLD). Recent data, however, have shown that each isoform can be regulated by more than one lipid second messenger (9) such as the activation of PKC  $\zeta$  by PIP3 (12–14). Multiple isoforms can be expressed in different cell types, but despite extensive study, the attribution of a specific function to a specific isoform cannot be consistently established, suggesting that several isoforms can possibly mediate a similar range of functions and that their actions may be cell specific (12,15).

## II. MECHANISMS OF HYPERGLYCEMIA-INDUCED PKC ACTIVATION

In association with the diabetic vascular complications, increased total DAG content has been demonstrated in a variety of tissues, including retina (16), aorta, heart (17), and renal glomeruli (18,19), in both diabetic animal models and patients (Table 1). This has also been observed in classically termed "in-sulin-sensitive" tissues such as the liver and skeletal muscle (20,21). In all vascular cells studied, increasing glucose levels from 5 to 22 mM in the media elevated the cellular DAG contents (17). Increased DAG levels in response to elevated glucose may not occur immediately and can take as long as 3–5 days to reach a maximum after elevating glucose levels (18,22). Xia et al. (22) also showed that increased DAG content was chronically maintained in the aorta of diabetic dogs even after 5 years of disease. In fact, Inoguchi et al. (17) reported that euglycemic control by islet cell transplant after 3 weeks was not able to completely reverse the increases in DAG level or PKC activation in the aorta of diabetic rats. These results clearly indicate that the activation of the DAG–PKC pathway can be chronically sustained.

Cellular DAG content can also be increased by agonist-stimulated hydrolysis of PI or PC such as PLC or PLD (11–13). Because inositol phosphate products are not increased by hyperglycemia in aortic cells and glomerular

**Table 1** Summary of DAG Level and PKC Activity in Cultured Cells Exposed to High Glucose and Tissues Isolated from Diabetic Animals

	Diacylglycerol	Protein kinase C
Cultured cells		
Retinal endothelial cells	↑	↑
Aortic endothelial cells	↑	↑
Aortic smooth muscle cells	↑	↑
Renal mesangial cells	↑	↑
Pericytes	→	↑
Tissues		
Retina (diabetic rats and dogs)	↑	↑
Heart (diabetic rats)	↑	↑
Aorta (diabetic rats and dogs)	↑	↑
Renal glomeruli (diabetic rats)	↑	↑
Brain (diabetic rats)	ND	Not changed
Peripheral nerve	→↑	→↓

ND, not determined.

mesangial cells, increases in PI hydrolysis are most likely not involved in diabetes (22,23). Increases in DAG content could also arise from PC metabolism since Yasunare et al. (24) reported that PLD activity was increased by elevated glucose levels in aortic smooth muscle cells. They did not, however, quantitate the amount of total DAG. Most studies, however, have shown that the source of glucose-induced DAG increases were through the de novo synthesis pathway. In this case, labeling studies using [6-<sup>3</sup>H]- or [U-<sup>14</sup>C]-glucose demonstrated that elevated glucose levels increased the incorporation of labeled glucose into the glycerol backbone of DAG in aortic endothelial cells (25), aortic smooth muscle cells (22), and glomeruli (18). These studies clearly established that the increased DAG content was partially derived from glycolytic intermediates (26–28). Palmitic acid and oleic acid are the predominate fatty acids incorporated into DAG through the de novo pathway and from the metabolism of PC, which is consistent with the findings in vascular tissues from diabetic animals (25). In contrast, DAG derived from PLC activation consists mainly of 1-stearoyl, 2-arachidonyl fatty acid, which was not altered by glucose (25,29).

The activation of PKC by hyperglycemia may be tissue specific because it was noted in the retina (16), aorta, heart (17), and glomeruli (8,18) but not clearly demonstrated in the brain (16) and peripheral nerves (30) (Table 1). Similar increases in DAG levels and PKC activation have also been shown in multiple types of cultured vascular cells in response to increased glucose levels (Table 1) (8,16,22,31). Thus, it is likely that the DAG–PKC pathway is activated by the hyperglycemic-diabetic state in all vascular cells. Among the various PKC isoforms in vascular cells, PKC  $\beta$  and  $\delta$  isoforms appear to be preferentially activated as shown by immunoblotting studies in aorta and heart of diabetic rats (17) and in cultured aortic smooth muscle cells exposed to high levels of glucose (32). However, increases in other isoforms such as PKC  $\alpha$ ,  $\beta$ 2, and  $\epsilon$  in the retina (18) and PKC  $\alpha$ ,  $\beta$ 1, and  $\delta$  in the glomerular cells from diabetic rats have also been noted (33,34). These results demonstrate that diabetes and hyperglycemia will activate the DAG–PKC pathways in many tissue types, including vascular tissues, and thus glucose and its metabolites can cause many cellular abnormalities. However, for a hyperglycemia-induced change to be credible as a causal factor of diabetic complications, it has to be shown to be able to be chronically altered, difficult to reverse, to cause similar vascular changes when activated without diabetes, and to be able to prevent complications when it is inhibited. Thus far, based on the evidence presented, DAG–PKC pathway activation appears to fulfill the first two criteria. In the following sections, data are presented to support a fulfillment of the final two criteria with respect to the DAG–PKC pathway.

### III. FUNCTIONAL ALTERATIONS IN VASCULAR CELLS MEDIATED BY DAG-PKC ACTIVATION

Multiple cellular and functional abnormalities in the diabetic vascular tissues have been attributed to the activation of DAG-PKC pathways, including vascular blood flow, vascular permeability,  $\text{Na}^+\text{-K}^+$  ATPase, and extracellular matrix components.

#### A. Vascular Blood Flow

Abnormalities in vascular blood flow and contractility have been found in many organs of diabetic animals or patients, including the kidney, retina, peripheral arteries, and microvessels of peripheral nerves. In the retina of diabetic patients without clinical retinopathy (35–38) and animals with short durations of disease (39–43), retinal blood flows have been shown to be decreased. However, retinal blood flow may be normal or increased with longer duration of retinopathy (37,38,44). Multiple lines of evidence have supported the hypothesis that the decreases in retinal blood flow are due to PKC activation. For example, introduction of a PKC agonist such as a phorbol ester into the retina will decrease retinal blood flow (16). On the other hand, decreases in retinal blood flow in diabetic rats have been reported to be normalized by PKC inhibitors (16,19). In nondiabetic animals, the intravitreal injection of a DAG kinase inhibitor resulted in increased retinal DAG levels, activation of PKC, and a concomitant reduction in retinal blood flow (1,31). DAG kinase metabolizes DAG to phosphatidic acid, so DAG kinase inhibition will result in an increase in the available DAG pool. The results from this study showed that increased retinal DAG levels resulted in retinal blood flow decreases comparable with those measured in the diabetic rats. In addition to the retina, decreases in blood flow have also been reported in the peripheral nerves of diabetic animals, which were normalized by PKC inhibition (45,46), although some reports have noted increases in neuronal blood flow in diabetic rats (6).

One of the potential mechanisms by which PKC activation could be causing vasoconstriction in the retina is by increasing the expression of endothelin-1 (ET-1) (47). We reported that the expression of ET-1, a potent vasoconstrictor, plays a primary role in the regulation of retinal hemodynamics (48) and is increased in the retina of diabetic rats. Additionally, intravitreal injection of an ET-A receptor antagonist BQ123 prevented the decrease in retinal blood flow in these diabetic rats (47). The decrease in blood flow to the retina could lead to local hypoxia. Hypoxia is known to be a potent inducer

of vascular endothelial growth factor (VEGF), causing increases in permeability and microaneurysm formation (49,50).

Abnormalities in hemodynamics have been clearly documented to precede diabetic nephropathy (51,52). Elevated renal glomerular filtration rate (GFR) and modest increases in renal blood flow are characteristic findings in insulin-dependent diabetes mellitus (IDDM) patients (51,52) and experimental diabetic animals (53). Diabetic glomerular hyperfiltration is likely to be the result of hyperglycemia-induced decreases in arteriolar resistance, especially at the level of afferent arterioles (54,55), resulting in an elevation of glomerular filtration pressure. Multiple mechanisms have been proposed to explain the increases in GFR and glomerular filtration pressure, including an enhanced activity of angiotensin (56) and culturation in prostinoid productions (57–59). It is possible that the activation of DAG–PKC may also play a role in the enhancement of angiotensin actions because angiotensin mediates some of its activity by the activation of the DAG–PKC pathway (57). In addition, increases in vasodilatory prostanoids such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and prastafandin I<sub>2</sub> could also be involved in causing glomerular hyperfiltration in diabetes (58,59). The enhanced production of PGE<sub>2</sub> induced by diabetes and hyperglycemia could be the result of sequential activation of PKC and cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), a key regulator of arachidonic acid synthesis (60–63).

In the microvessels, increases in nitric oxide (NO) activities, a potent vasodilator, may also enhance glomerular filtration (64). Urinary excretions of NO<sub>2</sub>/NO<sub>3</sub>, stable metabolites of NO, have been reported to be increased in diabetes of short duration (64–66), possibly due to enhanced expression of inducible NO synthase (iNOS) gene and increased production of NO in mesangial cells (67). In addition, both increases in iNOS gene expression and NO production can be mimicked by PKC agonist and inhibited by PKC inhibitors when induced by hyperglycemia (67), suggesting that NO production might be increased in diabetes through PKC-induced iNOS overexpression. In addition, Graier et al. (68) suggested that NO production was enhanced by the elevation of glucose levels, possibly by the increased flux of Ca<sup>2+</sup> and its activation of eNOS. However, Craven et al. (69) reported that the production of glomerular NO and its second messenger, cGMP, in diabetic rats in response to cholinergic agents were decreased and that PKC inhibitors restored the glomerular cGMP production. Several authors also reported that elevated levels of glucose decreased NOS expression in vascular smooth muscle cells and that these effects of glucose were reversed by PKCβ inhibitors (70,71). Thus, PKC can regulate renal hemodynamics by increasing or decreasing NO production dependent on the cell type and tissue location.

In the macrovessels, increases in contractility found in diabetes are due to a delay in the relaxation response after contraction induced by cholinergic agents (72–75). These abnormal responses can also be prevented by PKC inhibitors (76), suggesting that PKC activation plays a general role in causing abnormal peripheral hemodynamics in diabetes.

## **B. Vascular Permeability and Neovascularization**

Increased vascular permeability is another characteristic systemic vascular abnormality in diabetic animals, in which increased permeability to albumin can occur as early as after 4–6 weeks of diabetes (77), suggesting endothelial cell dysfunctions. PKC activation can directly increase the permeability of albumin and other macromolecules through barriers formed by endothelial cells (78,79) and skin chamber granulation tissues (80), probably by phosphorylating cytoskeletal proteins forming the intracellular junctions (81,82). Interestingly, phorbol ester-induced increases in endothelial permeability may be regulated by PKC  $\beta$ 1 activation (83), which is consistent with the preferential activation of PKC  $\beta$  isoforms in diabetes.

PKC activation could also regulate vascular permeability and neovascularization via the expression of growth factors, such as the VEGF/vascular permeability factor (VPF), which is increased in ocular fluids from diabetic patients and has been implicated in the neovascularization process of proliferative retinopathy (49). We reported that both the mitogenic and the permeability-inducing actions of VEGF/VPF are due in part to the activation of the PKC  $\beta$  isoform via tyrosine phosphorylation of PLC (84). Further, *in vivo* studies in the rat have shown that VEGF-associated increases in retinal vascular permeability are mediated through the PKC pathway (85). Inhibition by the PKC  $\beta$  isoform selective inhibitor LY333531 resulted in the decrease of both endothelial cell proliferation and angiogenesis (84) and permeability increases induced by VEGF (85). In addition, Williams et al. (50) showed that the expression of VEGF was increased in aortic smooth muscle cells by elevating glucose concentration and was inhibited by PKC inhibitors.

In the kidney, the expression of transforming growth factor- $\beta$  (TGF- $\beta$ ) has been shown to be increased in the glomeruli of diabetic patients and experimental animals. Similar increases of TGF- $\beta$  have also been reported in cultured mesangial cells exposed to high glucose levels (9). Because TGF- $\beta$  can directly cause the overexpression of extracellular matrix, PKC inhibitors have been shown both to inhibit TGF- $\beta$  expression by hyperglycemia and to prevent the mesangial expansion observed in diabetic nephropathy (7,9,11).

### C. $\text{Na}^+\text{-K}^+$ ATPase

$\text{Na}^+\text{-K}^+$  ATPase, an integral component of the sodium pump, is involved in the maintenance of cellular integrity and functions such as contractility, growth, and differentiation (5). It is well established that  $\text{Na}^+\text{-K}^+$  ATPase activity is generally decreased in the vascular and neuronal tissues of diabetic patients and experimental animals (5,86–88). However, studies on the mechanisms by which hyperglycemia inhibited  $\text{Na}^+\text{-K}^+$  ATPase activity have provided some conflicting results regarding the role of PKC.

Phorbol esters, activators of PKC, have been shown to prevent the inhibitory effect of hyperglycemia on  $\text{Na}^+\text{-K}^+$  ATPase (5), which suggested that PKC activity might be decreased in diabetic conditions. Recently, however, we showed that elevated glucose level (20 mM) will increase PKC and  $\text{cPLA}_2$  activities, leading to increases of arachadonic acid release and  $\text{PGE}_2$  production resulting in decreases in  $\text{Na}^+\text{-K}^+$  ATPase activity. Inhibitors of PKC or  $\text{PLA}_2$  prevented glucose-induced reduction in  $\text{Na}^+\text{-K}^+$  ATPase activities in aortic smooth muscle cells and mesangial cells (61). The apparent paradoxical effects of phorbol ester and hyperglycemia are probably due to both the quantitative and the qualitative differences of PKC stimulation induced by these stimuli. Phorbol ester, which is not a physiological activator, can increase the activity of many PKC isoforms and overall PKC activity by 5–10 times, whereas hyperglycemia only increases PKC by twofold, a physiological relevant change (61) that appears to affect only a few isoforms. Thus, the results derived from the studies using phorbol esters are difficult to interpret with respect to their physiological significance.

### D. Extracellular Matrix Components

The thickening of the capillary basement membrane is one of the early structural abnormalities observed in almost all the tissues, including the vascular system, in diabetes (89). Because the basement membrane can affect numerous functions such as structural support, vascular permeability, cell adhesion, proliferation, differentiation, and gene expressions, alterations in its components may cause vascular dysfunction (90).

Histologically, increases in type IV and VI collagen, fibronectin, and laminin and decreases in proteoglycans are observed in the mesangium of diabetic patients (91,92). These observations can be replicated in mesangial cells incubated in media of increasing glucose levels (5–20 mM). These mesangium changes could be prevented by general PKC inhibitors (93–98). As described above, the increased expression of TGF- $\beta$  has been implicated in the development of mesangial expansion and basement membrane thickening in diabetes (99–104). Ziyadeh et al. (105,106) reported that neutralizing TGF-

$\beta$  antibodies significantly reduced collagen synthesis and gene expression of type (IV) collagen and fibronectin in the renal cortex of diabetic rats and in cultured mesangial cells exposed to high glucose levels. Because PKC activation can increase the production of extracellular matrix and TGF- $\beta$  expression, it is not surprising that several reports have shown that PKC inhibitors can also prevent hyperglycemia- or diabetes-induced increases in extracellular matrix and TGF- $\beta$  in mesangial cells or renal glomeruli (32).

### E. Selective PKC $\beta$ Isoform Inhibition

Numerous studies have used PKC inhibitors, such as staurosporine, H-7, and GF109203X, to characterize the role of PKC activation in diabetic vascular complications. Long-term studies involving PKC inhibitors, however, have not been possible due to their toxicity, which is associated with their nonspecificity with respect to inhibition of other kinases (19,107). Because analyses of retina, kidney, and cardiovascular tissues from diabetic rats showed that the PKC $\beta$  isoforms were preferentially activated (17,19,32), a specific inhibitor for the PKC $\beta$  isoforms could potentially be more effective and less toxic than the general isoform nonspecific PKC inhibitors.

Recently, we reported that increases in albuminuria and abnormal retinal and renal hemodynamics in diabetic rats can be ameliorated by an orally available PKC $\beta$  isoform selective inhibitor, LY333531. These physiological changes are concomitant with the inhibition of diabetes-induced PKC activation in retina and renal glomeruli (19). LY333531 prevented the overexpression of TGF- $\beta$ ,  $\alpha$ 1(IV) collagen, and fibronectin in renal glomeruli of diabetic rats (33). These results suggested that activation of PKC $\beta$  isoforms are involved in the development of some of the early abnormalities of diabetic vascular complications. PKC inhibitors could also mediate their effect by the inhibition of angiotensin actions. Angiotensin action appears to be increased because angiotensin-converting enzyme inhibitors have been shown to delay the progression of nephropathy (107). However, long-term studies are needed to clarify the usefulness of LY333531 to prevent the chronic pathological changes of diabetic vascular complications.

## IV. VITAMIN E AND PKC INHIBITION

Oxidative stress has been postulated as an underlying cause of diabetic vascular complications (108–112). Antioxidants such as vitamin E have been the subject of considerable interest with respect to their potential ability to ameliorate diabetic complications. There has also been considerable interest in the

use of vitamin E as an antioxidant agent for potential beneficial effects in coronary disease and cancers. Results from large multicenter clinical trials are now becoming available. A study on coronary heart disease in women (113) and in men (114) showed that increased vitamin E intake was associated with a significant risk reduction for coronary heart disease. Additionally, a recently published study involving male smokers in Finland (115) showed a 32% decrease in the incidence of prostate cancer in subjects taking 50 mg vitamin E/day. Interestingly, in this study there was a 23% increase in prostate cancer in those subjects randomized to  $\beta$ -carotene. Clinical studies aimed at characterizing the effect of vitamin E in the eye have focused primarily on the potential benefit of vitamin E in age-related macular degeneration (116,117), retinitis pigmentosa (118), and retinopathy of prematurity (119). The results from these studies are suggestive that vitamin E specifically and other antioxidants in general may be beneficial in treating macular degeneration and retinopathy of prematurity. There have been, however, no clinical studies aimed at investigating the effect of vitamin E in diabetes.

In the rat retina, vitamin E levels were fivefold higher than in other tissues such as the aorta (120). Vitamin E supplementation further increased these retinal vitamin E levels. Other investigators have shown that vitamin E is present in primate and human retinas (121–123), that the regional retinal distribution of vitamin E suggests an antioxidant protective effect against age-related macula degeneration, and that the level of vitamin E in the retina correlates with serum vitamin E levels (121).

Vitamin E, in addition to its antioxidant potential, has the other interesting property of being effective in inhibiting the activation of the DAG–PKC pathway in vascular tissues and cultured vascular cells exposed to high glucose levels (32,120). When retinal vascular endothelial cells exposed to high glucose were treated with vitamin E (*d*- $\alpha$ -tocopherol), DAG decreased and PKC activation was normalized (32,120). We reported that vitamin E can inhibit PKC activation, probably by decreasing DAG levels (32,120), because the direct addition of vitamin E to purified PKC  $\alpha$  or  $\beta$  isoforms in vitro did not have any inhibitory effect (120). These results are consistent with other studies demonstrating that *d*- $\alpha$ -tocopherol will inhibit PKC activation (124–126). Boscoboinik et al. (124) first demonstrated in 1991 that PKC activation was inhibited by *d*- $\alpha$ -tocopherol in a manner unrelated to *d*- $\alpha$ -tocopherol's antioxidant action (124–127). They also showed that the magnitude of the inhibition was related to the level of PKC activation (128) with little effect of *d*- $\alpha$ -tocopherol if cellular PKC was not activated.

Recently, the activation of DAG kinase has been suggested to be one potential site of action for vitamin E to inhibit PKC. Results indicate an indirect effect through activation of DAG kinase and increased metabolic break-

down of DAG to phosphatidic acid that resulted in decreased DAG levels and decreased PKC activation (129). Koya et al. (130) confirmed these results in the kidney and showed that glomerular dysfunction in diabetic rats could be prevented by *d*- $\alpha$ -tocopherol treatment through PKC inhibition, most likely mediated through increased DAG kinase activity.

In vivo studies in the diabetic rat have shown that the decreased retinal blood flow is related to elevation of retinal DAG levels, inhibition of DAG kinase (131), and the activation of PKC (16,32), particularly the  $\beta$  isoform of PKC (19,131). The results from these studies showed that the effects of increased DAG levels and PKC activation on retinal hemodynamics in nondiabetic rats can mimic the hemodynamic changes measured in untreated diabetic rats.

In diabetic rats, vitamin E treatment through regular intraperitoneal injections prevented the increases in both DAG levels and the activation of PKC in the retina, aorta, heart, and renal glomeruli (120,131). Functionally, vitamin E treatment prevented the abnormal hemodynamics in retina and kidney of diabetic rats in parallel with the inhibition of DAG-PKC activation (120,131). In addition, increased albuminuria was prevented by vitamin E treatment in diabetic rats (131). Normalization of the physiological parameters studied in these diabetic rats was achieved despite chronically maintained elevated blood glucose levels. Thus, it is possible that some of the PKC activation induced by diabetes could also be the result of excessive oxidants, which are known to activate PKC and can be produced by hyperglycemia, leading to the development of vascular dysfunction in the early stages of diabetes (132).

In diabetic patients with no or minimal diabetic retinopathy, retinal blood flow was reduced to an extent comparable with that measured in diabetic rats (35,37). Studies have shown that the reduction in retinal blood flow in these patients is associated with the level of glycemic control (35). These clinical results combined with prior animal studies provide the support for performing clinical studies aimed at evaluating whether vitamin E treatment is also effective in normalizing retinal blood flow and renal function in patients with IDDM. Additionally, multicenter clinical trials will need to be initiated to answer the question of whether high doses of vitamin E can prevent the development of microvascular complications in diabetes.

## V. SUMMARY

The results presented above are consistent with the activation of the DAG-PKC signal transduction pathway in diabetes. The initiating factors are chiefly metabolic with hyperglycemia as the main triggering element. Other metabolic

changes such as those associated with free fatty acids are also potentially involved. The finding that the secondary metabolic products of glucose such as glycation products and oxidants can also increase DAG-PKC suggest that the activation of DAG-PKC could be a common downstream mechanism by which multiple byproducts of glucose are exerting their adverse effects. It is not surprising that changes in the DAG-PKC pathway can play a role in diabetic microvascular complications as this signal transduction pathway is known to regulate many vascular actions and functions as described above (11,12). It is also likely that hyperglycemia and diabetes may affect other signal transduction pathways besides the DAG-PKC pathway because a number of these other pathways can also regulate vascular functions.

Hyperglycemia or diabetes has also been associated with the activation of more than one PKC isoform. Again, this is not surprising because many isoforms are DAG sensitive and each cell usually contains several PKC isoforms (11,12). However, it is surprising that the results of immunoblotting and the use of PKC $\beta$  isoform inhibitor appear to suggest that PKC $\beta$  isoforms are predominantly activated in all vascular tissues and may be responsible for many of the vascular dysfunctions.

The correlation between the activation of DAG-PKC and diabetic vascular and neurological complications are substantial in rodent models of diabetes (7,8,17-19,31); however, limited data are available to indicate that DAG-PKC levels are increased in the vasculature of diabetic patients. This is primarily due to the difficulty of obtaining fresh human vascular or neurological tissues for the measurement of DAG-PKC levels. Thus, further studies are needed to confirm whether DAG-PKC activation plays a role in the development of diabetic complications. First, the activation of the DAG-PKC pathway needs to be chronically inhibited in a long-term animal model of diabetes to demonstrate which of the various retinal and renal pathologies can be prevented. Long-term experiments to chronically inhibit PKC can be accomplished through the use of specific PKC isoform inhibitors or by characterizing the pathologic changes in transgenic mice strains lacking a specific PKC isoform. These experimental approaches are now possible because a specific and relatively nontoxic oral inhibitor of PKC $\beta$  isoforms is now available and can be used to test which of the vascular dysfunctions are due to PKC $\beta$  isoform activation (19).

Second, most of the reported findings to date have been performed in animal tissues and not in human vascular tissues. Thus, there may be differences between human and animal vascular tissue responses in relation to glucose metabolism and PKC isoform expression. A PKC $\beta$  isoform inhibitor will only be useful in diabetic patients if the same profile of PKC isoforms are

activated or expressed in diabetic patients as in the diabetic rodent models. In addition, the secondary markers of PKC activation need to be identified because they can be used to monitor the effectiveness of PKC inhibition when treated with intensive glycemic control or with PKC inhibitors. Progress has been made to identify some of these potential secondary parameters of vascular pathologies, such as the levels of VEGF, changes in retinal hemodynamics, and endothelial cell function (40,49,50,61).

The most important requirement for determining the role of activation of the DAG-PKC pathway in the vascular complication of diabetic patients has to be clinical trials using specific PKC isoform inhibitors. These trials are now in progress, specifically with the orally available PKC $\beta$  inhibitor. The need for clinical trials is vital as multiple agents have been shown to be capable of reversing vascular abnormalities induced by hyperglycemia in rodent models of diabetes. None of these agents, however, has been shown to be effective in clinical trials (133,134), clearly indicating the difficulties in extrapolating results to humans from those obtained using rodent models for diabetic complications. An additional potential problem with any therapeutic PKC inhibitor used clinically is the issue of toxicity because PKC activation is involved in so many vital functions of the cell. This is especially true in the clinical arena as these agents can be used by patients over long periods of time.

Thus, a large body of evidence has suggested that the activation of the DAG-PKC pathway by hyperglycemia and diabetes plays a role in the development of some vascular dysfunctions and neurovascular changes noted in diabetes. However, definitive studies as described above are ongoing and should determine clearly the role of DAG-PKC in the development of the various complications of diabetic patients.

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# 17

## Oxidative Stress and Pancreatic $\beta$ -Cell Destruction in Insulin-Dependent Diabetes Mellitus

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Insulin-dependent diabetes mellitus (IDDM) is considered to be an autoimmune disease (1,2). Recent reports suggest that reactive oxygen species (ROS) participate in the development of IDDM (3,4). Thioredoxin (TRX) is a small (12 kDa) reduction/oxidation (redox) protein (5,6) and has protective effects on cells against oxidative stress by scavenging ROS (5–7), by repairing DNA and proteins damaged by ROS (5–8), and by blocking apoptosis induced by ROS (6,9).

Nonobese diabetic (NOD) mice are well known as an excellent animal model for human IDDM (10–12). To elucidate the roles of oxidative stress in autoimmune diabetes, we generated NOD transgenic mice overexpressing TRX exclusively in pancreatic  $\beta$ -cells. Spontaneous diabetes was prevented or delayed in the NOD transgenic mice. The results indicate that ROS in pancreatic  $\beta$ -cells may play an essential role in the development of IDDM.

Here we describe the proposed mechanisms of the  $\beta$ -cell destruction by ROS in IDDM and the protective effects of the TRX system against  $\beta$ -cell destruction.

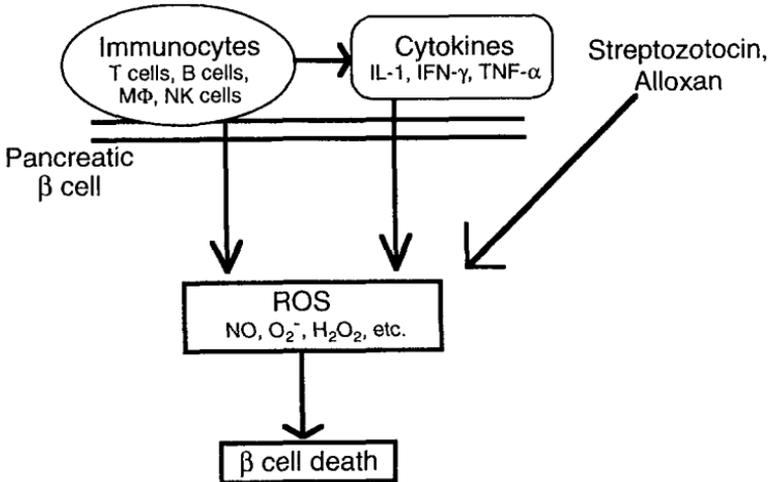
## I. PATHOGENESIS OF IDDM PATIENTS AND NOD MICE

IDDM is caused by autoimmune destruction of pancreatic  $\beta$ -cells (1,2). Because  $\beta$ -cells are almost completely destroyed at clinical onset of this disease, the affected individuals require daily injection of insulin to prevent diabetic ketoacidosis and to sustain their lives.

NOD mice spontaneously develop autoimmune diabetes (10–12) after the infiltration of inflammatory cells into pancreatic islets, termed “insulinitis,” and are known as an excellent animal model for studying human IDDM (10–12). Infiltrating cells are composed of T and B lymphocytes, macrophages (M $\Phi$ ), and natural killer (NK) cells. The  $\beta$  cells are thought to be destroyed by these immunocytes directly or via cytotoxic cytokines such as interleukin-1 (IL-1), tumor necrosis factor- $\alpha$ (TNF- $\alpha$ ), and interferon- $\gamma$  (3,4,13).

## II. OXIDATIVE STRESS AND IDDM

Recent studies have indicated that ROS such as nitric oxide (NO), superoxide anion radical ( $O_2^-$ ), and hydrogen peroxide ( $H_2O_2$ ) are generated by M $\Phi$  in islets or induced in  $\beta$ -cells by cytotoxic cytokines secreted from immunocytes (3,4) (Fig. 1). It has been shown that the expression of inducible nitric oxide



**Figure 1** A proposed mechanism of pancreatic  $\beta$ -cell destruction by oxidative stress in autoimmune diabetes and drug-induced diabetes.

synthase (iNOS) is augmented in  $\beta$ -cells of NOD mice (14). It has also been suggested that NO was induced by iNOS in the  $\beta$ -cells of human IDDM patients (15). These data indicate that oxidative stress may be one of the effector mechanisms of  $\beta$ -cell destruction by infiltrating inflammatory cells in autoimmune diabetes.

It has previously been suggested that pancreatic  $\beta$ -cells are especially vulnerable to oxidative stress. In fact,  $\beta$ -cells are selectively destroyed by ROS-generating agents, streptozotocin (STZ) and alloxan (16–18). Such susceptibility to ROS is probably due to low levels of key enzymes scavenging ROS, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (19–22). Overexpression of *Drosophila* Cu/Zn SOD in  $\beta$  cells has been shown to confer resistance to alloxan-induced diabetes (23). Antioxidative agents such as nicotinamide (24,25) and vitamin E (26) have been shown to have protective effects against diabetes of NOD mice. Recently, it was reported that transgenic mice overexpressing iNOS in  $\beta$  cells develop insulin-dependent diabetes without insulinitis (27). These results suggest that ROS and the antioxidative systems in  $\beta$ -cells play pivotal roles in the destruction of  $\beta$ -cells in the development of autoimmune diabetes.

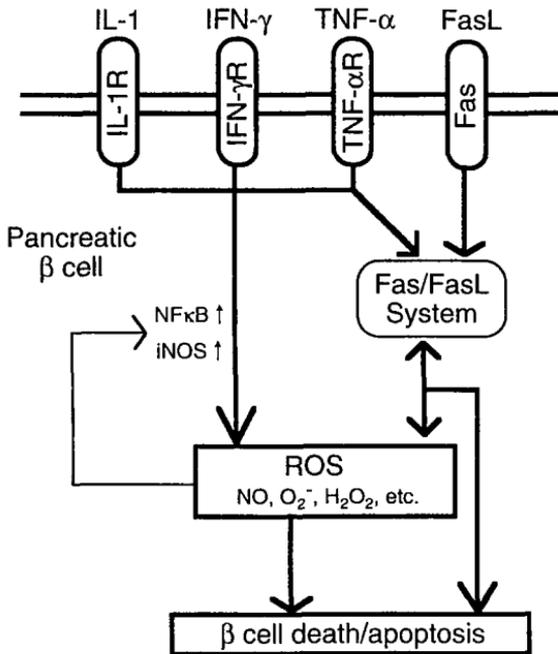
### III. OXIDATIVE STRESS AND APOPTOSIS

Recent histochemical analysis suggested that apoptosis is the mechanism of  $\beta$ -cell destruction in autoimmune diabetes (28,29). The Fas/Fas ligand (FasL) system is profoundly related to apoptosis (30) (Fig. 2). Transgenic mice overexpressing FasL in  $\beta$ -cells were reported to be highly susceptible to autoimmune diabetes (31), whereas Fas deficient (*lpr/lpr*) mice were resistant to autoimmune diabetes (31,32). Fas/FasL system was also suggested to mediate  $\beta$ -cell destruction in human IDDM patients (15).

NF- $\kappa$ B has been implicated in apoptosis, although the functions of NF- $\kappa$ B are complicated and there is controversy about the functions of NF- $\kappa$ B in apoptosis (33,34). It has recently been reported that NF- $\kappa$ B is induced by IL-1 in rat islets and RINm5F cells (35). Because NF- $\kappa$ B is known to activate iNOS (33,34), these results indicate that excessive NO may be generated by IL-1 (36) via activation of NF- $\kappa$ B (33,34), resulting in  $\beta$ -cell destruction, possibly due to apoptosis (37).

### IV. TRX AND ANTIOXIDATIVE EFFECTS

TRX is a small reduction/oxidation (redox) protein present in both prokaryotic and eukaryotic cells (5,6). In human, this protein was found as an adult T-



**Figure 2** A proposed mechanism of pancreatic  $\beta$ -cell death/apoptosis by cytotoxic cytokines in autoimmune diabetes.

cell leukemia-derived factor (6). TRX is induced by various types of stress, such as viral infection, ischemic insult, ultraviolet light, x-ray irradiation, and H<sub>2</sub>O<sub>2</sub> (5,6). Recently, TRX has been shown to have protective effects on cells against oxidative stress. TRX itself can function as an ROS scavenger (6,7). TRX, originally studied as a cofactor of ribonucleotide reductase in DNA synthesis, has been revealed to be a substrate of the antioxidative and antiapoptotic enzyme, TRX peroxidase (38). Recent studies have indicated that TRX plays essential roles in the repair of DNA and proteins damaged by ROS (5–8,39–43).

Augmented expression of TRX is often seen in neoplastic cells (5,6,44–46) and appears to protect neoplastic cells against the cytotoxicity of ROS-generating antineoplastic agents such as *cis*-diaminedichloroplatinum (II), adriamycin, etoposide, and mitomycin C (6,9,45,46). Recombinant TRX prevents cell death induced by ROS (6). TRX also protects cells against apoptosis induced by TNF and Fas-agonistic antibody (6,47). A number of *in vitro* stud-

ies have suggested that the TRX system plays an important role in the protection of cells against the cytotoxicity of oxidative stress (6,9,44–48).

## **V. TRX AS A REGULATOR OF INTRACELLULAR SIGNALING AGAINST OXIDATIVE STRESS**

Recent reports revealed that TRX also functions as a regulator of intracellular signalings (6). NF- $\kappa$ B (6,49,50) and Ref-1 (6,8,43), which have TRX binding sites, are regulated by TRX. Ref-1 regulates activator factor (AP)-1 (6,8,43). NF- $\kappa$ B and AP-1 have been implicated in apoptosis (33,51), although the precise functions and mechanisms are complicated and still unknown. It has been reported that overexpression of TRX inactivates NF- $\kappa$ B and activates AP-1 (49). Inactivation of NF- $\kappa$ B by TRX overexpression may reduce excessive NO production under oxidative stress.

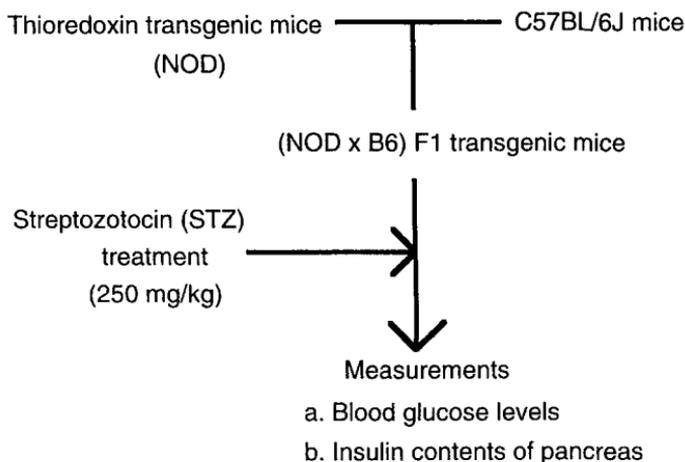
Very recently, mammalian TRX has been shown to function as a direct inhibitor of apoptosis signal-regulating kinase (ASK)-1 (52). Das et al. (53) suggested that TRX can induce antioxidative proteins such as MnSOD. These results indicate that TRX acts as a regulator of intracellular signalings against oxidative stress.

## **VI. ENDOGENOUS TRX EXPRESSION IN PANCREATIC $\beta$ -CELLS**

In our study using immunoblot analysis, we found the expression of endogenous TRX to be much lower in islet cells than in pancreatic exocrine cells. Based on immunohistochemical analysis, Hansson et al. (54) also suggested that TRX expression of islet cells was lower compared with pancreatic exocrine cells. Their result was consistent with our study. This attenuation of TRX expression in  $\beta$ -cells may be one of the reasons why  $\beta$ -cells are vulnerable to oxidative stress.

## **VII. PREVENTIVE EFFECTS OF TRX OVEREXPRESSION AGAINST AUTOIMMUNE DIABETES IN VIVO**

To directly assess the roles of oxidative stress in  $\beta$ -cell destruction in autoimmune diabetes, we generated NOD transgenic mice overexpressing TRX exclusively in  $\beta$ -cells. The incidence of diabetes in NOD transgenic mice was



**Figure 3** The generation of (NOD  $\times$  B6) F1 transgenic mice and the evaluation of pancreatic  $\beta$ -cell damage after streptozotocin treatment.

remarkably reduced compared with their negative littermates. Although the incidence of diabetes was reduced, the severity of insulinitis before overt diabetes was not significantly different between NOD transgenic mice and their negative littermates. To study the protective effects of TRX against ROS-generating agents, we produced (NOD  $\times$  C57BL/6J (B6)) F1 transgenic mice and injected the ROS-generating agent STZ into them (Fig. 3). The elevation of blood glucose levels in (NOD  $\times$  B6) F1 transgenic mice was reduced and the reduction of insulin contents was suppressed compared with their negative littermates. These results suggest that ROS play pivotal roles in  $\beta$ -cell destruction, in autoimmune diabetes and in STZ-induced diabetes.

## VIII. SUMMARY

TRX overexpression has preventive effects on both autoimmune diabetes and ROS-generating agent-induced diabetes as described. Our data suggest that the pancreatic  $\beta$ -cell-targeted control of redox systems utilizing TRX and/or the antioxidative therapies targeted to  $\beta$ -cells are anticipated to prevent or delay the overt diabetes even after the development of insulinitis.

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# 18

## Interrelationship Between Oxidative Stress and Insulin Resistance

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### **I. INSULIN RESISTANCE: A KEY FACTOR IN TYPE 2 DIABETES**

Insulin is the predominant hormone responsible for the maintenance of glucose homeostasis through its regulation of metabolic activities in muscle, liver, and adipose tissue. Insulin causes an increase in glucose uptake into peripheral tissues, specifically muscle and fat cells; conversely, in the liver, the hormone decreases gluconeogenesis, thereby reducing hepatic glucose output. Insulin is also responsible for the promotion of protein synthesis. These effects result from both rapid and long-term metabolic actions of the hormone (1).

One hundred million people worldwide suffer from type 2 diabetes (2), yet despite intense research, the primary lesion(s) responsible for type 2 diabetes remains unknown. The genetic susceptibility of this disease fails to follow simple Mendelian inheritance but is of a polygenic nature with superimposed environmental influences (3). An aggregation of small genetic effects rather than the effect of one single gene, gene-to-gene, and gene-to-environment interactions are thought to contribute to the development of this disease, yet most genes involved in the origin of type 2 diabetes remain unknown (R. Heggele, personal communication). The environmental factors that favor the development of this disease in genetically predisposed individuals include high-fat diets, low levels of physical activity, and increasing age (3).

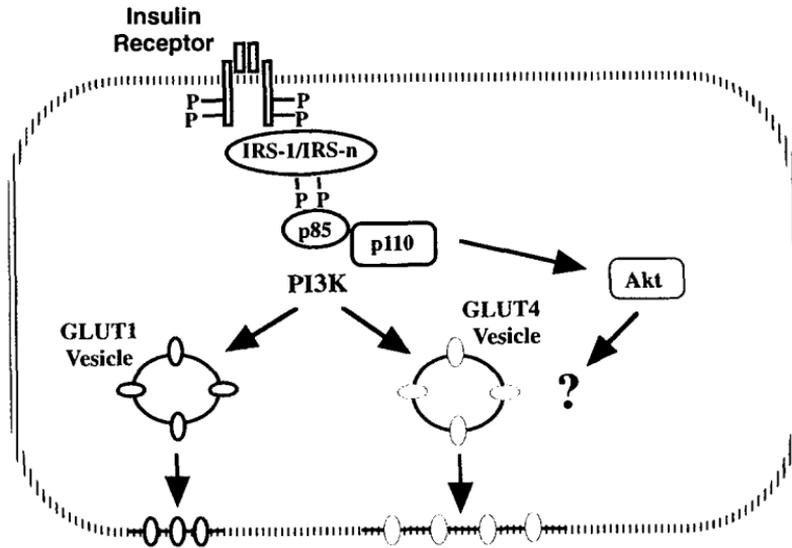
Type 2 diabetes is characterized by resistance to the insulin stimulation of glucose uptake in skeletal muscle and adipose tissue, by impaired insulin-dependent inhibition of hepatic glucose production, and by dysregulated insulin secretion (3). Both the insulin resistance and insulin secretory defects appear to be the result of genetic and environmental factors associated with the disease.

It is largely acknowledged that insulin resistance is a primary factor responsible for glucose intolerance in the prediabetic state. Initially, to compensate for the insulin resistance, insulin secretion increases to maintain normal glycemic levels. However, when the insulin secretory capacity fails to adequately compensate for the impaired insulin action, hyperglycemia ensues. This in turn further exacerbates the primary insulin resistance through the effects of high glucose (collectively known as glucose toxicity) and through the increased circulation of fatty acids and triglycerides (4). Hence, insulin resistance has primary and secondary causes in type 2 diabetes (Fig. 1).

### **A. Molecular Basis of Insulin Action and Insulin Resistance in Peripheral Tissues**

To understand the molecular basis of insulin resistance (whether primary or secondary) in muscle and fat cells, it is imperative to gain knowledge of the normal mechanisms of insulin action. In these tissues, insulin stimulates glucose uptake by rapidly mobilizing preexisting glucose transporters (primarily the GLUT4 isoform) from an intracellular storage organelle (or vesicle) to the plasma membrane (5,6). This is achieved by a series of signals elicited from the receptor, which are detected in an unknown fashion by the intracellular organelle. The latter is then free to find and interact with docking sites on the plasma membrane (7) that will ultimately enable fusion of the two membranes to provide functional glucose transporters. Detailed knowledge has emerged on the signals emanating from the receptor that are essential for GLUT4 translocation and the nature of the proteins engaged in vesicle docking and fusion with the plasma membrane.

The signaling events involved in the insulin-mediated GLUT4 translocation include autophosphorylation of the insulin receptor, tyrosine phosphorylation of docking proteins known as insulin receptor substrates (IRS 1–4), their subsequent binding to the enzyme phosphatidylinositol 3-kinase (PI 3-kinase), and the resultant activation of PI 3-kinase to produce phosphorylated phosphoinositides. How these signals then translate into translocation of the GLUT4 vesicle is the subject of vigorous study. A serine/threonine kinase identified

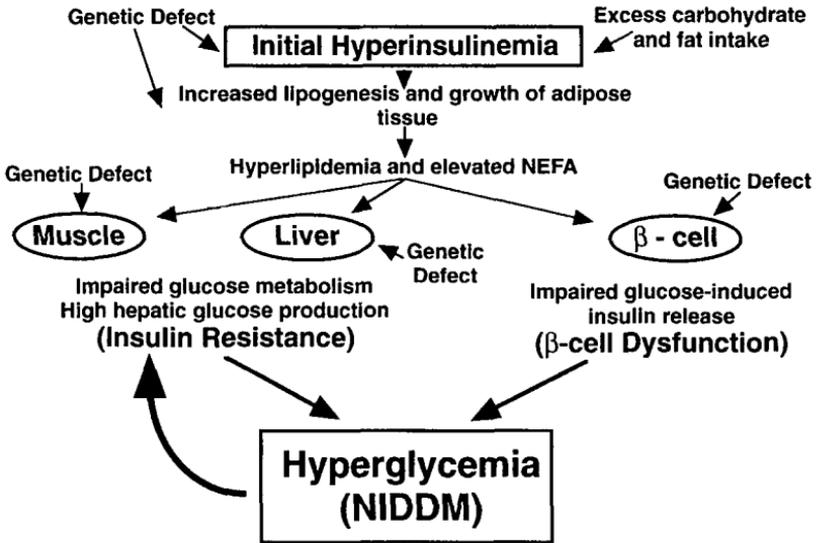


**Figure 1** Proposed model for the development of insulin resistance and type 2 diabetes. It is proposed that elevations in insulin levels, as a result of genetic defects and excess carbohydrate and fat intake, lead to increased levels of nonesterified fatty acids (NEFA) and hyperlipidemia. These elevated levels of lipids result in impaired  $\beta$ -cell function and induce insulin resistance through changes in glucose metabolism in skeletal muscle and in the liver. These changes in metabolism in insulin responsive tissues lead to hyperglycemia and the diabetic state ensues.

as protein kinase B, also referred to as Akt, has been shown to be activated by these phosphoinositides in vitro (8,9) and in response to insulin in vivo (10) and has been proposed to be a mediator of GLUT4 translocation (11,12) (Fig. 2).

Among the proteins involved in vesicle docking and fusion with the target plasma membrane are the vesicular proteins of the synaptobrevin family and the plasma membrane proteins syntaxin4 and SNAP23 (13–17). These proteins are isoforms of functional equivalents that participate in synaptic vesicle docking and fusion with the presynaptic plasma membrane of neurons. In contrast to this knowledge, little is known about how the glucose transporter-containing vesicles sense the insulin signal or travel to the plasma membrane.

It follows that the diminished response to insulin of glucose uptake into muscle and fat could result from any of the following possibilities: defects in



**Figure 2** Schematic diagram of the glucose transporter translocation hypothesis. Insulin-responsive tissues, specifically adipose tissue and skeletal muscle, contain intracellular stores of glucose transporter proteins (GLUT). The binding of insulin and the subsequent increase in tyrosine kinase activity of the insulin receptor initiates a signaling cascade, which results in the tyrosine phosphorylation of insulin receptor substrates (IRS 1-n), their binding to the enzyme phosphatidylinositol 3-kinase (PI 3-kinase), and the resultant activation of PI 3-kinase to produce phosphorylated phosphoinositides. This signaling cascade leads to the mobilization and insertion of stored glucose transporters into the plasma membrane, allowing for increased glucose influx into the cell in response to insulin.

insulin signals, defects in detecting/transducing the signal, a reduction in the total amount of glucose transporters, and/or inability of the transporters to properly dock with and incorporate into the plasma membrane. Of these, defects in insulin signaling and glucose transporter levels have been amply explored, and emerging evidence is being provided for defects in glucose transporter translocation. No studies have examined the mechanism of glucose transporter interaction with the plasma membrane in either humans or animals with diabetes. Finally, because the mechanism whereby the intracellular organelle detects the insulin signal is largely unknown, the possibility that this step is defective remains unexplored. A brief account of the status of glucose transporter levels, GLUT4 translocation, and defects in the insulin signaling pathway in diabetes is provided below. It is important to realize that, for the

most part, these studies do not distinguish whether the defects found relate to the primary or secondary insulin resistance.

### 1. GLUT4 Expression

The levels of expression of the GLUT4 glucose transporter have been analyzed in a variety of animal models of type 2 diabetes and in tissue from individuals with type 2 diabetes (for review see 18,19). GLUT4 protein content is markedly diminished in adipose tissues of human and most animal models. It has been found that GLUT4 expression in adipocytes decreases as diabetes develops in older Zucker rats (18,19), and adipose cells taken from humans with type 2 diabetes also show a reduction in GLUT4 content (20). However, this change in GLUT4 levels is restricted to adipose tissue and is not seen in skeletal muscle of these animal models of type 2 diabetes as normal expression of GLUT4 is observed in muscle of *db/db* mice and Zucker rats (21–24). Muscle biopsies taken from individuals with type 2 diabetes also show normal skeletal muscle GLUT4 content (25). However, a small number of studies have examined the amount of GLUT4 protein on the plasma membrane of muscle from diabetic animals, and it was found to be abnormal (18,19). This may suggest that sorting of the transporter is a key factor in muscle, whereas net synthesis of the transporter is more pertinent in fat. Whether these changes cause the diabetic state or ensue from hyperglycemia, hyperinsulinemia, and/or hypertriglyceridemia remains to be established. In a study attempting to shed light on this question, brown adipose tissue was ablated in transgenic mice, resulting in a decrease in the total GLUT4 protein in adipocytes. This led to the development of diabetes, suggesting that a reduction in the level of GLUT4 could be causative in this disease (26). However, genetic knockout of the GLUT4 gene in muscle and fat did not create a phenotype of diabetes, although glucose intolerance was generated (27).

### 2. GLUT4 Translocation

In skeletal muscle of humans with type 2 diabetes, the plasma membrane does not show a reduction and in fact may show a small increase in the amount of GLUT4 transporters. Yet the stimulation of glucose uptake by insulin is totally blunted. Zierath et al. (28) showed that the membranes of these muscles display a diminished gain in glucose transporters in response to an insulin clamp. A similar observation was also made in two animal models of diabetes (24,29). Defective GLUT4 translocation is also seen in fat cells from these animals (30) and in fat cells from humans with type 2 diabetes (20). As a result, several explanations have been put forward to account for this reduced translocation

of GLUT4 to the cell surface in skeletal muscle and adipocytes. These include topics that have been briefly mentioned, such as impaired translocation machinery and an inability of the transporters to functionally incorporate into the plasma membrane, in addition to the next topic to be discussed, an alteration in the signaling emerging from the insulin receptor.

### 3. *Insulin Signaling*

In animal models of type 2 diabetes and in humans with type 2 diabetes, there is considerable evidence for defects in the early stages of insulin action (31–33). There is an approximately 50% decrease in insulin receptor phosphorylation and an 80% decrease in IRS-1 phosphorylation in liver and skeletal muscle of *ob/ob* mice (34). This was associated with a more than 90% decrease in insulin-stimulated PI 3-kinase activity associated with IRS-1 and no detectable stimulation of total PI 3-kinase activity. In addition, insulin-stimulated Akt kinase activity in skeletal muscle of the lean diabetic Goto-Kakizaki rat was reduced by 68% (35). Skeletal muscle isolated from individuals with type 2 diabetes also show defects at the level of the insulin receptor tyrosine kinase activity, IRS-1 expression and phosphorylation, and IRS-1-associated PI 3-kinase activity (36). A reduction in IRS-1 expression (by 70%) and IRS-1-associated PI 3-kinase activity has also been reported in adipose cells isolated from individuals with type 2 diabetes (20). Thus, in type 2 diabetes, there are defects at four early steps of insulin action. Whether there are also defects distal to the initial signaling events that contribute to impaired translocation of GLUT4 remains to be determined.

In addition to alterations in the level of expression or activation of the signaling molecules in type 2 diabetes, the isoform selectivity of signaling also changes. In adipose cells isolated from humans with type 2 diabetes, IRS-2 becomes the main docking protein for PI 3-kinase and Grb2 in response to insulin (20). This is not surprising because expression of IRS-2 increases, and this protein predominates as the main insulin receptor substrate in mice lacking IRS-1 (37,38). Importantly, mice genetically manipulated to lack IRS-1 do not develop diabetes (38), whereas mice lacking IRS-2 do (39).

In summary, changes in the levels of glucose transporter expression, defects in the insulin signaling pathway, and alterations in pattern of signaling molecules may all contribute to either or both primary and secondary insulin resistance in type 2 diabetes.

## **B. Factors That May Trigger Insulin Resistance**

It has been suggested that circulating and metabolic factors could play an important role in the etiology of insulin resistance. This is supported by the

observation that insulin resistance of in vitro muscle preparations can be reversed by incubation in solutions of normal insulin and glucose levels (40). Circulating factors, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and free fatty acids (FFA), and intracellular metabolites, such as glucosamine, can induce an insulin-resistant state in vitro and may contribute to the development of insulin resistance in vivo.

### 1. *Tumor Necrosis Factor- $\alpha$*

The level of expression of adipose tissue TNF- $\alpha$ , a multifunctional cytokine, rises as a consequence of obesity. It is also closely correlated with circulating insulin levels, which serve as an index of insulin resistance, (41) and is expressed in increased levels in skeletal muscle of individuals with insulin resistance (42). Mice homozygous for a targeted null mutation in the TNF- $\alpha$  gene were significantly less insulin resistant compared with normal obese mice (43), suggestive of a causative role of TNF- $\alpha$  in obesity-related insulin resistance. The mechanism by which TNF- $\alpha$  exerts an impairment of insulin action remains incompletely understood. In adipocytes, chronic TNF- $\alpha$  exposure induced serine phosphorylation of IRS-1, inhibiting the insulin receptor tyrosine kinase (44). However, acute exposure of TNF- $\alpha$  in rat hepatoma cells induced serine phosphorylation of IRS-1 without any inhibitory effect on the insulin receptor tyrosine kinase, yet IRS-1 tyrosine phosphorylation and association with the p85 regulatory subunit of PI 3-kinase was blunted (45). Greater confusion on the TNF- $\alpha$  mechanism of action was seen in 3T3-L1 adipocytes, whereby TNF- $\alpha$  increased IRS-1 tyrosine phosphorylation and association with p85 (46). Further studies are required to elucidate the mechanism by which TNF- $\alpha$  acts as a mediator, directly or indirectly, of obesity-related insulin resistance.

### 2. *Free Fatty Acids*

It has been demonstrated that increased levels of circulating FFA correlate with peripheral insulin resistance in humans (47,48). Animal models of hyperlipidemia lend support to a correlation between increased FFA and insulin resistance (49,50). In addition, infusion of intralipid in vivo was shown to inhibit insulin-stimulated glucose uptake (48,51), and numerous studies also demonstrate that elevated plasma FFA levels decrease insulin-stimulated glucose uptake in skeletal muscle (48,51,52). However, the underlying mechanism of how FFA induce insulin resistance remains unknown. Elevated plasma FFA levels have been suggested to have an inhibitory effect on glucose oxidation, via the classic glucose-fatty acid cycle (53) and have been linked to impaired glycogen synthesis (54). It has also been suggested that the decrease

in insulin-stimulated glucose uptake in skeletal muscle mediated by elevated FFA occurs at early steps in glucose utilization, specifically at the level of glucose transport and/or glucose phosphorylation (51,55). In support of a mechanism whereby elevated FFA induces insulin resistance through inhibition of glucose transport/phosphorylation, it was recently reported that elevations in FFA induce insulin resistance in vivo via inhibition of components of the insulin signaling cascade, specifically inhibition of IRS-1-associated PI 3-kinase activity (56). Overall, these results suggest that elevated FFA may contribute to insulin resistance by action at several cellular processes that control glucose uptake.

### 3. *Glucosamine*

An intracellular mechanism that has received much attention lately as a possible generator of insulin resistance is increased flux of glucose through the hexosamine pathway (57). This pathway utilizes intracellular glucose and the amino acid glutamine to produce glucosamine, which is a precursor of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylgalactosamine (UDP-GalNAc) used in protein glycosylation. How glycosylation leads to insulin resistance is not known, but increasing evidence supports the concept that a rise in *N*-acetylglucosamine is linked to insulin resistance. The first observations were made in primary cultures of rat adipocytes, in which it was observed that increased glucose flux through the hexosamine biosynthetic pathway is the mechanism by which prolonged exposure to high levels of glucose and insulin resulted in impaired glucose transport (58,59). Induction of insulin resistance via increased glucosamine flux can be reproduced by administration of glucosamine in vitro and in vivo and is associated with increased accumulations of UDP-GlcNAc and UDP-GalNAc (60,61). These serve as an index of the amount of carbon flux through the pathway. Impaired insulin-mediated glucose disposal induced by elevated glucosamine levels is associated with decreased muscle GLUT4 translocation to the sarcolemmal fraction in response to insulin (62). Furthermore, overexpression of glucosamine fructose amido transferase, the rate-limiting enzyme for the hexosamine biosynthetic pathway, resulted in decreased GLUT4 translocation to the plasma membrane in response to insulin (63). Increased glucose flux through the hexosamine biosynthetic pathway could therefore be responsible for inducing insulin resistance through the downregulation of the glucose transport system.

In contrast to the evidence presented whereby circulating factors such as TNF- $\alpha$ , FFA, and the metabolite glucosamine may be involved in the induction of insulin resistance, there is no formal evidence to support or discard a

role of oxidative stress in the origin of insulin resistance. The following section examines the possibility that a link might exist between them.

## II. OXIDATIVE STRESS IN RELATION TO DIABETES AND INSULIN RESISTANCE

### A. Methods of Detection of Oxidative Stress

Oxidative stress is defined as the oxidative damage inflicted by an excess of reactive oxygen species (ROS) on a cell or organ (64). As the natural balance between toxic oxidants and protective antioxidant defenses is altered, oxidative stress results. Such conditions include an increase in free radical concentration or a decrease in the antioxidant capacity—or oxidant scavenging ability—of the cell (65).

Free radicals are highly reactive atoms or molecules containing one or more unpaired electrons (64). These toxic metabolites, such as peroxides, superoxides, and hydroxyl radicals, can be generated through both essential and nonessential oxidation-reduction reactions of the cytosol and mitochondria (66). Free radical-mediated oxidative damage has been observed in a variety of pathological conditions, including type 2 diabetes. However, difficulties arise in measuring oxygen radicals due to the highly reactive nature of these species, because they rapidly react with various substrates, including themselves, and have short lifespans ( $10^{-6}$ ,  $10^{-9}$  s) in aqueous systems (65). Nonetheless, measurements of free radicals via direct and indirect measures, although complicated, allow for an approximate assessment of oxidative stress. These measurements include spin trapping to directly measure the levels of free radicals (65), direct measurement of superoxide radicals in plasma (64), and identification of products of the oxidation of polyunsaturated fatty acids (PUFA) by lipid hydroperoxide assays (65). In addition, the plasma GSH/GSSG ratio (reduced glutathione/oxidized glutathione) serves as an indirect measurement of free radical reactions and as an indicator of the oxidative stress that may occur under physiological and pathological conditions (65).

Several of these methods have been used to measure oxidative stress in diabetes (67), as discussed elsewhere in this book. A few examples follow.

Animal models have been used to address the possible *in vivo* relationship between oxidative stress and diabetes. Accelerated accumulation of advanced glycation end products (AGEs) was measured as the amount of fluorescent protein adducts with lipoperoxidative aldehydes (malondialdehyde [MDA] and 4-hydroxynonenal [HNE]) in rat skin collagen of diabetic BB rats

(68). The diabetic rats had significantly higher levels of autoantibody against albumin modified by the lipoperoxidative aldehydes, MDA, and HNE, in addition to reactive oxygen species ROS (69). This suggests an increased generation of oxygen free radicals and lipoperoxidative aldehydes in these diabetic rats. Also, the duration of diabetes correlated significantly with the development of antibodies directed against MDA and HNE. The presence of antibodies against oxidatively modified proteins in spontaneously diabetic rats provides indirect evidence of the occurrence of oxidative modification of proteins *in vivo* (69). In addition, decreased plasma concentrations of ascorbic acid (AA) have been reported in individuals with diabetes (70). In a recent study, lens and renal AA levels were partially restored when normal glycemia was approached by insulin treatment in streptozotocin-diabetic BB rats (71). These *in vivo* studies provide evidence for the coexistence of oxidative stress associated with the increased level of oxidative damage in diabetes.

Studies of humans with type 2 diabetes also reveal the coexistence of oxidative stress with the disease. Serum has antioxidant activity against transition metal ion-catalyzed reactions, a result of the ferrous iron oxidizing (ferroxidase) activity of caeruloplasmin and of the iron-free fraction of transferrin (72). Increased levels of transferrin, ferritin, ferroxidase activity of caeruloplasmin, and iron-binding capacity were described in the serum of 67 subjects with type 2 diabetes (73). This observation has been interpreted to represent a protective response to the increased level of oxidative stress in subjects with diabetes.

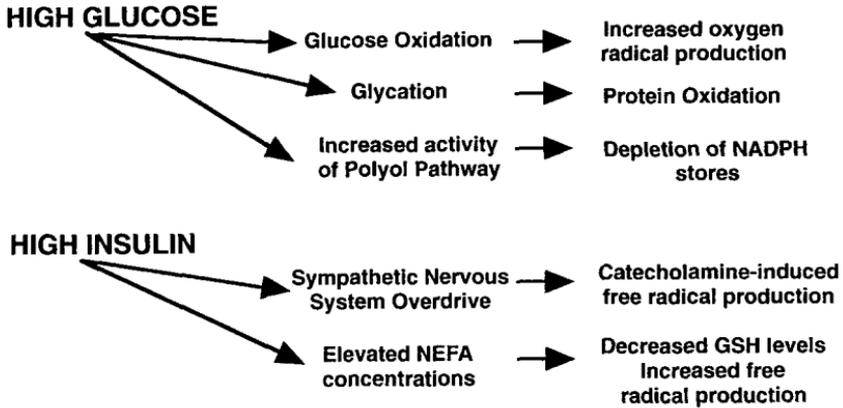
Free radical-mediated oxidative stress has been implicated in the pathogenesis of complications associated with type 2 diabetes. This wide field is not discussed here because it is treated amply elsewhere in this book. The following paragraphs address the origin of oxidative stress in diabetes and its possible link to insulin resistance.

## **B. Origin of Oxidative Stress in Type 2 Diabetes**

Suspected causative agents of the increased level of oxidative stress associated with type 2 diabetes are hyperglycemia, hyperinsulinemia, and an alteration of serum antioxidant activity (Fig. 3).

### *1. Hyperglycemia*

Hyperglycemia has been strongly implicated in the development of diabetic complications, an effect also known as glucose toxicity. The mechanisms of glucose toxicity are the subject of extensive investigation and include glucose



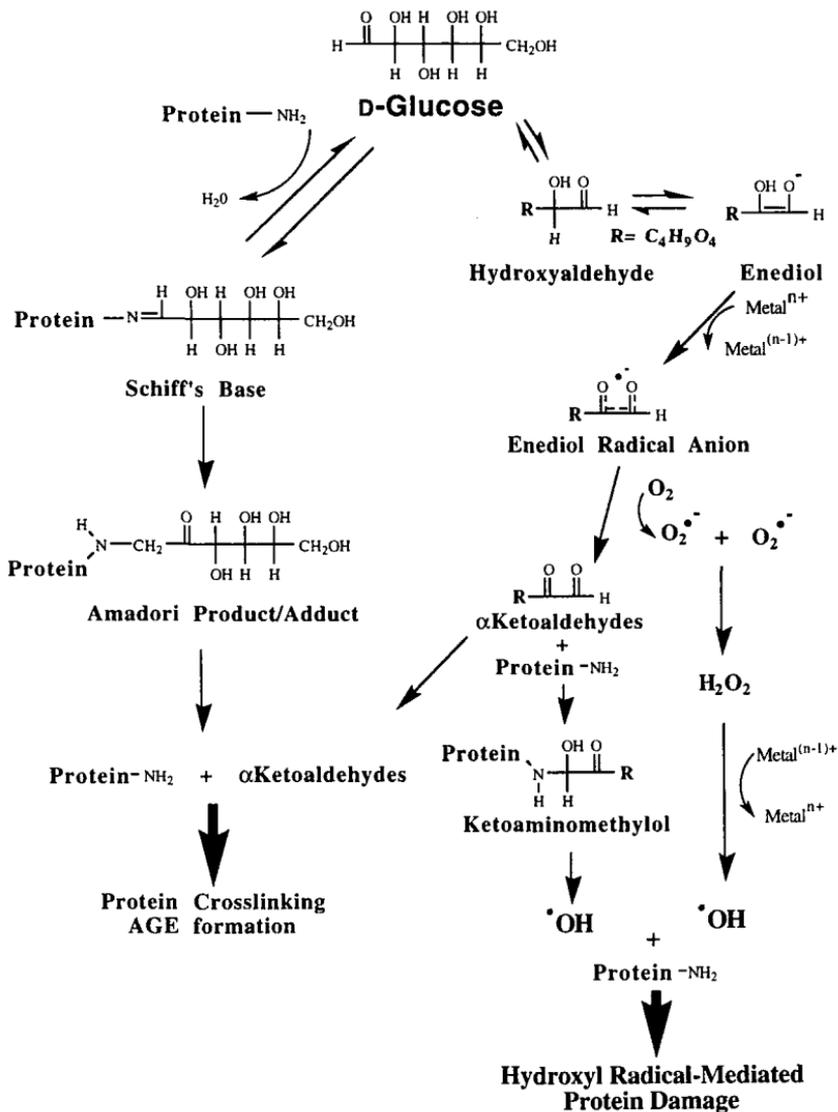
**Figure 3** How oxidative stress may arise in diabetes. The characteristic high levels of glucose and insulin observed in type 2 diabetes may contribute to oxidative stress through the production of free radicals, protein oxidation, and by depletion of intracellular reductants and antioxidants. This increased level of oxidative stress may be attributed to the increased levels of glucose oxidation, glycation, increased activity of the polyol pathway, sympathetic nervous system overdrive, and elevated nonesterified free fatty acid (NEFA) concentrations resulting from high levels of glucose and insulin.

oxidation, glycation, increased levels of polyols, and elevation of the hexosamine biosynthetic pathway. Most of these actions can contribute to oxidative stress.

**Glucose Enolization.** Glucose enolizes *in vitro*, reducing molecular oxygen catalyzed by transition metals, to yield oxidizing intermediates and  $\alpha$ -ketoaldehydes (74) (Fig. 4). Hydroxyl radicals, superoxide anions, and hydrogen peroxide are the reduced oxygen products formed in this glucose oxidation reaction, all of which are capable of protein damage through cross-linking, fragmentation, and lipid oxidation (64,75).

**Glycation and AGEs.** The protein reactive  $\alpha$ -ketoaldehydes, which are formed via protein glycation, are in turn protein reactive and are thus responsible for the formation of AGEs and protein cross-linking (74) (Fig. 4). AGEs have been implicated in the generation of ROS, activation of the transcription factor NF- $\kappa$ B, and in modulation of endothelial physiology (66).

**Sorbitol and Fructose Levels.** Intracellularly, elevated glucose levels can promote glucose reduction into sorbitol and fructose due to increased free glucose and an elevation in aldose-reductase and sorbitol-dehydrogenase activities (76). The rise in aldose-reductase activity diminishes NADPH cellular



**Figure 4** Glucose oxidation and glycation: glucose-induced protein damage and increased oxidative stress. Glucose, via glycative and oxidative processes, can result in protein damage and increased levels of oxidative stress as a result of the increased production of advanced glycation end products (AGEs) and hydroxyl radicals. These processes can therefore be attributed to type 2 diabetes due to the increased glucose levels (hyperglycemia) associated with this disease. (From Hunt JV, Dean RT, Wolff SP. Hydroxyl radical production and autoxidative glycosylation: glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. *Biochem J* 1998; 256:205–212. Copyright 1988 by The Biochemical Society and Portland Press.)

levels, thereby lowering the reducing power of the cell. It has been proposed that this leads to inhibition of the activity of NADPH-requiring enzymes including nitric oxide synthase and glutathione reductase (64). Vasoconstriction and tissue injury can result from the diminished nitric oxide synthase activity (77), and the low glutathione reductase activity could result in an increased cellular susceptibility to free radical damage (78) and oxidative stress.

## 2. *Hyperinsulinemia*

Hyperinsulinemia, a hallmark of insulin resistance, has recently been invoked as a plausible causative agent in the development of oxidative stress-induced diabetic complications. Experimental evidence suggests a relationship between hyperinsulinemia and increased free radical production (79). In human fat cells, increased accumulation of hydrogen peroxidase occurs upon exposure to elevated insulin levels (79). It has been hypothesized that the increase in free radical production associated with elevated levels of insulin may result from heightened sympathetic nervous system activity and from elevated FFA concentrations (64). High insulin leads to an overdrive of the sympathetic nervous system that results in increased catecholamine release (80,81). Catecholamines have been associated with an augmented production of free radicals in diabetic animals through a rise in metabolic rate and autooxidation (82).

In vitro and in vivo studies suggest that the hyperinsulinemia-induced rise in fasting FFA concentrations may be associated with oxidative stress. An in vitro study with cultured endothelial cells showed an association between increased fatty acid levels in the medium with both an increase in oxidative stress and a decrease in initial glutathione levels (83). Further studies in humans have supported a relationship between increased FFA levels and free radical-associated oxidative stress; the latter was inferred from the lower GSH/GSSG ratio in the plasma of subjects with diabetes compared with controls (64).

## 3. *Natural Antioxidant Activities*

A brief summary of certain properties of selected antioxidants is addressed in the following section. Antioxidants are scavengers of free radicals that constitute an important component of the cellular defense mechanism against oxidative stress. As a result, reduction or changes in the activity of these compounds result in deleterious effects to the cell due to the development of free radical-mediated oxidative stress. In previous and in following sections, evidence is provided to discuss the status of antioxidants in diabetes.

Vitamin E (tocopherol) is the predominant lipophilic antioxidant and is localized to the plasma membrane (66). It is one of the most important chain-breaking antioxidants responsible for the prevention of the propagation of free radical-induced reactions (84). Interestingly, vitamin E has been shown to decrease the covalent linking of glucose to serum proteins in vitro and to inhibit the glycation of serum proteins in vivo (85).

Glutathione is one of the most important water-soluble antioxidants. It is a tripeptide, and the reduced form is the major hydrophilic intracellular reductant responsible for protection and repair against oxidant damage (66).

Ascorbate (vitamin C), a key aqueous phase antioxidant, is involved in vitamin E reduction (86), thereby promoting many of the antioxidant properties of vitamin E, including the ability to prevent the propagation of free radical-induced chain reactions.

$\alpha$ -Lipoic acid is the most potent endogenous antioxidant and is also a natural cofactor of mitochondrial dehydrogenase complexes. The redox potential of the dihydrolipoate (DHLA)/ $\alpha$ -lipoic acid couple is  $-0.32$  mV (87). This strong reductant is responsible for the regeneration of reduced glutathione and vitamin E. It can also prevent lipid peroxidation, possibly through its ability to scavenge superoxide and hydroxyl radicals, and is involved in the inhibition of the activation of NF- $\kappa$ B by directly preventing its translocation from the cytoplasm to the nucleus (66). The ability of  $\alpha$ -lipoic acid to protect against oxidative stress is an important feature that might be applied to counteract the oxidative stress-mediated diabetic complications. This approach is discussed elsewhere in this book.

### C. Can Oxidative Stress Cause Insulin Resistance?

The question of precedence between hyperinsulinemia and oxidative stress into the subsequent development of diabetes remains unanswered (64). The studies mentioned above suggest that at least the secondary hyperinsulinemia could plausibly precede or cause increased free radical production and the resulting oxidative stress. Further studies are necessary to address whether oxidative stress, as a result of hyperinsulinemia-mediated increased free radical production, could precede insulin resistance and lead to the onset of diabetes. To date there is no evidence that primary insulin resistance is linked to oxidative effects. The following sections analyze two emerging lines of study in this direction.

#### 1. *Oxidative Stress, Antioxidants, and Insulin Resistance In Vivo*

There is little evidence for a role of antioxidant therapy in the prevention of insulin resistance and type 2 diabetes. Serum of individuals with type 2 diabe-

tes have a lower vitamin E level and a higher GSH/GSSG ratio compared with control subjects (88). Individuals with type 2 diabetes who received vitamin E supplementation had improved metabolic control, as indicated by the significant drop in circulating levels of glycosylated hemoglobin (89). In a small randomized trial with humans with or without type 2 diabetes, vitamin E supplementation reduced oxidative stress and improved insulin action (64,88), verifying an important role for antioxidant therapy in diabetes. This study involved the use of a euglycemic hyperinsulinemic clamp to study the effects of vitamin E supplementation on insulin sensitivity (90). The results showed a significant gain in insulin-mediated nonoxidative glucose disposal after supplementation of 900 mg vitamin E (90). In addition, the vitamin E supplementation significantly increased plasma vitamin E levels and significantly reduced GSH/GSSG ratios in all subjects (90). It was proposed that the mechanism by which vitamin E improves insulin responsiveness in individuals with and without diabetes was related to its role as an antioxidant (88). The hypothesis was put forward that increased lipid peroxidation, as a result of increased free radical-induced oxidative stress or a reduction in the antioxidant capacity of the cell, could cause changes in the fluidity of the membrane; this in turn would purportedly lower glucose uptake (91). The ability of an antioxidant to quench free radicals and reduce lipid peroxidation could presumably provide protection from changes to membrane fluidity and restore normal glucose transporter function.

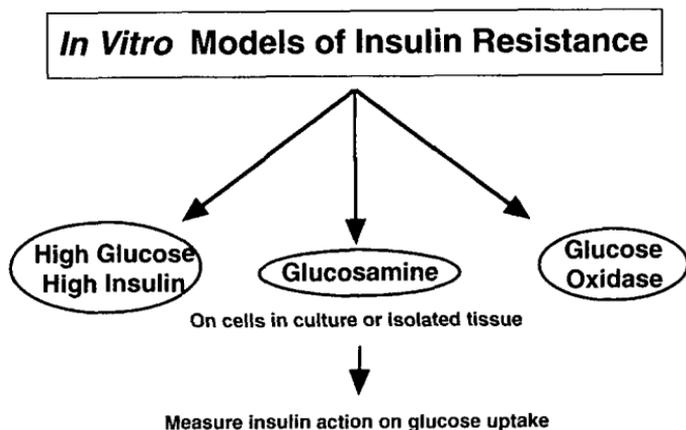
The first prospective population study undertaken to address the role of free radical stress and antioxidants in relation to the incidence of diabetes examined whether low vitamin E concentrations are a risk factor for the incidence of type 2 diabetes (92). The authors computed the levels of plasma vitamin E and the incidence of developing diabetes over a 4-year period in 944 men aged 42–60 who were determined not to have diabetes at baseline examination (92). Type 2 diabetes was defined by either a fasting blood glucose concentration of  $\geq 6.7$  mM, a blood glucose concentration  $\geq 10.0$  mM 2 h after a glucose load, or by a clinical diagnosis of diabetes with either dietary, oral, or insulin treatments. Forty-five men developed diabetes over the 4-year follow-up period (92). However, these 45 men also had a raised baseline body mass index, elevated blood glucose and serum fructosamine concentrations, a higher ratio of saturated fatty acids to the sum of monounsaturated and polyunsaturated fatty acids, and a higher serum triglyceride concentration (92). From the multivariate logistic regression model used in this study, it was found that the baseline body mass index was the strongest predictor of diabetes. Other factors with significant associations to an excess risk of diabetes included low plasma vitamin E concentrations, a high ratio of saturated to other fatty acids in serum, and a high socioeconomic status (92).

From this multivariate logistic regression model, a 3.9-fold risk of developing diabetes was associated with a low lipid standardized plasma vitamin E concentration (below median) (92). In addition, another model was used whereby lipid standardized vitamin E concentrations were replaced by unstandardized vitamin E concentrations when other risk factors such as serum low-density-lipoprotein cholesterol and triglyceride concentrations were taken as the strongest predictors of diabetes. From this model, a decrease of 1  $\mu\text{mol/L}$  of uncategorized vitamin E concentration was associated with an increment of 22% in the risk of developing diabetes (92). Hence, a significant relationship was proposed to exist between low vitamin E concentrations and an increased risk of diabetes. Clinical trials are needed to support the effect of antioxidants in the prevention of diabetes. It has been suggested that these trials should address the need for a "free radical initiative" (93) to understand how free radicals could affect the intrinsic mechanisms of diabetes (65). Also, trials are necessary to confirm a role of vitamin E, or other antioxidants, in the prevention of type 2 diabetes (92).

## 2. *Oxidative Stress and Insulin Resistance In Vitro*

In vitro, insulin resistance can be induced by prolonged insulin treatment, exposure of cells to high glucose concentrations, or by the preexposure of cells to glucosamine (Fig. 5). Insulin resistance at the level of glucose transport can result from various signaling defects as previously mentioned, including alterations in insulin receptor function, depletion of the GLUT4 transporter pool, and alterations in the postreceptor signaling pathway (94).

Prolonged insulin treatment of 3T3-L1 adipocytes induced an insulin-resistant state as a result of changes in the insulin signal transduction cascade (95). Treatment of 3T3-L1 adipocytes with 500 nM insulin for 24 h increased basal glucose transport and led to an insulin-resistant state for this transport (5,96,97). This treatment did not modify the insulin receptor or levels of GLUT4; however, it prevented GLUT4 translocation in response to acute insulin stimulation (95). Prolonged insulin treatment also induced a decrease in the level of IRS-1 expression and phosphorylation and a reduced ability of insulin to stimulate PI 3-kinase and MAP kinase (98). Thus, insulin resistance induced by prolonged insulin treatment of 3T3-L1 adipocytes is associated with multiple signaling defects, including defects at the level of IRS-1, PI 3-kinase, MAP kinase, and impaired GLUT4 translocation (98). These defects are similar to those observed in the hyperinsulinemic states of obese humans and rodents (98), validating this insulin-resistant model as an effective approach to study alterations of the insulin signaling pathway and insulin resistance.



**Figure 5** In vitro models of insulin resistance provide an effective tool to study alterations in the insulin signaling pathway and insulin resistance. Treatment of cells in culture or tissue with high levels of glucose, insulin, glucosamine, or glucose oxidase generate systems of insulin resistance, thus enabling further studies of insulin action in the insulin-resistant state.

Hyperglycemia has been linked to a worsening of insulin resistance (99–101) attributed to a disruption of normal cellular metabolism and of insulin-induced glucose disposal (102). In vitro, adipocytes exposed to high concentrations of glucose develop impaired insulin signaling, reduced insulin responsiveness, and diminished recruitment of glucose transporters to the plasma membranes in response to insulin (58,103). These alterations may be a result of the adverse metabolic consequences of the hyperglycemia.

Furthermore, it was discovered that an additional factor, glucosamine, was necessary for the glucose-induced desensitization of the insulin-stimulated glucose transport system (104). Glucosamine was also found to be more potent than glucose in the ability to induce insulin resistance and to decrease insulin responsiveness (104). It has been hypothesized from these findings that hexosamine metabolism may be the pathway by which cells sense and respond to ambient glucose levels and, when glucose flux is excessive, downregulation of glucose transport occurs and insulin resistance results (102,104). Thus, these models may lead to a better understanding of the mechanisms involved in the alterations of glucose metabolism seen in the insulin-resistant state.

To assess the role of oxidant stress and insulin resistance in vitro, Rudich et al. (105) used 3T3-L1 adipocytes preexposed to an enzymatic system capable of generating ROS. Exposure of the 3T3-L1 adipocytes to 25  $\mu\text{M}$

glucose oxidase for 18 h resulted in steady production of  $H_2O_2$ , with a concomitant threefold increase in basal 2-deoxyglucose uptake activity and a reduction in insulin-dependent 2-deoxyglucose uptake (105). The increase in basal transport as a result of increased oxidative stress was associated with an increase in GLUT1 mRNA and protein level (105). A reduction in GLUT4 protein and mRNA content was also observed, and this may account for a portion of the reduced insulin-stimulated glucose transport in the glucose oxidase-treated adipocytes (105). Basal lipogenesis was also enhanced by this treatment, whereas acute insulin stimulation of glucose oxidase-treated adipocytes significantly reduced lipogenesis activity (105). A further alteration of insulin-stimulated metabolism was also observed in these adipocytes as exposure to glucose oxidase lowered both the basal and insulin-stimulated glycogen synthase  $\alpha$  activity (105). Recently, Rudich et al. (106) observed that GLUT4 translocation was selectively impaired in glucose oxidase-treated 3T3-L1 adipocytes as there was impaired redistribution of PI 3-kinase to the LDM fraction of cells upon insulin stimulation. This suggests that oxidative stress could impair the insulin-mediated PI 3-kinase cellular redistribution, which results in the impaired GLUT4 translocation.

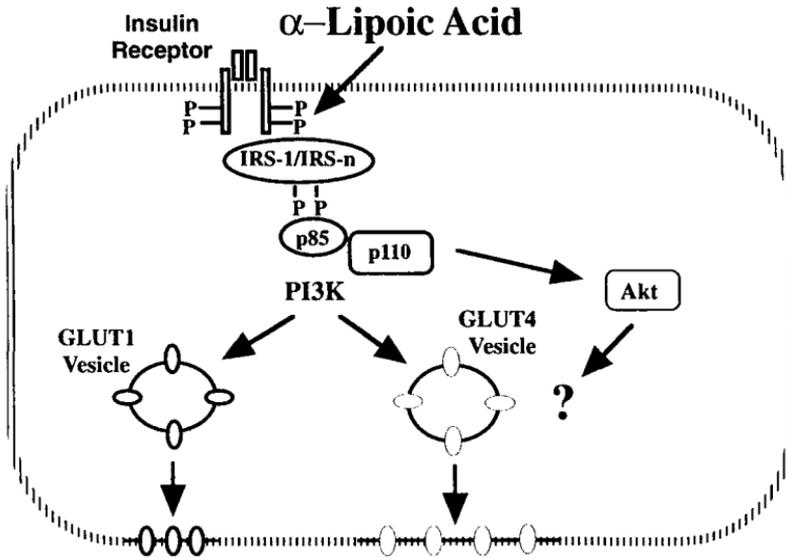
#### **D. $\alpha$ -Lipoic Acid: Stimulation of Glucose Uptake via Components of the Insulin Signaling Pathway**

As previously mentioned,  $\alpha$ -lipoic acid is a naturally occurring cofactor of oxidative metabolism, which is found as lipoamide covalently bound to a lysyl residue in five eukaryotic proteins, including mitochondrial dehydrogenase complexes (107). A natural antioxidant, lipoic acid has been used for the treatment of diabetic neuropathy (108) and ischemia-reperfusion injury (109) and has been indicated to improve glucose metabolism (110). In vitro and in vivo studies have demonstrated that exogenously supplied  $\alpha$ -lipoic acid is taken up and reduced to DHLA by NADH- or NADPH-dependent enzymes in a variety of cells and tissues (111,112). Furthermore,  $\alpha$ -lipoic acid has the ability to decrease the NADH/NAD<sup>+</sup> ratios elevated as a result of sorbitol oxidation to fructose under hyperglycemic conditions by the consumption of NADH (113). Further antioxidant properties attributed to  $\alpha$ -lipoic acid include its ability to directly scavenge ROS and to recycle thioredoxin, glutathione, vitamin E, and vitamin C (114). However, it is not known whether these potent antioxidant properties of  $\alpha$ -lipoic acid contribute to its ability to improve glucose utilization.

$\alpha$ -Lipoic acid has been shown in vitro to stimulate glucose utilization in isolated rat diaphragms (115), to enhance insulin-stimulated glucose metabolism in insulin-resistant skeletal muscle of obese Zucker rats (116), and to

stimulate glucose transport activity in skeletal muscle isolated from both lean and obese Zucker rats (117). In streptozotocin-diabetic rats, chronic  $\alpha$ -lipoic acid treatment reduced blood glucose concentrations by enhancement of muscle GLUT4 content and increased muscle glucose utilization (118). In addition, acute and repeated parenteral administration of  $\alpha$ -lipoic acid improved insulin-stimulated glucose disposal in individuals with type 2 diabetes (110,119), strengthening its therapeutic value as an antidiabetic agent. Estrada et al. (120) established the ability of  $\alpha$ -lipoic acid to stimulate glucose uptake into the insulin-responsive L6 skeletal muscle cells and 3T3-L1 adipocytes in culture. The naturally occurring (R)+ isoform of lipoic acid was shown to have a significantly greater effect on the stimulation of glucose uptake in L6 cells in comparison with the (S)- isoform or the racemic mixture (120). In addition, (R)+ lipoic acid had a positive effect on both basal and insulin-stimulated glucose uptake but did not improve the sensitivity of glucose uptake to submaximal concentrations of insulin (120). It was suggested that this increase in glucose uptake could not be entirely attributed to the antioxidant abilities of this agent alone. The increase in glucose uptake was mediated by a rapid translocation of the GLUT1 and GLUT4 glucose transporter isoforms from the internal membrane fraction to the plasma membrane of L6 myotubes (120).

We have recently shown that  $\alpha$ -lipoic acid similarly stimulates the translocation of GLUT1 and GLUT4 from the internal membrane fractions to the plasma membrane in 3T3-L1 adipocytes (K. Yaworsky, unpublished data). To account for the mechanism by which  $\alpha$ -lipoic acid could stimulate this increase in glucose uptake via rapid glucose transporter translocation, an inhibitor of PI 3-kinase, wortmannin, was used. As stated earlier, PI 3-kinase activity is essential for the propagation of the insulin signal responsible for the mediation of insulin-stimulated glucose uptake as a result of the translocation of glucose transporters (121). Wortmannin significantly lowered the  $\alpha$ -lipoic acid stimulated increase in glucose uptake in L6 myotubes, suggesting the involvement of PI 3-kinase in lipoic acid's mechanism of action (120). More recent evidence has shown that  $\alpha$ -lipoic acid directly stimulates IRS-1 immunoprecipitated PI 3-kinase activity in L6 myotubes, and this increase in PI 3-kinase activity is wortmannin sensitive (R. Somwar, K. Yaworsky, and A. Klip, unpublished data). These data suggest that  $\alpha$ -lipoic acid engages components of the insulin signaling pathway in its ability to stimulate glucose uptake in L6 myotubes. This differs from other stimuli of glucose uptake such as the exercise and/or hypoxia pathway that do not use PI 3-kinase in their ability to stimulate glucose uptake, although they require glucose transporter translocation (122,133). The unique action of  $\alpha$ -lipoic acid to increase glucose uptake



**Figure 6** Schematic diagram of the proposed  $\alpha$ -lipoic acid signaling mechanism.  $\alpha$ -Lipoic acid uses components of the insulin signaling pathway in its ability to stimulate glucose uptake via the rapid translocation of GLUT1 and GLUT4 to the plasma membrane. In L6 myotubes, it has been shown that lipoic acid activates phosphatidylinositol 3-kinase (PI3K) and Akt in a wortmannin-sensitive fashion. These results indicate that lipoic acid uses components of the insulin signal transduction cascade in its ability to increase glucose uptake into insulin-responsive cells in culture.

via an insulin-sensitive pathway was further exemplified by studies whereby  $\alpha$ -lipoic acid increased Akt activity in L6 myotubes (R. Somwar, K. Yaworsky, and A. Klip, unpublished data) (Fig. 6). These actions of  $\alpha$ -lipoic acid are distinctive from other currently used antidiabetic agents and highlight  $\alpha$ -lipoic acid as an attractive therapeutic strategy for the treatment of insulin resistance in type 2 diabetes.

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# 19

## Oxidative Stress and Antioxidant Treatment: Effects on Muscle Glucose Transport in Animal Models of Type 1 and Type 2 Diabetes

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By definition, diabetes mellitus is a group of pathophysiological conditions of varying etiologies that has as a common denominator—the derangement of blood glucose regulation (i.e., hyperglycemia). Two major forms of diabetes mellitus exist: type 1, a less common form in which there is an absolute deficiency of circulating insulin due to destruction of the  $\beta$ -cells of the pancreas, and type 2, the most common form, characterized primarily by a decreased ability of insulin to stimulate skeletal muscle glucose transport and metabolism. Although there is increasing information that oxidative stress, characterized by the localized production of free radicals and other reactive oxygen species, may be associated with metabolic abnormalities present in both type 1 and type 2 diabetes and several studies have been published recently supporting the effectiveness of antioxidant interventions in improving the defective metabolic state characteristic of diabetes, the relationship between oxidative stress and insulin resistance remains controversial (1,2).

The purpose of this chapter is to briefly review the regulation of skeletal muscle glucose transport by insulin under normal conditions and the underlying defects in this regulation present in type 1 and type 2 diabetes and the available information regarding the role of oxidative stress in diabetes and the

utility of antioxidant interventions, with a focus on the water-soluble antioxidant lipoic acid (3), in ameliorating these metabolic abnormalities associated with type 1 and type 2 diabetes. In this context, I discuss primarily evidence from animal model studies because Chapter 20 specifically addresses clinical investigations involving diabetes and antioxidant interventions.

## I. REGULATION OF MUSCLE GLUCOSE TRANSPORT

Skeletal muscle is the major tissue responsible for the peripheral disposal of glucose in the face of a glucose or insulin challenge or during exercise (4,5). Skeletal muscle glucose transport activity is acutely regulated by insulin through the activation of a series of intracellular proteins, including insulin receptor autophosphorylation and tyrosine kinase activation, tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), and activation of phosphatidylinositol 3-kinase (PI 3-kinase), ultimately resulting in the translocation of a glucose transporter protein isoform, the GLUT4 protein, to the sarcolemmal membrane where glucose transport takes place via a facilitative diffusion process (for a review, see 6). Recent evidence from rodent studies indicates that the amount of GLUT4 protein incorporated into the sarcolemmal membrane correlates closely with the degree of insulin-stimulated glucose transport (7,8) and supports the idea that GLUT4 translocation represents the major mechanism for insulin stimulation of glucose transport in skeletal muscle.

Skeletal muscle glucose transport is also stimulated by an insulin-independent process that is activated by contractions (9–11) via a GLUT4 translocation mechanism (7,12). Evidence that these insulin-dependent and insulin-independent pathways for stimulation of glucose transport are mediated by different mechanisms came initially from studies demonstrating that the maximal effects of the two pathways are completely additive (11,13–15). Moreover, the additive effect of insulin and contractions in combination is due to an additivity of the effects of these stimuli on GLUT4 translocation into the sarcolemma (7). More recently, studies by Goodyear et al. (16) have provided a molecular basis for these two distinct pathways for activation of glucose transport in skeletal muscle. Although insulin increases tyrosine phosphorylation of the insulin receptor and IRS-1 and activates IRS-1-associated PI 3-kinase, muscle contraction alone has no effect on these factors, indicating that distinct intracellular pathways exist in muscle for activation of GLUT4 translocation and glucose transport by insulin and contractions (16).

## II. INSULIN RESISTANCE IN DIABETES

Insulin resistance is defined as a reduced ability of insulin to activate specific insulin-dependent biological processes in cells of target organs. In poorly controlled type 1 diabetes, insulin resistance is thought to be a secondary effect of the dyslipidemic state (elevated free fatty acids) and the prolonged hyperglycemic state. In type 2 diabetes, insulin resistance of skeletal muscle glucose disposal is generally considered to be a primary factor in the etiology of this disease. In this latter state, the skeletal muscle insulin resistance is often accompanied by a variety of other metabolic abnormalities, including obesity, dyslipidemia, hypertension, and atherosclerosis (17–19), a condition referred to variously as “syndrome X” (18,19) or the “insulin resistance syndrome” (17). The link among these disorders has been attributed to hyperinsulinemia, a consequence of the insulin resistance (17). Indeed, the increased cardiovascular mortality associated with this condition has been directly attributed by some leading investigators to the hyperinsulinemia itself (20–22).

Interventions that improve insulin action on skeletal muscle glucose metabolism in insulin-resistant individuals are therefore expected to decrease conversion rates to overt diabetes and to reduce cardiovascular mortality in diabetic populations. Therefore, an understanding of the pathophysiology underlying this insulin resistance and the search for optimal interventions for improving insulin action on skeletal muscle are of substantial interest.

## III. ANIMAL MODELS OF TYPE 1 AND TYPE 2 DIABETES

### A. Type 1 Diabetes

The most widely used animal model of type 1 diabetes is the streptozotocin-induced diabetic rat. Streptozotocin is a compound that causes hypersecretion of insulin from the pancreatic  $\beta$ -cells, resulting in their eventual dysfunction and leading to a hypoinsulinemic state (23). The streptozotocin-diabetic rat is characterized by marked postprandial hyperglycemia and by an elevation in free fatty acids without ketoacidosis (23). Skeletal muscle from the streptozotocin-diabetic rat is markedly insulin resistant for stimulation of glucose transport (24,25) and expresses a significantly reduced protein expression of the GLUT4 glucose transporter isoform (24,25).

### B. Type 2 Diabetes

Although numerous rodent models of type 2 diabetes exist, the focus here is on the obese Zucker (*fa/fa*) rat. The obese Zucker rat is an animal model of

severe skeletal muscle insulin resistance also characterized by marked hyperinsulinemia (26), glucose intolerance (27,28), dyslipidemia (26), moderate hypertension (29), and central adiposity (30). It is therefore an excellent model with which to study the underlying pathophysiology and potential interventions in the insulin-resistance syndrome. Studies have identified at least one cellular locus for the insulin resistance of glucose transport in this animal model. Insulin-stimulated GLUT4 protein translocation (8,31) and glucose transport activity (8,32,33) are substantially impaired in isolated skeletal muscle from these obese animals.

Anai et al. (34) have very recently shown that in skeletal muscle from obese Zucker rats, there are significant defects in crucial aspects of the insulin signaling cascade. Compared with age-matched lean Zucker rats, in hindlimb muscle from the obese Zucker rats, there is a 60% smaller IRS-1 protein level, and insulin-stimulated IRS-1 phosphorylation is only 72% of control values, despite elevated basal levels. The amount of the regulatory subunit of PI 3-kinase (detected using a p85 $\alpha$  antibody) associated with the tyrosine-phosphorylated IRS-1 in the insulin-stimulated state is only 29% of control. Finally, IRS-1-associated PI 3-kinase activity in muscle immunoprecipitates from the obese animals is 54% of the level observed in lean animals (34). These findings likely represent the molecular basis for the skeletal muscle insulin resistance present in the obese Zucker rat.

#### **IV. OXIDATIVE STRESS, INSULIN RESISTANCE, AND ANTIOXIDANT TREATMENT IN DIABETES**

##### **A. Streptozotocin-Diabetic Rat**

There is ample evidence that markers of oxidative stress are increased in the most widely accepted rodent model of type 1 diabetes, the streptozotocin-diabetic rat. For example, plasma and liver lipid peroxides, as measured by the thiobarbituric acid reactive substances assay, are elevated in the streptozotocin-diabetic rat (35). In addition, recent evidence indicates that in this model of type 1 diabetes, sciatic nerve levels of reduced glutathione (GSH) are lower and the ratio of oxidized to reduced glutathione (GSSG/GSH) is elevated compared with tissue from normoglycemic control animals (36). Chronic treatment with the antioxidant lipoic acid brings about a nearly complete normalization of the GSH and GSSG/GSH profiles in sciatic nerve from the streptozotocin-diabetic rats and also significantly improves nerve blood flow and conduction velocity (36).

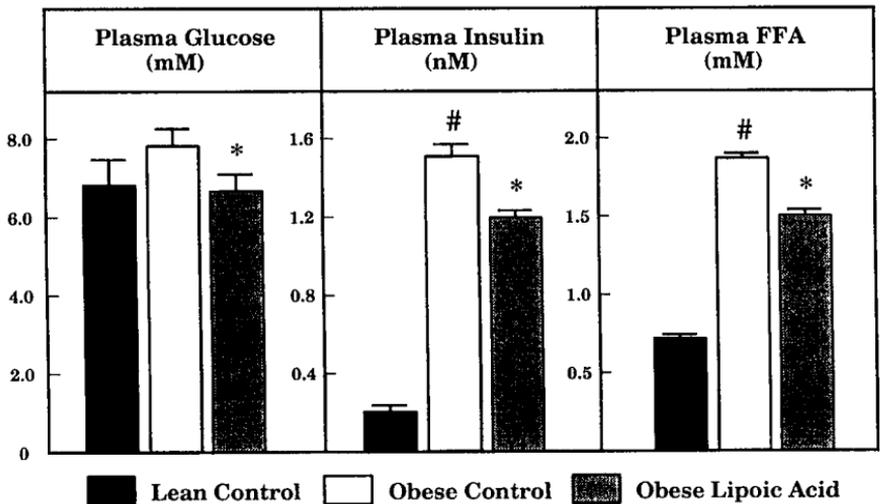
Plasma glucose is markedly elevated and insulin action on skeletal muscle glucose transport activity is substantially reduced in the streptozotocin-diabetic rat, possibly as a result of reduced muscle GLUT4 protein levels (25). Acutely, lipoic acid can cause a marked lowering of plasma glucose in these diabetic animals (25). Chronically, a 10-day treatment period of these diabetic animals with lipoic acid also results in a significant lowering of plasma glucose levels and causes profound increases in both skeletal muscle GLUT4 protein levels and insulin-stimulated glucose transport activity (25). Collectively, these results provide evidence that the beneficial metabolic effects of lipoic acid in this severely hyperglycemic diabetic animal model may be associated with an improvement in the oxidant/antioxidant status of the animal.

## B. Obese Zucker Rat

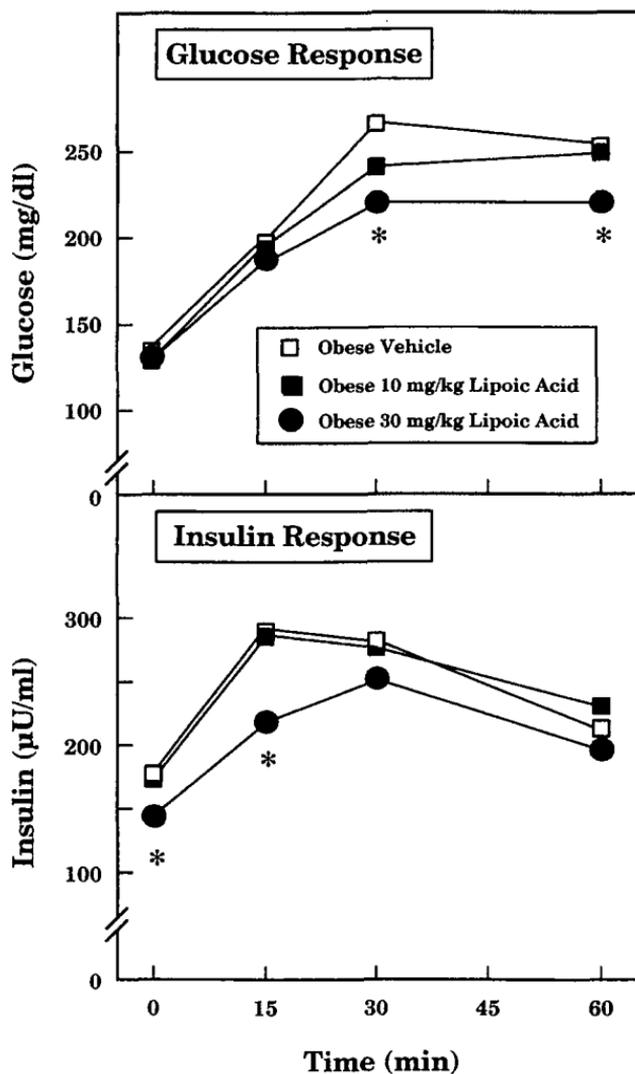
Much less information regarding the oxidant/antioxidant status is presently available for the obese Zucker rat. It should be stressed that this animal model displays only mild fasting hyperglycemia, with more severe abnormalities observed when the animal is presented with a glucose load (27,37,38). Nevertheless, Nourooz-Zadeh (39) reported that the isoprostane 8-epi-PGF<sub>2α</sub>, a marker of oxidative stress, is elevated in the plasma of the diabetic Zucker rat compared with lean controls. Interestingly, these elevated levels of oxidative stress are significantly reduced with antioxidant treatment, such as  $\alpha$ -tocopherol (39). These results concerning oxidative stress in the diabetic Zucker rat are consistent with observations of human type 2 diabetes. During a euglycemic hyperinsulinemic clamp, a significant inverse relationship has been observed between insulin action on nonoxidative glucose disposal and plasma superoxide ion, and a significant positive relationship has been seen between insulin action on nonoxidative glucose disposal and plasma GSH/GSSG ratio in type 2 diabetic patients (40). Patients with impaired glucose tolerance (a prediabetic state) or overt type 2 diabetes have significantly reduced erythrocyte levels of the antioxidant enzymes catalase and superoxide dismutase and diminished plasma GSH (41). Decreased serum vitamin E content, a marker of impaired oxidant/antioxidant status, was recently reported to be associated with increased risk of developing type 2 diabetes in a Finnish population (42), and type 2 diabetic patients themselves display significantly reduced plasma vitamin E levels (43). Finally, plasma hydroperoxides, another marker of oxidative stress, are higher in subjects with type 2 diabetes compared with healthy control subjects and are significantly inversely correlated with the degree of metabolic control (43).

The effectiveness of antioxidant interventions, particularly chronic treatment with lipoic acid, in ameliorating the metabolic abnormalities present in the obese Zucker rat has been demonstrated in a series of studies from our laboratory. The results of these studies are summarized below. In these studies, the obese Zucker rats were treated intraperitoneally with a racemic mixture (50% R- and 50% S-enantiomers) of lipoic acid for 10–12 days and were investigated after an overnight fast (food restricted to 4 g at 5 P.M. of the previous evening). As shown in Figure 1, the obese Zucker rat displays only mild hyperglycemia, and this slight elevation in plasma glucose is completely reversed with chronic lipoic acid treatment (30 mg/kg) (44,45). More striking is the marked hyperinsulinemia and dyslipidemia of the obese Zucker rat compared with the lean Zucker rat (44,45). Chronic lipoic acid treatment leads to significant reductions in both plasma insulin ( $\sim 20\%$ ) and free fatty acids ( $\sim 15\%$ ) (Fig. 1). It should be noted that these alterations due to the racemic mixture of lipoic acid are entirely due to the R-enantiomer, as treatment with the S-enantiomer actually exacerbates the hyperinsulinemia and has no significant effect in lowering plasma free fatty acids (45).

More recently, we have shown that glucose tolerance after a 1-g/kg oral glucose feeding is improved by lipoic acid in a dose-dependent fashion (Fig.



**Figure 1** Effect of chronic treatment of obese Zucker rats with lipoate on plasma glucose, insulin, and free fatty acids. Values are means  $\pm$  SE. \* $p < 0.05$  vs. obese vehicle-treated control; # $p < 0.05$  vs. lean control. (From Ref. 44.)



**Figure 2** Effect of chronic treatment of obese Zucker rats with lipoate on glucose and insulin responses to a 1-g/kg oral glucose tolerance test. Values are means  $\pm$  SE. \* $p < 0.05$  vs. obese vehicle-treated control.

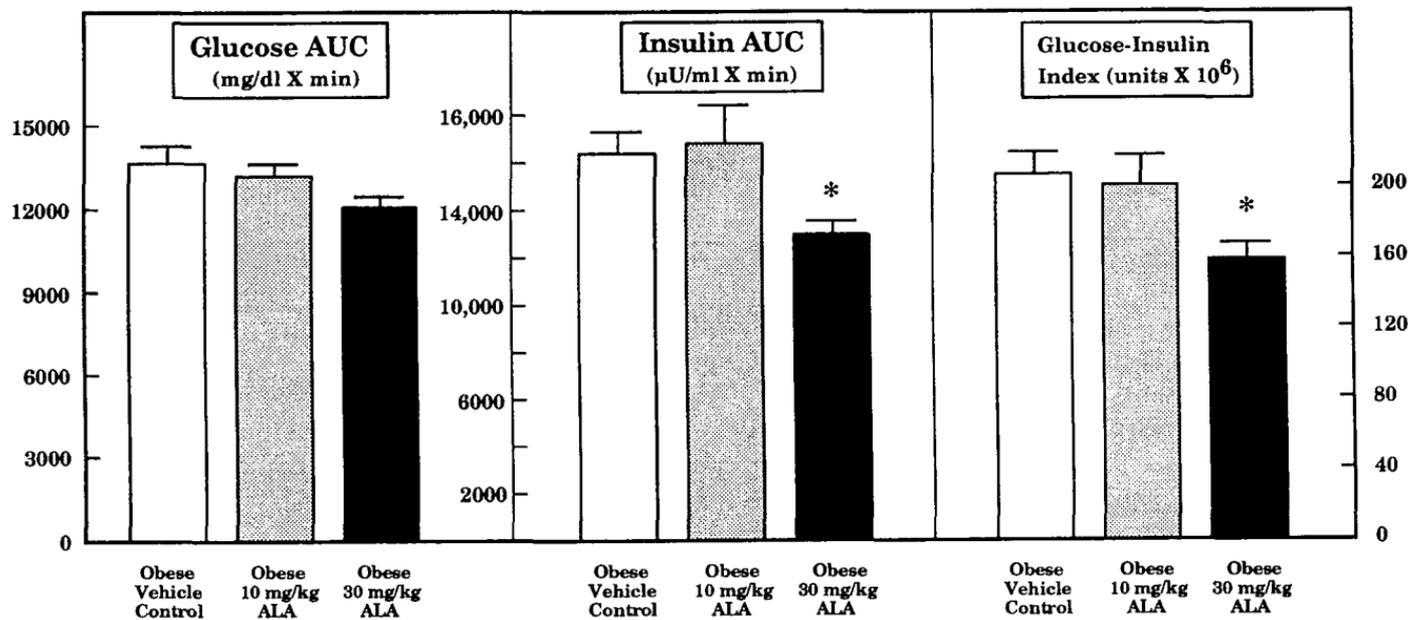
2), with a significantly smaller area under the curve (AUC) of the glucose response in a group of obese animals treated for 10 days with 30 mg/kg lipoic acid compared with control (Fig. 3, left). Moreover, this improved glucose response was seen in the face of a reduced insulin response during the test (Fig. 2) and a smaller insulin AUC (Fig. 3, middle). The glucose–insulin index, the product of the glucose and insulin AUCs and an indirect index of in vivo insulin action, was significantly lower in the 30 mg/kg lipoic acid-treated obese group compared with the obese control group, implying that peripheral insulin action was enhanced by lipoic acid. Consistent with this finding was our observation that insulin-mediated glucose transport activity in both fast glycolytic muscle (m. epitrochlearis, Fig. 4) and slow oxidative muscle (m. soleus, Fig. 5) was improved in the 30 mg/kg lipoic acid-treated obese group compared with the obese control group.

To determine the functional relevance of this improvement of insulin-mediated glucose transport, we assessed the correlation between insulin-mediated glucose transport activity in either the epitrochlearis or the soleus and the glucose–insulin index in obese animals treated with either vehicle, 10 mg/kg lipoic acid, or 30 mg/kg lipoic acid (Fig. 6). The correlation coefficients between the glucose–insulin index and insulin action on glucose transport in the epitrochlearis ( $r = -0.598$ ,  $p < 0.05$ ) and in the soleus ( $r = -0.654$ ,  $p < 0.05$ ) were statistically significant, indicating that the improved insulin action on muscle glucose transport was, at least in part, responsible for the improvement in whole-body glucose tolerance observed after lipoic acid treatment.

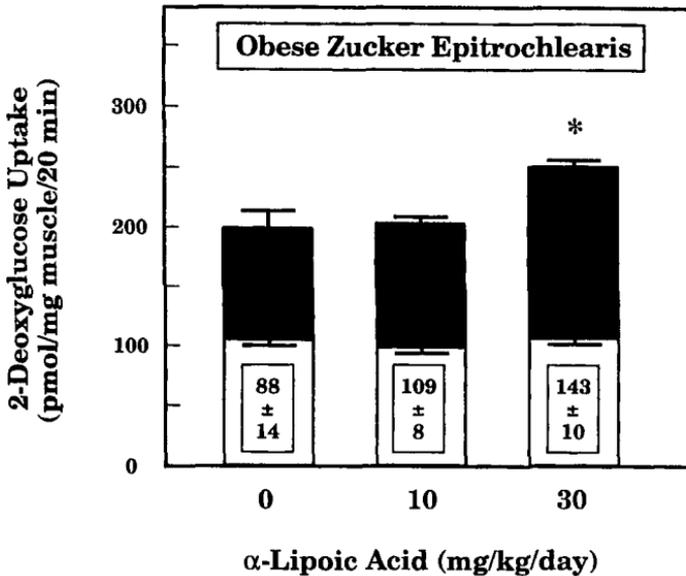
Because the whole homogenate level of GLUT4 protein in skeletal muscle from lipoic acid-treated obese Zucker rats is not significantly elevated compared with obese controls (44), this would imply that lipoic acid enhances the ability of insulin to activate translocation of intracellular GLUT4 protein into the sarcolemmal membrane, a process that is defective in obese Zucker rats (8,31). This hypothesis, however, remains to be tested experimentally.

## V. PERSPECTIVES: ANTIOXIDANTS AND INSULIN RESISTANCE

A growing body of knowledge supports a role of oxidative stress in the complications associated with the hyperglycemic state of diabetes. Moreover, increasing evidence, though still fairly limited at this point, indicates that oxidative stress may be associated with the skeletal muscle insulin resistance inherent to both type 1 and type 2 diabetes. Therefore, interventions that can



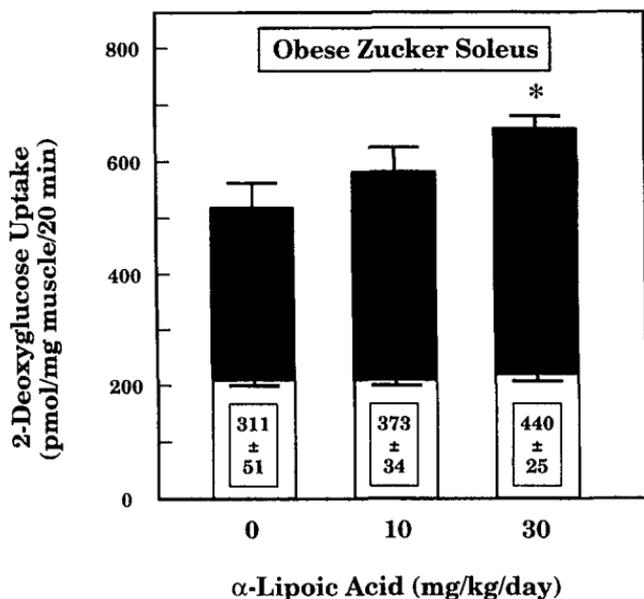
**Figure 3** Areas under the curve (AUC) for the glucose and insulin responses to an oral glucose tolerance test in control and chronic lipoate-treated obese Zucker rats. The glucose-insulin index represents the product of the glucose AUC and the insulin AUC. Values are means  $\pm$  SE. \* $p < 0.05$  vs. obese vehicle-treated control.



**Figure 4** Effect of chronic treatment of obese Zucker rats with lipoate on in vitro insulin-stimulated glucose transport activity in the isolated epitrochlearis muscle. □, Basal 2-deoxyglucose uptake; ■, increase in 2-deoxyglucose uptake due to insulin (2 mU/mL). This increase is shown in the box for each bar. Values are means  $\pm$  SE. \* $p < 0.05$  vs. 0 mg/kg lipoate.

ameliorate the oxidant/antioxidant imbalance in this condition will be helpful in improving peripheral insulin action on glucose transport and metabolism in skeletal muscle. Indeed, several animal model and clinical investigations support the beneficial effects of antioxidants, particularly lipoic acid, in the diabetic state.

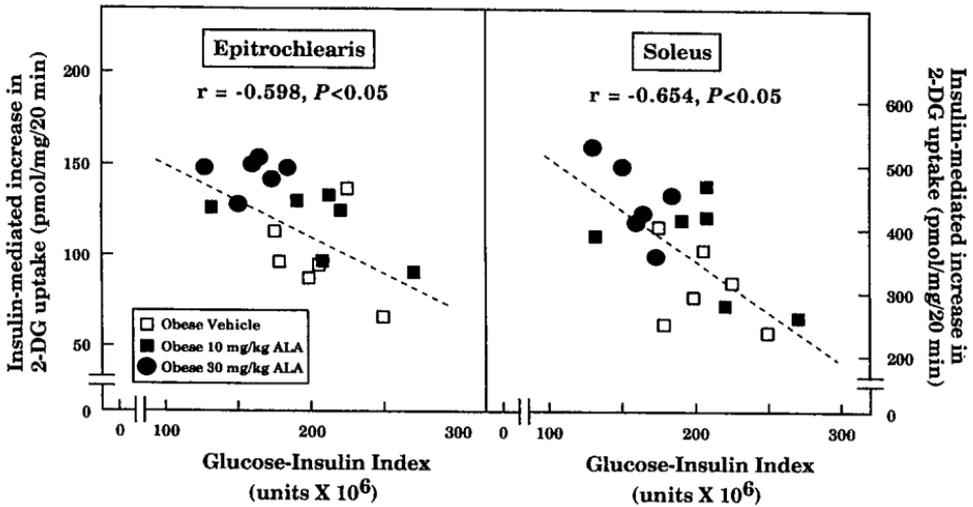
Ample evidence now exists in the literature indicating that one locus of action of lipoic acid in improving metabolic control in animal models of insulin resistance is at the level of the skeletal muscle itself. Chronic treatment of the streptozotocin-diabetic rat, a model of type 1 diabetes, leads to a reduction in blood glucose that is associated with an increase in muscle GLUT4 protein expression and insulin-stimulated muscle glucose transport. Likewise, chronic treatment of obese Zucker rats, an animal model of the insulin resistance syndrome, with lipoic acid enhances whole body glucose tolerance and is associated with significant improvements in insulin action on skeletal muscle glu-



**Figure 5** Effect of chronic treatment of obese Zucker rats with lipoate on in vitro insulin-stimulated glucose transport activity in the isolated soleus muscle strips. □, basal 2-deoxyglucose uptake; ■, increase in 2-deoxyglucose uptake due to insulin (2 mU/mL). This increase is shown in the box for each bar. Values are means  $\pm$  SE. \* $p < 0.05$  vs. 0 mg/kg lipoate.

glucose transport and metabolism, and with reductions in plasma insulin and free fatty acids.

There are, however, areas where our knowledge of lipoic acid action on metabolism is incomplete. For example, we still need more information on the underlying molecular mechanism(s) responsible for the lipoic acid-induced improvement in insulin action. Some limited evidence indicates that there may be some interaction between lipoic acid and the insulin signaling cascade in the L6 muscle cell line (46) and in isolated muscle from the Zucker rat (47); however, a more complete characterization of this interaction in skeletal muscle is necessary. In addition, the relationship between lipoic acid action on skeletal muscle metabolism and its effects on cell oxidant/antioxidant status need to be more thoroughly investigated. It is clear that although investigations of the metabolic actions of lipoic acid have yielded much important information over the last few years, there is still much more work to be done



**Figure 6** Correlations between the glucose-insulin index and skeletal muscle insulin-mediated glucose transport activity in epitrochlearis (left) or soleus (right) muscles from obese Zucker rats treated chronically with lipoate.

in the future to further our understanding of this important antioxidant compound.

## ACKNOWLEDGMENTS

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## Oxidative Stress and Insulin Action: A Role for Antioxidants?

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There is increasing evidence that alterations in the capacity to reduce oxidants, like superoxide anion radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^-$ ), nitric oxide (NO), and alkyl or peroxy radicals, could play an important role in the pathogenesis of various diseases. The imbalance between oxidants and antioxidants in favor of the oxidants, so-called oxidative stress, results in a nonenzymatic free radical-mediated oxidation of biological molecules, membranes, and tissues associated with a variety of pathological events.

Although it is generally acknowledged that oxidative stress plays a role in the development of angiopathy in diabetes mellitus and its vascular complications (1–4), there is little information available about the impact of radical oxygen species in the pathogenesis of insulin resistance and the development of diabetes mellitus (5). Several groups have shown that the levels of free radicals are increased when metabolic control is poor. This is found in both type 1 (6) and type 2 diabetes (7,8).

Indices of this augmented oxidative stress are reduced or even reversed to normal when glycemia is well controlled, and this can be shown even after a very short period of time of improved glycemic control (9). Better metabolic control was also clearly shown to be associated with a drastic reduction of diabetic complications (10–12). These observations could suggest that oxidative stress does not play a role in the nonhyperglycemic/euglycemic state.

However, oxidative stress also seems to be present in uncomplicated type 2 (8,13) or type 1 (6) diabetes.

Furthermore, few reports describe increased radical formation in several conditions without clinical diabetes mellitus, such as dyslipidemia, impaired glucose tolerance, hypertension, coronary artery disease, aging, and smoking (1,2,14–20). These conditions have also been found to be associated with a decrease in insulin sensitivity (21–24), but contrary to the diabetic state, effects mediated by hyperglycemia can be excluded. Therefore, it seems that the origin of oxidative stress cannot be solely explained by hyperglycemia.

## **I. OXIDATIVE STRESS AND INSULIN SENSITIVITY— CLINICAL OBSERVATIONS**

Paolisso et al. (25) demonstrated close correlations between the presence of  $O_2^-$  and insulin sensitivity in an elderly nondiabetic population. Epidemiological studies found a close correlation between low levels of antioxidants, such as vitamin E or vitamin C, and a high risk of developing frank type 2 diabetes (26,27). Several groups report a higher prevalence of radical oxygen species in prediabetic individuals who had an impaired oral glucose tolerance test (17–19).

An increase in plasma thiobarbituric acid reactive substance (TBARS) was found in healthy subjects when free fatty acids (FFA) were experimentally kept elevated by an infusion of intralipid and heparin (28); under these experimental conditions, insulin sensitivity was markedly reduced (28). It could thus be speculated that the elevation of FFA seen in patients with type 2 diabetes or with insulin resistance (29) could be a source for such an augmented oxidative stress.

## **II. ROLE OF IMPAIRED INSULIN ACTION IN THE PATHOGENESIS OF TYPE 2 DIABETES MELLITUS**

In type 2 diabetes mellitus, plasma glucose levels are elevated as a result of an impairment of several metabolic pathways (29–31) (Table 1). Skeletal muscle is the principal organ for postprandial glucose uptake (29,30). In the pathogenesis of diabetes mellitus type 2, reduced insulin-stimulated glucose disposal (insulin resistance) plays a key role (21,29). When clearance of plasma glucose is impaired, blood glucose after a meal will remain slightly elevated

**Table 1** Metabolic Alterations in Type 2 Diabetes

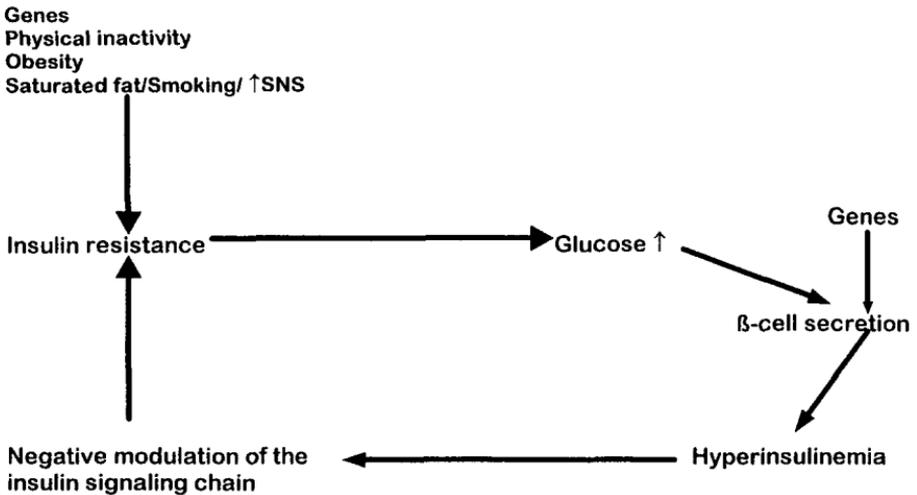
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Diminished insulin-mediated peripheral glucose disposal and metabolism (insulin resistance)
Impaired insulin secretion (reduced first-phase response, prolonged second phase)
Decreased insulin-mediated inhibition of lipolysis
Increased gluconeogenesis

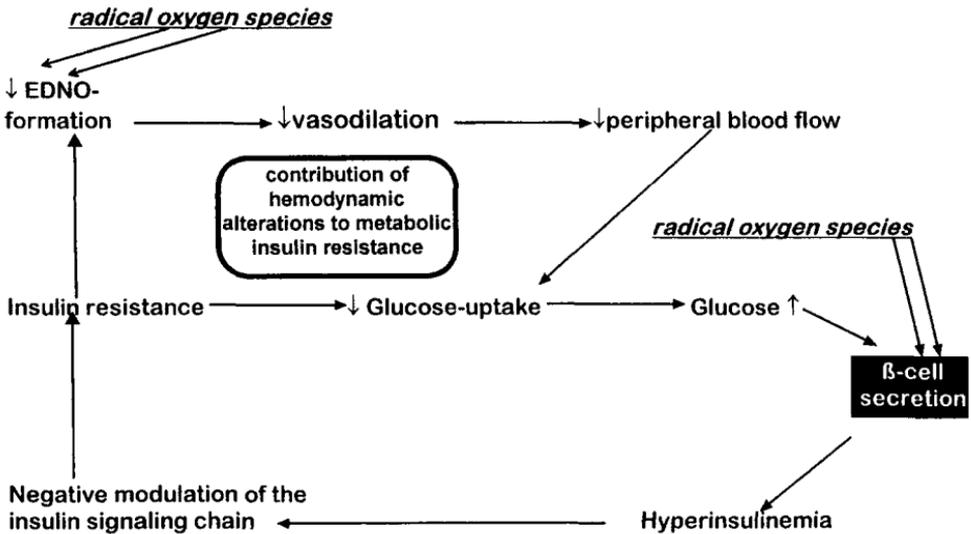
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and will thus induce hyperinsulinemia to overcome resistance (Fig. 1). Hyperinsulinemia, however, will evoke an alteration of the insulin-signaling cascade, which will further augment insulin resistance, thus leading to a vicious cycle (29,30).

There is an ongoing scientific discussion as to whether insulin resistance of skeletal muscle or an impairment of insulin secretion is the first and principal disorder. However, epidemiological data indicate that reduced insulin sensitivity can already be demonstrated when insulin secretion is still adequate



**Figure 1** Vicious cycle of insulin resistance and hyperinsulinemia. Due to the reduced insulin sensitivity, peripheral glucose uptake is diminished. Therefore, plasma glucose clearance is reduced, and postprandial blood glucose will remain slightly higher. This will consequently induce hyperinsulinemia to overcome the insulin resistance. Hyperinsulinemia, however, will evoke a negative modulation of the insulin signaling chain, which will further exacerbate the insulin resistance, hence leading to a vicious cycle. SNS, sympathetic nervous system.



**Figure 2** Model of the potential pathomechanism induced by oxidative stress. Interplay between hemodynamic and metabolic alterations. EDNO, endothelium-derived nitric oxide.

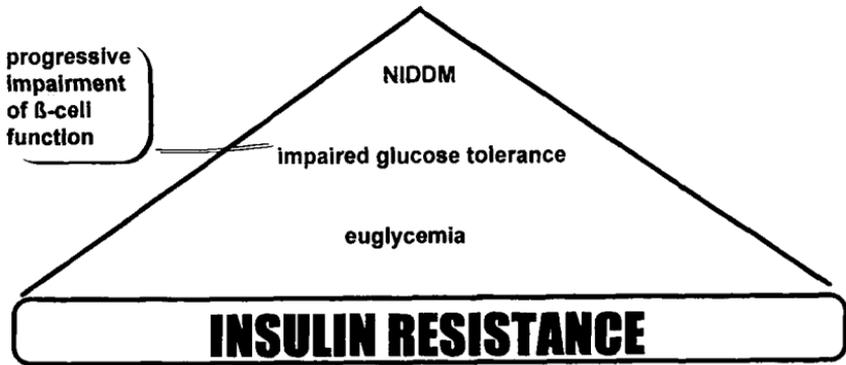
(29). Thus, it is currently believed that as long as hyperinsulinemia can compensate for insulin resistance, glucose tolerance will be normal; with progressive impairment of  $\beta$ -cell function, impaired glucose tolerance (iGT) or frank type 2 diabetes will be the consequence (29) (Fig. 2).

### III. NITRIC OXIDE AND INSULIN SENSITIVITY

#### A. Nitric Oxide and Endothelial Dysfunction

Several groups have shown that insulin-resistant subjects have a reduced insulin-stimulated increase in leg blood flow; the changes in peripheral blood flow were closely associated with the degree of insulin resistance (22,32–35). This endothelial dysfunction is demonstrated not only in patients with type 2 diabetes but also in nondiabetic subjects, such as obese adolescents or first-degree relatives of patients with type 2 diabetes (33,35,36).

It seems that peripheral blood flow is augmented via an insulin-mediated NO-dependent process (Fig. 3), and inhibition of NO formation prevents the



**Figure 3** Progress from insulin resistance to type 2 diabetes mellitus.

insulin-induced increase in blood flow (37). Thus, NO could indirectly affect insulin sensitivity as it increases peripheral blood flow and subsequent substrate delivery to the skeletal muscle. However, there is still a debate about the clinical relevance of these findings.

### **B. Intracellular NO—A Direct Modulator of Glucose Uptake?**

Very recently it was shown that NO synthase is also present within the skeletal muscle (38,39), and NO was found to increase glucose uptake by an insulin-independent mechanism (40–43). Furthermore, Roberts et al. (40) reported that exercise-stimulated glucose transport in the skeletal muscle of rats is NO dependent; this group provides evidence that NO is markedly involved in the regulation of exercise-induced glucose uptake. Exercise-induced skeletal muscle glucose uptake is normal, even in insulin-resistant animals (44). Thus, the exercised-induced elevation of NO availability (40,42) seems to provide an important alternative pathway. Animal studies indicate an improvement of endothelial function after exercise training (45).

### **C. NO—A Radical Scavenger**

NO is also known to act as a radical scavenger itself (46,47). An increased availability of NO was associated with a decrease in the levels of superoxide

(47). Furthermore, exercise training was not only found to improve metabolism and endothelial function (40,42,45) but also antioxidant defense (48); therefore, one of the beneficial effects of exercise could be mediated by the increase of antioxidant defense mechanism. Indirect support for the association between NO availability and insulin sensitivity emerges from a clinical study, in which L-arginine, the substrate required for NO synthesis, was infused and insulin sensitivity and endothelial function was improved (49); the increase of insulin sensitivity, however, remained significant after adjustment for changes in blood flow (49).

It is thus conceivable that an increase in radical oxygen species may reduce the availability of NO, and this consequently would contribute to the endothelial dysfunction and possibly to the development of insulin resistance (Fig. 3).

#### **D. Homocysteine, NO, and Insulin Sensitivity**

Homocysteine levels were found to be significantly higher in patients with coronary artery disease and in those with diabetes mellitus (50–54). Elevated homocysteine levels induce oxidative stress and reduce NO availability (55); this can contribute to endothelial dysfunction. It seems possible that hyperhomocysteinemia could also alter insulin sensitivity by this mechanism. Therefore, it remains to be clarified whether there are any interactions between elevated homocysteine levels and the development of insulin resistance in nondiabetic subjects.

The interesting observation of an augmented oxidative stress, insulin resistance, and elevated homocysteine levels in smokers (14,23,24,50) suggests some interactions. However, this still remains to be evaluated.

### **IV. INTERVENTIONS KNOWN TO ALTER RADICAL OXYGEN SPECIES**

#### **A. Lifestyle**

A diet high in saturated fat and low in fiber and a low level of physical activity are associated with an increased risk of developing type 2 diabetes mellitus (26,27). Modifications of these factors are known to improve metabolic control and insulin sensitivity (56) but should be also expected to reduce oxidative stress. Experimental data suggest that exercise training can improve insulin resistance in parallel with a better antioxidant defense (45). One explanation

for this could be the improvement of NO availability in the skeletal muscle (see above).

Epidemiological data support the protective role of regular exercise: Greater physical activity (57–60) reduces the risk of developing type 2 diabetes mellitus, even in those with a family history of type 2 diabetes. Because smoking is known to be associated with an increased oxidative stress (14) and insulin resistance (23,24), this association could be one mechanism by which smoking cessation improves insulin sensitivity (61).

## B. Pharmacological Intervention

Several compounds with an antioxidant potential were found to modify insulin sensitivity.

### 1. Troglitazone

Troglitazone, a thiazolidinedione which is the first compound of the new class of insulin sensitizers (62,63), improves glycemia and dyslipidemia by reducing insulin resistance and hyperinsulinemia in type 2 diabetes mellitus (64) and also in normoglycemic subjects with insulin resistance (65,66). Experimental data indicate that troglitazone improves insulin action by various mechanisms (67–69) (Table 2).

Troglitazone has a similar structure to vitamin E and is also known to be a potent radical scavenger (70–72). At present, it is not known whether the radical scavenging ability of troglitazone is relevant for its beneficial effect on insulin resistance. Pioglitazone, another thiazolidinedione, is also a potent insulin sensitizer, but an experimental study recently reported that it had no radical scavenging property (72).

### 2. Glutathione

Administration of glutathione was found to be advantageous in type 2 diabetics and those with impaired glucose tolerance (Table 2). Glutathione improved insulin secretion in patients with iGT (73). Because insulin secretion is impaired in type 2 diabetes (29) and because recent data suggest that lipotoxicity might play a role in decreasing  $\beta$ -cell function (74,75), glutathione could improve  $\beta$ -cell function by protecting the  $\beta$ -cell. However, this hypothesis still needs to be tested.

Glutathione also improves insulin sensitivity in patients with type 2 diabetes after acute (76) and chronic administration (49). In type 2 diabetes, a

**Table 2** Synopsis of the Effects of Interventions Improving Antioxidant Capacity and Insulin Sensitivity

Intervention	Direct	Indirect	Other effects	Ref.
Diet	yes			1
Exercise	yes	endothelial function	↑ NO-availability	2–9
Smoking cessation	?	endothelial function		10,11
Troglitazone	yes	?endothelial function	PPAR $\gamma$ ↓ glucose toxicity ↑ LDL-oxidation	12–19
Glutathione	?yes	endothelial function	↑ insulin secretion in iGT ↑ microviscosity ↓ “lipotoxicity”	20–24
Vitamin E	?	endothelial function	↑ vasodilation no effect on insulin secretion	25–32
Vitamin C	?	endothelial function	↑ insulin-stimulated glucose uptake ↑ vasodilation no effect on insulin secretion	31,33–35
Thioctic acid	? yes	↑ insulin-independent glucose uptake endothelial function ↓ of adhesion molecules NF- $\kappa$ B↓	↑ insulin-stimulated glucose uptake ↑ vasodilation ↑ microcirculation	36–45
Others				
ACE inhibitors	yes	endothelial function	insulin-stimulated glucose uptake is kinin-mediated	46–48
Vasodilating beta-blockers	?	endothelial function		49,50

reduced plasma GSH/GSSG ratio was found; this was negatively associated with the levels of fasting FFA ( $r = -0.53$ ;  $p < 0.05$ ) (49). In an experimental study with healthy volunteers, "metabolic oxidative stress" was induced by infusion of intralipid and heparin, resulting in a marked rise of FFA levels. This was associated with an increase of indicators of oxidative stress, as reflected by increased TBARS and a reduced GSH/GSSG ratio (28). In contrast, the infusion of glutathione diminished the negative effect of the sustained elevation of FFA; although FFA were elevated, the alteration of both oxidative stress and insulin-stimulated glucose uptake were markedly attenuated when glutathione was coinfused with intralipid. In addition, glutathione even improved insulin sensitivity and oxidative stress in the control experiment in which no intralipid was given (28).

Ammon et al. (77) showed that the administration of acetyl-cysteine, a compound that could increase endogenous formation of glutathione, improved glucose disposal in healthy volunteers; this was associated with an improved GSH/GSSG ratio. Furthermore, experimental studies indicate a protective role of glutathione on endothelial function (78).

### 3. Vitamin E

A large epidemiological study indicates that a low level of vitamin E confers a marked risk for the development of a type 2 diabetes mellitus (26). Low levels of vitamin E are also documented in patients with coronary artery disease (20). A regular intake of higher doses of vitamin E was associated with a marked decline in vascular events in coronary artery disease patients (79). These observations suggest a role for vitamin E and/or oxidative stress in these chronic diseases.

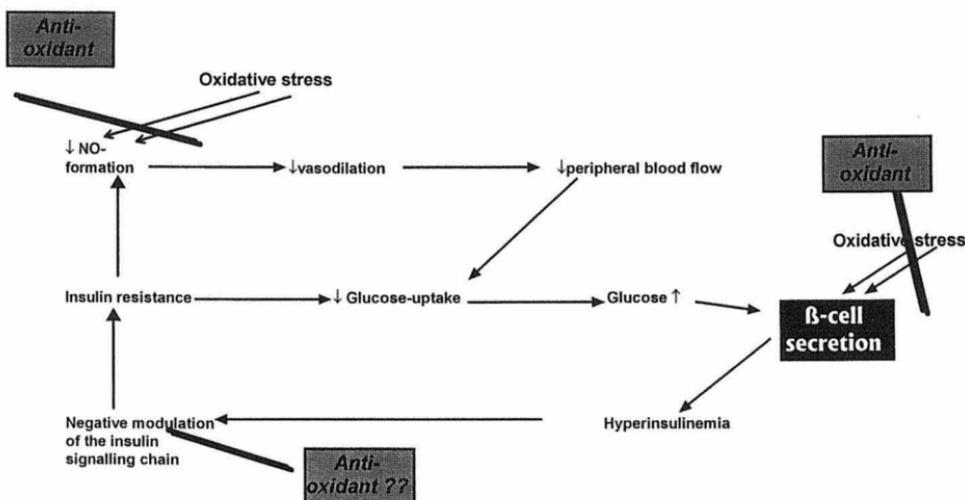
In experimental studies, it was shown that vitamin E has beneficial effects on insulin sensitivity. Fructose feeding induces insulin resistance and hypertension in rats; this is also associated with an increase in radical oxygen species formation (80). Vitamin E administration prevented not only the diet-induced alterations in insulin sensitivity but also reduced oxidative stress. Finally, several clinical studies by Paolisso's group suggest that vitamin E intake improves insulin sensitivity as measured by the glucose clamp technique in healthy and diabetic subjects (81–84).

Vitamin E could also modulate insulin sensitivity by indirect effects because it can improve endothelial function. It was shown that in diabetes mellitus endothelial dysfunction is present (1,4), and it is suggested that oxidative stress reduces vasodilatation (3,85,86). Vitamin E administration restores this defect (3,85,86).

If endothelial dysfunction is involved in modulating insulin's action, a restoration of endothelial function should also augment insulin sensitivity (see III.A and Table 2, Fig. 4). Furthermore, vitamin E improves insulin secretion in experimentally induced (type 1) diabetes mellitus (87). At present, however, there are no data concerning the effect of vitamin E on  $\beta$ -cell function in type 2 diabetes mellitus.

#### 4. Vitamin C

There are a few studies indicating a role for vitamin C in modulating insulin sensitivity. Epidemiological data identify low serum levels of the vitamin as a risk factor for the development of type 2 diabetes mellitus (27). Experimental data describe a protective role of vitamin C on the age-associated deterioration of insulin sensitivity (88). Clinical studies describe an enhanced insulin-stimulated glucose uptake in a glucose clamp study after acute vitamin C treatment in healthy subjects and in those with type 2 diabetes (89,90); the beneficial effects on insulin sensitivity were closely associated with the increases in the plasma levels of the vitamin C (90). Experimental studies indicate also a beneficial effect on endothelial function in experimental polyneuropathy (3). Clinical studies also describe an improvement of endothelial dysfunction (91).



**Figure 4** Decrease of insulin resistance by antioxidants (hypothetical mechanisms).

### 5. $\alpha$ -Lipoic Acid

Thioctic acid, also known as  $\alpha$ -lipoic acid, was found to improve insulin action in various experimental models (92–95). Its action seems to involve interaction with the insulin receptor signaling cascade and potentially also insulin-independent steps (see Chaps. 18 and 19). In vivo experimental studies found an improvement of insulin sensitivity and glucose tolerance after the administration of the racemic mixture (95,96).

Clinical pilot trials suggest that this compound might also have beneficial effects in humans, because insulin resistance was improved after acute or chronic intravenous administration of  $\alpha$ -lipoic acid (97–99). Recently, a small placebo-controlled pilot trial found an improvement of insulin sensitivity in patients with type 2 diabetes mellitus after oral administration (100). The changes in insulin sensitivity seen after the active treatment was significantly different from that seen in placebo: whereas insulin sensitivity decreased in the control group, it improved after  $\alpha$ -lipoic acid. Furthermore, thioctic acid improves endothelial function (101) and microcirculation by reducing adhesion molecules (102) and preserves endothelial structure (103).

### 6. Other Compounds

There are several other compounds with antioxidant activity also shown to have a beneficial effect on insulin sensitivity. Two groups of antihypertensive agents are known to improve insulin sensitivity and oxidative stress.

Angiotensin-converting enzyme inhibitors increase the availability of kinins by inhibition of kininase II (104) and consequently NO (47). They were shown to augment insulin-stimulated glucose uptake in clinical and experimental studies (104,105). Recent data suggest that treatment with Ramipril has pronounced effects on NO synthase expression and NO formation and a concomitant decrease in superoxide accumulation, which was associated with an extended lifespan in the angiotensin-converting enzyme treated rats (47).

The vasodilating beta-blockers carvedilol and celiprolol have a marked antioxidant capacity (46,106,107) and were found to improve endothelial function (108). In clinical studies they increased insulin sensitivity as documented by the glucose clamp (109–111).

## V. CONCLUSION AND OUTLOOK

At present, experimental and clinical data suggest but do not prove an association between insulin sensitivity and oxidative stress. Furthermore, experimen-

tal and some clinical studies suggest a beneficial effect on insulin secretion or insulin action after treatment with certain antioxidants. Currently, it is still unknown whether the effects on insulin sensitivity are modulated by direct mechanisms, for instance on the insulin receptor-signal transduction cascade, or whether metabolism improves indirectly such by an improvement of endothelial function.

It is necessary and seems to be promising to analyze further the association between oxidative stress and insulin action (112); this involves both the quantitative assessment of insulin sensitivity and the radical oxygen species respective of the oxidant defense system.

If an antioxidant is supposed to improve insulin sensitivity by decreasing oxidative stress, this should be associated with a decrease in radical oxygen species. Furthermore, it would be important to show a dose-response relationship, that is, the more the oxidative stress is reduced, the better the insulin resistance is improved.

To date, only small clinical trials with a short duration of treatment have been conducted (Table 2). It is absolutely necessary in the near future to conduct larger trials involving intensive assessment of the oxidative stress and antioxidant defense and the exact analysis of insulin sensitivity and metabolic control. Therefore, there is a need for a "radical initiative" (113).

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