Antioxidants and Diabetes

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The American Diabetes Association Research Symposium on the Role of Oxidants and Antioxidant Therapy on Diabetic Complications was held in Orlando, FL, in November 1996. Among the presentations was that of Lester Packer, Berkeley, CA, who discussed potential sources of oxidant stress in biological systems and presented an overview of the role of oxidants in aging and the mechanisms of antioxidant action. Sources of free radicals in biological systems include radiation, environmental chemicals, and many biological stressors, and there are a number of endogenous antioxidant proteins, such as superoxide dismutase, glutathione peroxidases, and metal-binding proteins, to protect the body against the potent oxidant properties of free metals, such as iron and copper. Packer mentioned a number of antioxidants, including vitamins C and E, carotenoids, flavenoids, and protein-bound selenium, copper, and zinc. α-Lipoic acid also may have great potential as an antioxidant. It exists in oxidized and reduced forms and plays a role in a number of essential intermediate metabolic processes by regenerating NAD from NADH. Packer brought up a theme mentioned subsequently by other researchers in the field: multiple antioxidants may interact additively in biological systems. For example, reduced dehydrolipoic acid, in combination with vitamin C and vitamin E, may participate in an "antioxidant network" that allows ongoing regeneration of the reduced forms of vitamins C and E after they have acted. α-Lipoic acid inhibits intercellular adhesion molecule 1 and increases levels of glutathione, another endogenous antioxidant, by its action in reducing the oxidized amino acid cysteine to cystine. Mortality levels due to experimental stroke decrease with α-lipoic acid treatment, and benefits have been shown clinically with administration of 600 mg daily of the drug to patients with neuropathy. Preliminary studies have also shown decreased glycation of proteins in animals treated with this agent.

Metal ions play a role in the generation of oxidants, and this topic was discussed by John Gutteridge, London, U.K. He defined a free radical as "any chemical species capable of independent existence that contains one or more unpaired electrons." Thus, oxygen plays a major role as an oxidant in the form of superoxide (O_2^-), hydroxyl (OH^-), and peroxyl ($R\text{-}O_2^-$) radicals and in many other forms having free radical properties. O_2 metabolism relies on the transition metals iron and, to a lesser extent, copper in oxidases, oxygenases, and many other enzymes. A normal cell's DNA sustains more than 10,000 "radical hits" daily, almost all of which are readily repaired, but under conditions of increased free radical generation, damage levels exceed the reparative capacity. Thus, several epidemiological studies have suggested a correlation between increased iron levels and increased rates of carcinogenesis. There is also an association between increased transferin saturation and increased concentrations of coagulation factors, and Gutteridge emphasized his feeling that the common use of iron supplements in foods, appropriate for growing children and for menstruating women, leads to adverse consequences for adult men. Furthermore, hemochromatosis is "not a rare disease, but is just rarely diagnosed," Gutteridge stated, pointing out that the gene frequency for this disorder is 0.3% in Celtic populations.

Joseph Beckman, Birmingham, AL, spoke on the role of NO and peroxynitrite. NO is produced by the oxidation of arginine and plays an important role in the endothelium, where it acts as an endogenous vasodilatory substance via cyclic GMP production. It is also produced in large quantities by neurons. NO is approximately as reactive as O_2, but it acts as a chain terminator ($^{\bullet}N\text{-}O$ rather than $^{\bullet}O\text{-}O^{\bullet}$) and binds readily to metals. In vivo, NO is removed by diffusion into erythrocytes, where it slowly binds to hemoglobin to produce methemoglobin. It binds readily to O_2^-, producing peroxynitrite ($O\text{-}N\text{-}O_2^-$), a highly reactive and toxic intermediate. NO diffuses farther than the hydroxyl radical and "disarms its major antioxidant defense" by binding to superoxide dismutase and also by competing for O_2^-. Structural proteins are readily nitrated, and nitration changes their biological activity. As an example of this, Beckman presented evidence of actin nitration at two tyrosine sites.

Rajindar Sohal, Dallas, TX, discussed mitochondrial oxygen radical generation and the aging process. Chronic oxidative stress increases with age due to increased levels of reactive oxygen species, generated particularly in the mitochondria, and this leads to a decline in the efficiency of a variety of homeostatic mechanisms. Increased oxidative stress with age is also caused by decreased antioxidant defense levels and decreased removal of damaged molecules.

There are a number of antioxidant substances available to the organism. Etsuo Niki, Tokyo, Japan, spoke on the dynamics of action of vitamin E as an antioxidant. Epidemiological studies have suggested a role of vitamin E as a factor protecting against coronary disease. The chemical structure of vitamin E includes a phenolic OH attached to a chromatin ring, which increases its reactivity, and a long fatty acid–like tail, which leads to incorporation of vitamin E into lipid membranes. Vitamin E scavenges peroxy radicals approximately 10,000-fold more avidly than these react with fatty acids, so in theory if one vitamin E molecule is present for every thousand fatty acids, their oxidation will be decreased by a factor of 10. Other factors, however, lessen this benefit. Vitamin E is the most abundant antioxidant in LDL but is located in the more rigid outer layer of the particle, while free radicals may exist in the more fluid core and therefore not be affected by the antioxidant's presence. Thus, in homogeneous solutions β-carotene is only 1/30 as active as vitamin E in scavenging free radicals, whereas in models of LDL, such as liposomal membranes, β-carotene is actually consumed more rapidly. Most likely there is an interaction between antioxidants such as vitamins C and E, one regenerating the other, so that combinations are more effective than individual agents. Angelo Azzi, Bern, Switzerland, spoke further on

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potential benefits of vitamin E. In addition to discussing its antioxidant effects, he discussed an effect of α-tocopherol (vitamin E) in inhibiting smooth muscle cell proliferation. Such inhibition appears to be stereospecific to a single isomer, suggesting that it is a receptor-mediated effect, perhaps acting in part to prevent autophosphorylation of protein kinase C (PKC)-α and thus leading to a decrease in transcription of factors that promote cellular proliferation. After discussing evidence that aldose reductase may be induced by α-tocopherol, Azzi also showed fascinating data suggesting that aldose reductase "is an ambivalent enzyme" that, although it may potentially cause adverse effects by producing sorbitol from glucose, also converts 4-hydroxynonenol, a free radical promoting cell lysis, to the nontoxic 4-hydroxynonenal.

Vitamin C is another antioxidant present in the body. Balz Frei, Boston, MA, spoke on the antioxidant actions of this substance, which acts as a potent electron donor and is then reduced back to ascorbic acid primarily by glutathione. Ascorbate, the most effective water-soluble antioxidant, is the first antioxidant to be consumed in plasma exposed in vitro to aqueous peroxy radicals, with sulfhydryl groups, bilirubin, urate, and α-tocopherol being consumed more slowly. It is interesting that after an acute oral dosage of 2 g of vitamin C, plasma vitamin C levels are increased, the lag time to generation of free radicals after administration of aqueous peroxy radicals is increased, and lipid peroxidation in lipoproteins is delayed. Comparison of patients with and without diabetes undergoing angiography showed that those with diabetes had lower ascorbic acid levels and greater susceptibility of their LDL to copper-induced oxidation. Other studies have shown improvements in vascular function in patients with coronary disease after vitamin C administration, perhaps by a glutathione-sparing effect.

The amino acid methionine may play a role as an antioxidant. Rodney Levine, Bethesda, MD, termed methionine residues in proteins "an additional type of antioxidant" that is often located near crucial amino acids, such as tryptophan, that are susceptible to oxidation and are located on the outer portion of proteins. Thus methionine acts as a "macromolecular bodyguard" or as "a pawn on the battlefield to protect critical sites." The enzyme methionine sulfate reductase reduces these methionines back to their original state to protect against proteolysis. Oxidized methionine levels appear to increase with normal aging and may increase further with diabetes.

George King, Boston, MA, discussed the relationship between diacylglycerol (DAG), activation of PKC, and antioxidants. Hyperglycemia increases DAG levels, leading to activation of PKC. This may explain some of the complications of diabetes, because a particular isoform of PKC, PKC-β, increases levels of growth factors, which then contribute to increased basement membrane and mesangial connective tissue deposition. King described studies with LY333531, a specific inhibitor of PKC-β, and vitamin E. LY333531 prevents the increased PKC-β activity seen in renal glomeruli of diabetic animals and normalizes the increased glomerular filtration and albuminuria seen in the diabetic state. In contrast, in a nondiabetic transgenic animal model with overexpression of PKC-β, cardiac complications similar to those in diabetes are seen. In the aorta and retina hyperglycemia activates PKC-β, and in vivo treatment with vitamin E normalizes PKC-β activity. Similarly, markers of PKC-β activity are increased in diabetic rat heart and can be normalized with vitamin E, as can the hyperfiltration seen with diabetes. Vitamin E also restores elevated DAG levels to normal in models of diabetes such as cultured cardiac myocytes and aortic smooth muscle under conditions of hyperglycemia. In these models, vitamin E does not appear to act directly on PKC. Probucol also is active, but vitamin C is not active, so it is not clear if antioxidant mechanisms per se are responsible for these effects on DAG. Fumio Umeda, Fukuoka, Japan, discussed the effects of diabetes in increasing levels of vasoconstrictors, such as thromboxane A_2, while decreasing levels of vasodilators, such as prostaglandin I_2 (PGI_2). These effects may be mediated by increased levels of free radicals, and specific factors produced by smooth muscle cells show decreased transcription in models of diabetes. Vitamin E, particularly *d*-α-tocopherol, shows specific binding to aortic smooth muscle cells and increases PGI_2 production and decreases lipid peroxidation in cultured cell systems. Vitamin E treatment of patients with diabetes restores vessel wall and plasma vitamin E levels and vessel wall prostaglandin levels.

Several consequences of oxidant action have been documented in vivo. David Hockenbery, Seattle, WA, spoke on mechanisms of oxidative stress from mitochondrial dysfunction in apoptosis, or programmed cell death, which is associated with increased lipid peroxidation and can be inhibited by antioxidants, and Naoyuki Taniguchi, Osaka, Japan, discussed the induction of apoptotic cell death by oxidants such as NO and by glycation. Alan Fogelman, Los Angeles, CA, reviewed the relationship between oxidized lipids and atherosclerosis. After non–receptor-mediated uptake of LDL into the arterial wall, oxidation leads first to "mild modification" of LDL, which increases chemotaxis of macrophages. As LDL continues to be changed into "highly oxidized" forms, it is taken up by the scavenger receptor of the macrophages, which then become foam cells. This process may be decreased by administration of antioxidants in animal models. Further, an enzyme contained in normal HDL, paraoxanase, inhibits LDL modification, but after acute stresses (e.g., surgery), HDL changes so that it potentiates the oxidative modification of LDL. This change may occur because of the presence of ceruloplasmin or other acute-phase reactants. Jay Heinecke, St. Louis, MO, further discussed the generation of reactive aldehydes and advanced glycation end products (AGEs) by activated phagocytes and the role of these substances in atherogenesis. Foam cells possess oxidative pathways, including membrane-associated NADPH-linked enzymes that convert O_2 to O_2^-, leading to generation of H_2O_2, which interacts with the form of iron associated with the enzyme myeloperoxidase (MPO), Fe(III), to produce the highly cytotoxic Fe(IV)=O. MPO can be detected by immunostaining in areas of atherosclerotic lesions similar to those containing oxidized LDL. The enzyme also catalyzes production of HOCl from H_2O_2, Cl^-, and H^+. 3-Chlorotyrosine is a marker of this effect on tyrosine residues of proteins, and levels of this are increased in human atherosclerotic aorta samples. The MPO system leads to the generation of reactive intermediates that cause protein cross-linking and AGE formation. Heinecke commented that he believes that this, rather than free metal ions, is a major source of oxidative damage in vivo.

Specific information on oxidant formation in diabetes is limited. John Baynes, Columbia, SC, addressed the crucial question of whether oxidant formation is actually increased in diabetes per se. Measuring specific oxidized molecules rather than relying on nonspecific assays of oxidative products, he found evidence that the level of oxidation in patients with diabetes is similar to that in age-matched controls as

long as patients with renal insufficiency and vascular disease, both of which are known to increase oxidation, are not included. Other presenters had different views. Michael Brownlee, New York, NY, discussed glycation and intracellular oxidants and showed evidence obtained with the use of the less specific thiobarbituric acid–reacting substances (TBARS) assay that there is increased oxidation in patients with diabetes and that administration of a number of antioxidants can decrease production of AGEs intracellularly. Richard Bucala, Manhasset, NY, discussed the interaction of AGEs with LDL. AGEs can be shown to form on the LDL of patients with diabetes, where they are linked to amine groups on apolipoprotein B (apoB), although they are not present to nearly as great a degree as is seen in end-stage renal disease with or without diabetes. AGE-LDL is not recognized by the LDL receptor, and its clearance from plasma is delayed. Bucala showed that serum total AGE and apoB-AGE are also increased in tobacco smokers and noted that cigarette tobacco is highly enriched in AGEs, so presumably smoke-derived AGEs enter the circulation through the lungs. It is interesting that AGEs are apparently also present in the circulation, although to a much lesser extent, after ingestion of foods such as soy sauce that are highly enriched in these products. Although AGEs may contribute to cardiovascular disease associated with end-stage renal disease, aging, and cigarette smoking, as well as with diabetes, by the same token this argues that the microangiopathic complications of diabetes may not be due to AGEs, because one might then expect to be see them associated with these other conditions.

Joseph Williamson, St. Louis, MO, discussed the role of oxidant formation in diabetic complications and pointed out that the increased NADH/NAD^+ ratio invariably seen in the cytoplasm of diabetic cells reflects cytosolic reductive stress. He suggested that rather than referring to increased levels of oxidants it might be less confusing to refer to "free radical stress," which does not imply that increased cellular oxidation is the primary abnormality. He termed this phenomenon "pseudohypoxia" and stated that it is linked to increased tissue blood flow, which perhaps occurs in response to increased cytosolic lactate levels. In actual hypoxia there is decreased oxidation of NADH to NAD^+, leading to decreased NADH transfer from the cytosol to the mitochondria and to mitochondrial NADH/NAD^+ ratios 2 orders of magnitude greater than seen in diabetes. When hypoxia and hyperglycemia coexist, they have additive effects. Consequences of the increased NADH/NAD^+ ratio include altered enzyme activity, increased nonenzymatic glycosylation of proteins, increased levels of cytokines and growth factors, and increased oxidant production, the effects of which may be favorably modulated by activators or mimickers of superoxide dismutase.

Alan Chait, Seattle, WA, discussed the roles of oxidized lipids and lipoproteins in diabetic macrovascular disease. After reviewing the events following LDL entry into the vascular wall, he discussed the production of monocyte chemoattractant protein-1, which follows the initial oxidation of LDL and leads to entry of monocytes into the vascular wall, where they become macrophages and take up more extensively oxidized LDL to become foam cells. At the same time, smooth muscle cells release cytokines and growth factors, and plasminogen activator inhibitor-1 and tissue factor are released, as well as extensively oxidized LDL, which causes direct cellular toxicity. Small dense LDL from patients with diabetes (both type II and poorly controlled type I) shows less resistance to oxidation than that from nondiabetic individuals, which increases the amount of oxidized LDL produced. Furthermore, the total peroxyl radical–trapping capacity of plasma is decreased in people with poorly controlled diabetes, and, with the use of TBARS and measurement of conjugated diones and lipid peroxides, it was determined that glucose increases LDL lipid peroxidation in vitro. Chait concluded that increased oxidative stress in diabetes facilitates LDL oxidation, with contributions by glucose autooxidation and decreased antioxidant defenses and by increased levels of small dense LDL. He then presented data addressing the question of the mechanism of trapping of LDL in the arterial wall. Vascular proteoglycans include heparan sulfate proteoglycans and other compounds that are negatively charged and therefore interact with positively charged groups on apolipoproteins. It is interesting, however, that as LDL is oxidized it loses its positive charge and is released from the proteoglycan matrix, presumably then becoming free to interact with macrophages. Guy Chisolm, Cleveland, OH, discussed the effect of oxidized lipids on cell growth. He mentioned that although death of cellular elements is characteristic of the atherogenic plaque, there is also proliferation of intima and smooth muscle cells. Oxidized LDL (Ox-LDL) is cytotoxic to vascular smooth muscle and endothelial cells, whether oxidized in cell-free systems with copper or iron, in systems with endothelial cells or activated monocyte-derived macrophages, or in vivo. The major cytotoxic component of oxidized LDL is 7β-hydroperoxycholesterol. This substance is seen in specimens of atheromas and appears to kill cells by inducing peroxidation of cellular lipids. Ox-LDL, when injected in vivo, produces endothelial injury and also dose-related endothelial proliferation at lower doses, with the latter phenomenon being mediated by basic fibroblast growth factor. After injection, ox-LDL can be detected in the arterial wall, particularly in the intima, where it causes alterations in endothelial barrier function and increased endothelial cell DNA synthesis. This process can be inhibited by vitamin E. Chisolm showed that lysophosphatidyl choline, a byproduct of LDL oxidation, is a mediator of this process and is present in atherosclerotic lesions. Ox-LDL and 7β-hydroperoxycholesterol injure cells by peroxidation of cellular lipids, and their presence may be related to the increased atherosclerosis of diabetics.

Oxidative effects of glucose on vascular function were discussed by Richard Cohen, Boston, MA. Normal endothelial regulation of vascular tone requires the release of NO for vasodilation. Oxidative stress lowers NO levels, and in experimental models, with administration of NO synthase inhibitors, lowered NO leads to intimal proliferation. Diabetes or in vitro hyperglycemia reduces NO-induced vasodilation in several experimental models, although baseline levels of blood flow may be increased. Decreased NO production does not appear to be the main problem in diabetes, but there may be increased NO degradation en route to sites of action, perhaps by O_2^- or by AGEs, and there also may be decreased NO activity at the level of cellular protein targets, including guanylate cyclase and ion channels. There are data suggesting a direct effect of NO in activating K^+ channels. Oxidation of sulfhydryl groups of the channel interferes with NO activation of the channel. Cohen hypothesized a positive feedback loop of lipid peroxidation leading to PKC activation and then to phospholipase A_2 activation. This would increase arachidonic acid release,

leading to NADPH oxidation, then to increasing levels of free radicals such as O_2^-, and to restarting the cycle. It is interesting that inhibition of thromboxane receptors and inhibition of prostaglandin synthesis with indomethacin have effects similar to antioxidants in outcomes such as decreasing albuminuria.

Timothy Kern, Madison, WI, commented on the paucity of experimental and clinical data on the role of oxidative stress in diabetes. He showed that diabetic rats develop many of the markers of background diabetic retinopathy, including the presence of acellular capillaries and pericyte ghosts, thickening of capillary basement membranes, and accumulation of lipids, and described studies in rats of the effects of antioxidant therapy on diabetic retinopathy and the related model of galactosemia. Both conditions show metabolic markers of oxidative stress, such as increased levels of retinal lipid peroxides measured as TBARS and decreased levels of reduced glutathione. In an 18-month study in which marked hyperglycemia was maintained without ketosis, administration of a combination of α-tocopherol and ascorbic acid to half of the animals normalized the oxidative markers as well as levels of superoxide dismutase and Na^+-K^+ ATPase and Ca^{2+}-ATPase. Diabetes also increased erythrocyte osmotic fragility, an indirect measure of oxidative stress, and this was normalized with antioxidant treatment. In addition, the number of acellular capillaries and pericyte ghosts were decreased with treatment, although the increased basement membrane thickness and lipid accumulation were not improved. The antioxidant treatment did not improve the increased glomerular volume, mesangial volume, or kidney weight. There was no delay in cataract formation or decrease in sorbitol levels. Thus, experiments with this animal model suggest that treatment with vitamins C and E, although it has antioxidant effects, might not be of benefit in patients with diabetes.

Daisuke Koya, Boston, MA, gave contrasting information on the role of vitamin E treatment in diabetic nephropathy arising from studies of control and streptozocin-induced diabetic rats treated with or without vitamin E. Glomerular DAG and PKC activity were increased with diabetes, as was the glomerular filtration rate, and these were corrected with vitamin E treatment. Koya concluded that the increased DAG/PKC activity was relevant to glomerular hyperfiltration and to albuminuria and that treatment with α-tocopherol might be helpful.

Norman Cameron, Aberdeen, Scotland, U.K., discussed the role of antioxidant treatment in neuropathy. Reactive oxygen species are increased in diabetes, with direct and indirect damaging effects on nerves, the latter mediated by decreased perfusion. Both lipophilic free radical scavengers such as vitamin E and probucol and hydrophilic scavengers such as ascorbate, glutathione, acetyl cysteine, and α-lipoic acid have been shown to have beneficial effects. For example, the fall in motor and sensory nerve conduction velocities that occurs in diabetes can be corrected with probucol, as can the decreased nerve blood flow and increased plasma ACE levels. The fall in endoneurial O_2 tensions seen with diabetes is restored with probucol as well. Interestingly, a variety of vasodilators, including α-blockers, β-antagonists, and ACE inhibitors, have similar beneficial effects on nerve blood flow and conduction velocity. Cameron asked rhetorically why it appears so much easier to treat diabetic rats than people, and answered, "What's really been tried in humans? Very little." He showed data suggesting that regeneration following nerve damage is decreased in diabetic rats and can be restored with acetyl cysteine, vitamin E, β-carotene, or vasodilator treatment. Diabetes decreases nerve glutathione levels, and these can be restored with α-lipoic acid treatment. Such treatment restores nerve conduction velocity in rather low doses, whereas the dosages of vitamin E and vitamin C required are quite high. Fairly low doses of metal chelators also restore nerve conduction abnormalities. For example, norepinephrine responsiveness is restored by desferoxamine treatment. Similar results are seen in galactosemic rat models of neuropathy. Cameron also described studies of γ-linoleic acid (GLA), a metabolite of linoleic acid whose formation is reduced in diabetes, which can be administered to diabetic animals and patients and interacts additively with antioxidants. He described a newly synthesized molecule, ascorbyl-6-GLA, which has both a GLA side chain and an ascorbyl-6 antioxidant component and which may deserve further investigation. There may also be benefits of administering antioxidants with ω-6 fatty acids. He concluded that the possibility of genuine effects leads one to hope for further research developments in this potentially important field.