The Role of Oxidative Stress in Neuropathy and Other Diabetic Complications

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1. INTRODUCTION

Reactive oxygen species (ROS) have been implicated in the pathophysiology of a large number of diseases, and can be harmful due to their high reactivity and deleterious effects on cell structures.[1] Activation of phagocytes, associated with increased production of ROS, may lead to important structural and functional changes of adjacent cells with peroxidation of lipid membranes, DNA and proteins. However, a limited and controlled concentration of free oxygen species is essential for physiologic intracellular mechanisms like oxidative phosphorylation and the cellular function of phagocytes.

As ROS can be both essential and highly toxic to cellular homeostasis, their physiological range is extremely limited. This is illustrated by the numerous antioxidant mechanisms that protect cells against oxidative stress. An imbalance between pro- and antioxidant factors plays an important role in many disease processes. Manipulation of the oxidative balance can also be considered as a key for therapeutic interventions; in the last decade several antioxidant drugs have been tested for prevention and treatment of oxidative tissue damage.[2]

The increased presence of ROS has been suggested to be one of the major causes of diabetic complications[3,4] and has also been implicated in the pathogenesis of type 1 diabetes.[5] The cause of increased pro-oxidant activity in diabetes is multifactorial and not completely understood. Theoretically, hyperglycemia can lead to both a rise in ROS production and to the attenuation of free radical scavenging compounds. In this review, we will focus on the relation between high glucose levels and their possible consequences for the oxidative balance. Furthermore, the role of antioxidant treatment in the prevention of diabetic complications, and especially of neuropathy, will be evaluated.

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2. HYPERGLYCEMIA AND INCREASED PRODUCTION OF REACTIVE OXYGEN SPECIES

Several hypotheses have been tested to evaluate the possible causal mechanism of increased ROS in diabetes. Some studies suggest enhanced free radical production due to elevated glucose concentrations, others focus on reduced antioxidant activity in diabetes. Both mechanisms are probably of importance in the pathogenesis of diabetic complications.

A. Lipid Peroxidation

As the reactive oxygen compound superoxide ($O_2^{-\cdot}$) and hydroxyl radical ($^{\cdot}OH$) have very short half-lives, their direct measurement in tissues of diabetic subjects or animals is virtually impossible. Hydrogen peroxide (H_2O_2)

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can be determined, but does not necessarily reflect the level of oxidative stress, as it is not a free radical and it is only toxic if a transition metal is available to catalyse the production of ·OH. In order to assess free radical activity other parameters which reflect ROS damage have been used. The cornerstone of the assessment of oxidative damage *in vivo* is the measurement of parameters of lipid peroxidation. Lipid peroxides, conjugated dienes and malonyldialdehyde (MDA) are most frequently used for the assessment of oxidative stress.

In both experimental and clinical diabetes, increased lipid peroxidation has been demonstrated. In diabetic animals different organs as well as erythrocytes have been studied for the presence of oxidative damage to lipid membranes. Jain *et al.*[6] measured thiobarbituric acid (TBA) reactivity, which reflects MDA production, in red blood cells of non-diabetic and both untreated and insulin-treated rats. The rise in TBA reactivity in diabetic rats after 2 and 4 months of diabetes was partly corrected by insulin treatment. In a study of untreated diabetic rats[7] MDA production in renal tissue was increased after two weeks but was reduced in heart and liver. These changes were also reversed by insulin. Mukherjee *et al.*[8] found highly significant increases in MDA in liver, brain and kidney as well as in red blood cells of rats two to five weeks after streptozotocin (STZ) induced experimental hyperglycemia. Except for hepatic tissue, all changes were also observed after four days of diabetes; in kidney MDA was increased already after 24 hours. Some changes occurred already after one or four days of diabetes. Kumar *et al.*[9] studied MDA, hydroperoxides and conjugated dienes in heart, liver and kidney after three weeks and three months of STZ-induced diabetes. In liver and kidney MDA and hydroperoxides were increased after three weeks of diabetes. Conjugated dienes were increased in cardiac tissue while MDA and hydroperoxides were decreased. After three months all parameters for lipid peroxidation were increased in all tissues, except for lower cardiac MDA and hydroperoxide levels. In another study with STZ-diabetic rats[10] MDA was increased in plasma and erythrocytes after six weeks of diabetes. Conjugated dienes were increased in plasma only. Insulin treatment normalised these values, but treatment with ascorbate had no effect.

All these data show that changes in lipid peroxidation occur under hyperglycemic conditions. As most changes are reversible following insulin treatment, the role of high glucose levels is evident. The reduced MDA levels in cardiac tissue observed by two authors[7,9] need further explanation. It seems that the heart is better protected against oxidative stress than other tissues. Kumar *et al.*[9] suggest that this is due to increased activity of glutathione-s-transferase observed in diabetic cardiac tissue or, alternatively, due to a modification in energy consumption as glucose is the preferred fuel to fatty acids. However, these data appear to contradict the elevated conjugated diene levels in cardiac tissue.

In clinical studies, lipid peroxidation has only been studied in erythrocytes and plasma. Matkovics *et al.*[11] and Uzel *et al.*[12] found increased MDA levels in erythrocyte hemolysates from diabetic patients. In the study by Uzel *et al.*, patients with retinopathy had higher MDA levels than those without. Lipid peroxides and conjugated dienes were found to be increased in diabetic and non-diabetic subjects with peripheral vascular disease in a study by MacRury *et al.*[13]; they concluded that diabetes had no additive effect on the ROS activity other than that related to angiopathy. The association between increased lipid peroxidation and microvascular disease was also observed by Jennings *et al.*[14], who noted that conjugated dienes were increased in diabetic subjects with microangiopathy, while no increase was found in diabetic patients without vascular disease. A positive correlation was observed between glycated hemoglobin and TBA reactivity.[15] In the same study the authors demonstrated that erythrocytes from healthy volunteers incubated with glucose showed increased lipid peroxidation and membrane fragility. Pretreatment with antioxidants (among which vitamin E) protected red blood cells against the toxic effect of glucose, suggesting that this mechanism is mediated by ROS.

As no other data from human studies, especially on lipid peroxidation in the organs mainly affected by diabetes, are available, it remains unclear whether a local increase in oxidative stress occurs. Microangiopathy may be the final common pathway leading to tissue damage, possibly mediated by tissue hypoxia or hypoxia/reperfusion and subsequent production of ROS. However, other mechanisms,

especially local high glucose concentrations leading to protein glycation and glucose autooxidation are probably of equal or greater importance.

B. Glucose Autooxidation and Protein Glycation

The production of ROS in diabetes is a consequence of the process of autooxidative glycosylation; this process has been extensively studied by Wolff *et al.*[16–22] The mechanism traditionally proposed in these studies is based on non-enzymatic glycation of proteins and amino acids. The consequent formation of Amadori products leads to alpha-ketoaldehyde compounds and produces increased cross-linking of proteins. However, a probably more important mechanism is the transition of glucose into enediols in the presence of transition metals. This mechanism, called glucose autooxidation, yields superoxide and hydroxyl radicals, and has been shown to cause protein oxidation[16] (Figure 1).

Most studies examining the role of glucose in the pathogenesis of oxidative protein damage have used *in vitro* techniques. It has been suggested that the structural changes found in diabetes, like glycation and protein cross-linking, are the result of an accelerated ageing process due to hyperglycemia.[20] Inhibition of glucose-induced protein damage in the presence of antioxidants and metal chelators suggests that free radicals are important factors in this process. Increased carbohydrate-derived oxidation products, measured as N-(carbo-xymethyl)lysine, N-(carboxymethyl) hydro- xylysine and pentosidine, are found after the oxidation of Amadori adducts formed during glycation of collagen in the presence of ROS.[4] As these oxidation products are increased in collagen from diabetic patients, they may reflect increased oxidative damage due to hyperglycemia. However, this mechanism may not only be explained by an increased production of free radicals, but also by an increased susceptibility of proteins to oxidative damage due to their glycated state under diabetic conditions.

Fu *et al.*[23] showed that incubation of collagen with glucose leads to increased production of fructeolysine (an Amadori product), and the glycoxidation products pentosidine and N-(carboxymethyl)lysine. Simultaneous incubation with a metal chelator, with reducing and radical scavenging agents, or with aminoguanidine reduced the formation of glycoxidation products, but had no effect on fructeolysine production. Furthermore, they almost all reduced cross-linking of collagen. In a similar study[17] the metal chelating agent diethylenetriamine pentaacetic acid (DETAPAC) inhibited the formation of ketoaldehydes and glycation of albumin. These studies suggest that under hyperglycemic conditions a reduction in glucose autooxidation leads to decreased collagen damage.

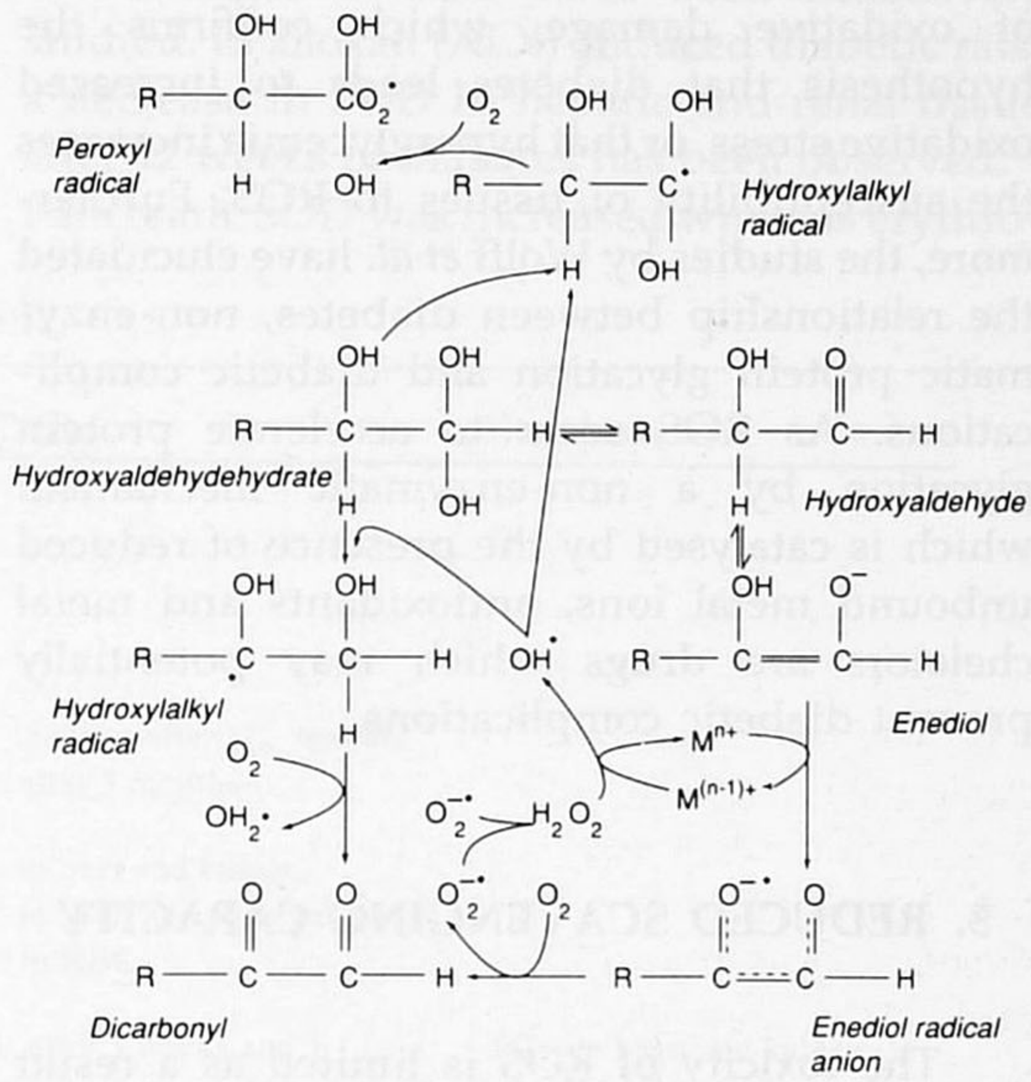

Figure 1. Monosaccharide autoxidation, adapted from Wolff *et al.* (20). The enolized monosaccharides can reduce transition metals (M^{o+}). In the presence of a reduced metal, superoxide ($0_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) form hydroxyl radical ($^{\cdot}OH$) (Haber-Weiss reaction). Hydroxyl radicals are scavenged by hydroxy-acids, leading to the formation of peroxyl radicals.

Although most studies have focused on lipid peroxidation and protein glycosylation, other parameters to measure increased oxidative damage in diabetes have been used. Ghiselli *et al.*[24] used 2,3-dihydrobenzoate (a hydroxylated form of ascorbate) as a parameter for oxidative damage, and showed that this was increased in diabetic subjects.

These studies show that hyperglycemia

leads to increased production of end-products of oxidative damage, which confirms the hypothesis that diabetes leads to increased oxidative stress, or that hyperglycemia increases the susceptibility of tissues to ROS. Furthermore, the studies by Wolff *et al.* have elucidated the relationship between diabetes, non-enzymatic protein glycation and diabetic complications. As ROS seem to accelerate protein glycation by a non-enzymatic mechanism which is catalysed by the presence of reduced unbound metal ions, antioxidants and metal chelators are drugs which may potentially prevent diabetic complications.

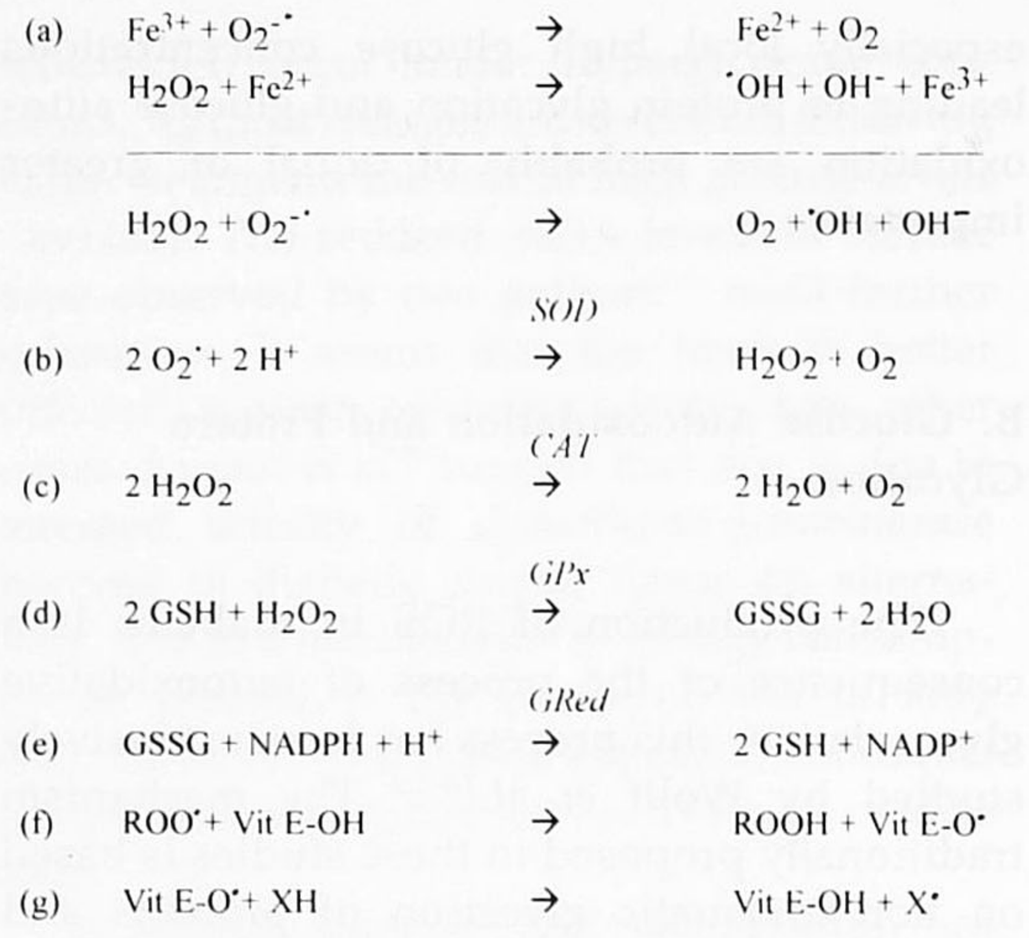

Figure 2. Production of hydroxyl radicals and reactive oxygen scavenging systems. (a) Haber-Weiss reaction, leading to the reduction of superoxide ($O_2^{-\bullet}$) by hydrogen peroxide (H_2O_2) and to the formation of hydroxyl radicals ($^{\bullet}OH$). This reaction is catalysed by free metal ions, mainly by iron (Fe) and copper (Cu) (Fenton mechanism). (b) Reduction of $O_2^{-\bullet}$ to H_2O_2 in the presence of superoxide dismutase (SOD). (c) Reduction of H_2O_2 in the presence of catalase (CAT). (d) Reduction of H_2O_2 by reduced glutathione (GSH) in the presence of glutathione peroxidase (Gpx). (e) Reduction of oxidised glutathione (GSSG) in the presence of glutathione reductase (GR) and NADPH. (f) Reduction of a lipid radical ($ROO^{\bullet}$) by vitamin E (Vit E-OH). (g) Reduction of oxidised vitamin E ($Vit\ E\text{-}O^{\bullet}$) by a reducing agent XH, probably ascorbic acid.

3. REDUCED SCAVENGING CAPACITY

The toxicity of ROS is limited as a result of the presence of radical scavengers, which transform $O_2^{-\bullet}$ and H_2O_2 into H_2O and O_2, and which have the capacity to reduce peroxyl radicals, products of lipid peroxidation (Figure 2). The transformation of superoxide into the less toxic hydrogen peroxide molecule is catalysed by superoxide dismutase (SOD). Hydrogen peroxide can be reduced to water by different mechanisms. Catalase catalyses the transformation of H_2O_2 into H_2O and O_2, mainly in the liver. Reduced glutathione (GSH) has the capacity to reduce H_2O_2, while glutathione is transformed into its oxidised form (GSSG). The GSH/GSSG redox couple has a high antioxidant capacity, as GSSG can be reduced enzymatically to GSH in the presence of glutathione reductase (GRed) and NADPH, which is oxidised to $NADP^+$. α-Tocopherol (vitamin E) is another non-enzymatic molecule which plays an important role in the protection against oxidative stress, as it can reduce peroxyl- and aloxy-radicals which are formed in the presence of $^{\bullet}OH$ and transition metals like copper and iron. The antioxidant function of α-Tocopherol is essential in lipid membranes. By reducing lipid radicals, α-Tocopherol is transformed into the tocopherol radical, which is returned into the reduced state by ascorbic acid (vitamin C), another important cofactor in the antioxidant system.

In the diabetic state, changes in the ROS scavenging system have been observed. However, the interpretation of changes in antioxidant status is not always unequivocal. As free radicals are difficult to measure directly *ex vivo*, and as parameters for tissue peroxidation like MDA and conjugated dienes are controversial, changes in antioxidant status are often used to reflect increased oxidative stress. Induction of catalase, SOD and GSH production by a higher level of oxygen radical formation, however, may reflect enhanced oxidative stress just as well as reduced antioxidant levels due to increased turnover. Furthermore, as glucose is transformed into ribose in the hexose monophosphate shunt, it is unclear whether hyperglycemia, by activating this pathway, induces the production of GSH, as the NADPH/GSH couple plays an important role in this mechanism.

Several groups have studied changes in antioxidant levels in diabetic animals and patients. The data from these studies have

been summarised in Table 1. Changes in antioxidant concentrations differ according to the tissue studied; these changes may be related to the capacity of different tissues to resist oxidative damage.

Changes in SOD have been extensively studied. In alloxan (ALX)-induced diabetic rats, a decrease in SOD in hepatic and renal tissue after 12 weeks of diabetes has been observed.[25] Pancreatic SOD was increased whereas erythro-

Table I.

	Superoxide Dismutase	Catalase	Glutathione
Asayama et al.[7] STZ rats, 2 weeks	CuZn-SOD ↓ in liver, = in heart and pancreas, Mn-SOD ↓ in heart, ↑ in liver, = in kidney		
Bitar et al.[32] STZ rats, 1-3 months		= in liver after 1-2 months, ↓ after 3 months	
Godin et al.[25] ALX rats,12 weeks	CuZn-SOD ↓ in liver and kidney,= in heart and RBC, ↑ in pancreas	↓ in liver and kidney, ↑ in heart and pancreas, = in RBC	
Kumar et al.[9] ALX rats, 3 weeks and 3 months	↓ after 3 weeks and 3 months in heart, liver, kidney	↓ after 3 weeks and 3 months in heart, liver, kidney	TG ↓ in heart and kidney after 3 weeks and 3 months, ↑ in liver after 3 months, = in liver after 3 weeks
Loven et al.[27] STZ rats, 10 days	Total SOD ↓ in small intestine, = in colon, CuZn-SOD ↓ in small intestine and colon		
Loven et al.[28] STZ rats, 10 days	CuZn-SOD ↓ in liver, kidney, RBC, Mn-SOD = in kidney and liver		GSH ↓ in liver, = in jejunal mucosa
Matkovics[11] STZ rats, duration ?	↓ in RBC, liver, muscle,kidney, spleen, brain, pancreas, = in lung	↓ in spleen and pancreas, ↑ in RBC, liver and kidney, = in brain, lung, muscle	
Mukherjee et al.[8] STZ rats, 1, 4, 15, 21 and 35 days		↑ in liver after 3-5 weeks, = in liver after 1-4 days, = in brain, RBC and kidney at all times	TG ↓ in RBC, kidney, liver and brain after 2, 3 and 5 weeks, = in RBC, kidney, liver and brain after 1-4 days
Tagami et al.[29] ALX rabbits, 17 days	CuZn-SOD ↓ in endothelium and liver, = in kidney, Mn-SOD ↓ in liver, = in kidney and endothelium	↓ in endothelium and liver, = in kidney	GSH ↓ in endothelium and liver, = in kidney
Wohaieb et al.[26] STZ rats,12 weeks	CuZn-SOD ↓ in liver and kidney, = in heart, ↑ in pancreas	↓ in liver and kidney, ↑ in heart and pancreas	GSH ↓ in liver, = in pancreas, heart and kidney
Wohaieb et al.[30] BB rats, 7-12 weeks	↑ in pancreas, = in liver, kidney and heart	↑ in pancreas and heart, = in liver and kidney	GSH ↓ in liver and pancreas, ↑ in heart, = in kidney

Influence of diabetes on copper-zinc- and manganese-superoxide dismutase, catalase, total and reduced glutathione in different organs. Summary of the studies which are mentioned in the text. In the first column, the reference is followed by the type of induction of diabetes, the type of animal and the duration of diabetes at the moment of measurement. The symbols ↑, = and ↓ refer to respectively increased, unchanged or decreased concentrations measured in diabetic animals as compared to controls.
ALX = alloxan; STZ = streptozotocin; BB = spontaneously diabetic BB Wor rats; SOD = superoxide dismutase; Cu,Zn-SOD = copper-zinc-superoxide dismutase; Mn-SOD = manganese superoxide dismutase; TG = total glutathione; GSH = reduced glutathione; RBC = Red blood cells.

cyte levels were unchanged. Pancreatic Cupper-Zinc SOD (CuZnSOD) was also increased in STZ-diabetic rats after 12 weeks of hyperglycemia.[26] This study confirmed the previously observed reduction in hepatic and renal SOD, but showed no changes in cardiac tissue. Matkovics *et al.*[11] also studied STZ-induced diabetic rats, and observed lower SOD levels in erythrocytes, liver, muscle, kidney and several other organs. In this study, pancreatic SOD was found to be reduced. The duration of diabetes in these animals was not mentioned. In shorter experiments with STZ diabetic rats, reduced total and CuZnSOD levels were observed after ten days of diabetes in small intestine[27] and in liver, kidney and erythrocytes (CuZnSOD only).[28] After two weeks of diabetes, lower CuZnSOD was also found in hepatic tissue.[7] Tagami *et al.*[29] found reduced SOD levels in aortic endothelium and liver of ALX-induced diabetic rabbits, which were corrected by insulin treatment. Renal SOD levels were unchanged. CuZnSOD was also decreased in sciatic nerve from STZ-diabetic rats after one month of diabetes.[30] This reduction was partly reversed by insulin. In a spontaneous diabetic rat model[31] increased pancreatic SOD was found after a diabetic period of seven to twelve weeks. In this study hepatic, renal and cardiac SOD remained unchanged.

CuZnSOD is probably more important as a superoxide scavenger than Manganese SOD (MnSOD). The data from animal studies consistently show a decreased CuZnSOD or total SOD in liver, and in many cases in kidney, as a consequence of hyperglycemia. This reduction is less evident in other organs; pancreatic SOD is mostly increased. As SOD, like most antioxidants, is mainly effective intracellularly, the tissue levels in those organs mainly affected by diabetes are more interesting than plasma or red blood cell concentrations. In this respect, the finding of reduced endothelial[29] and endoneurial[30] SOD may be important as a causal factor for increased tissue damage due to superoxide.

In humans, changes in SOD concentrations are not consistent. A small but non-significant reduction in erythrocyte SOD was found in type 2 diabetic patients.[13] In type 1 diabetes, erythrocyte SOD was attenuated in patients with and without retinopathy.[32] In another study a 25% increase in red cell SOD was observed in both type 1 and 2 diabetic patients.[25] In diabetic children, erythrocyte or lymphocyte SOD remained unchanged.[33] SOD tissue levels have not been reported in humans.

Similar studies can be cited for reports on catalase levels in diabetic animals and patients. Matkovics *et al.*[11] found important increases in catalase activity in red blood cells, liver and kidney in STZ-induced diabetes. In contrast, two studies[25,26] showed decreased levels in liver and kidney 12 weeks after ALX or STZ injection, but raised catalase in the heart and pancreas. In two longitudinal studies, the results were also controversial. A 35% reduction in hepatic catalase was observed after three months of STZ-induced diabetes, with no changes yet after one or two months.[34] In another STZ-rat model[8] catalase was increased in kidney after two, three and five weeks, and in liver after three and five weeks, while no changes were found in the first week. In ALX-diabetic rats reduced levels of hepatic, renal, and cardiac catalase have been reported after three weeks and three months.[9] In ALX-diabetic rabbits, hepatic and aortic endothelial catalase was reduced after 17 days, but renal levels were unchanged.[29] In human studies, no change in erythrocyte catalase was found in type 1[25,33] or type 2[11,25] diabetes.

It can be concluded from these data that changes in catalase are inconsistent. Again, the findings in endothelium by Tagami *et al.*[29] may be important and need further confirmation. At this moment, the significance of changes in catalase activity due to diabetes remains unclear.

Glutathione concentrations in experimental diabetes were found to be attenuated in most studies. Mukherjee *et al.*[8] observed decreased levels of total glutathione two, three and five weeks after STZ injection, but not yet after one or four days. The changes were shown in erythrocytes, liver, kidney and brain. Total glutathione levels were also decreased in ALX-rats after three weeks and three months of diabetes, whereas hepatic total glutathione was increased after three months.[9] An increase in hepatic glutathione in diabetes has been previously reported.[35] In contrast, after 12 weeks of STZ diabetes a reduction of hepatic GSH was found by Wohaieb *et al.*[26]; pancreatic, cardiac and renal GSH were unchanged. In a study from the same group[25] erythrocytes from 12 week ALX-diabetic rats were incubated with

H_2O_2. The decrease in GSH levels that is observed in cells from non-diabetic or insulin-treated animals did not occur, whereas increased levels of MDA were reported, reflecting a reduced capacity of GSH to prevent lipid peroxidation. Loven *et al.*[28] also showed decreased hepatic GSH after ten days of STZ diabetes in rats. Spontaneously diabetic BB/Wor rats were found to have lower GSH concentrations in liver and pancreas, but increased cardiac GSH.[31] In aortic endothelium of ALX diabetic rabbits, GSH was decreased 60% after 17 days.[29]

The measurement of the reduced form of glutathione is probably more informative than total glutathione. The synthesis of glutathione may be stimulated by chronic hyperglycemia, as GSH is an important co-factor in the hexose monophosphate shunt. The measurement of glutathione in different organs may be influenced by the high GSH concentrations in erythrocytes. Contamination by red blood cells of the tissues studied can lead to an increase in glutathione levels. GSH deficiency has been suggested to be an important factor in the increased ROS activity in diabetes. These experimental data show that not all organs are equally affected. Again, the finding that endothelial GSH was found to be reduced[29] may be crucial, and deserves further confirmation.

Type 2 diabetic patients showed a reduction of 12% in GSH in erythrocytes while GSSG was increased by 70%.[36] In an earlier study,[37] both total and reduced glutathione were decreased in type 2 patients. MacRury *et al.*[13] found no change in total thiol concentration in type 2 diabetes. A negative correlation was observed between glycated hemoglobin (HbA1c) and GSH levels in type 1 diabetic patients.[15] In two studies involving both type 1 and type 2 patients,[12,38] decreased erythrocyte GSH levels were found, while another study showed increased plasma GSSG, but no changes in GSH in erythrocytes from type 1 and 2 diabetic patients.[39]

The gluthathione related enzymes GRed and GPx have been less extensively studied. In animal studies, Mukherjee *et al.*[8] showed a reduction in erythrocyte, hepatic, renal, and cerebral GRed after three and five weeks of STZ-diabetes in rats. In the brain and kidney, GRed was also decreased after two weeks. Two studies[25,26] showed increased cardiac and pancreatic, but unchanged renal and hepatic GRed levels after 12 weeks of STZ- or ALX-induced diabetes; GPx was increased in kidney, decreased in liver, and unchanged in pancreas and heart. Identical GRed changes were observed in BB/Wor rats,[31] while GPx was increased in kidney and pancreas, but unchanged in heart and liver. In ALX-diabetic rabbits,[29] no GRed changes were observed in aortic endothelium, liver or kidney, but GPx was decreased in endothelium.

The significance of GRed and GPx changes remains unclear. As both enzymes are essential to ensure the optimal function of GSH, a decrease in activity may lead to a reduction in antioxidant capacity. More extensive studies on this subject, especially in order to clarify the importance of changes in the balances between GSH, GSSG, GRed and GPx are necessary.

In erythrocytes from type 2 diabetic patients, GRed levels were found to be reduced[12,36] or increased, while GPx remained unchanged. In combined type 1 and type 2 patients, GRed activity has been shown to be increased[25] or unchanged[40]; in these groups of patients GPx was increased[11] or decreased.[25]

Little is known about α-tocopherol and ascorbate in diabetes. Karpen *et al.*[41] showed that platelet vitamin E levels in STZ-diabetic animals on a low vitamin E diet were undetectable, in contrast to non-diabetic controls and diabetic animals on a high vitamin E diet. This study was performed after 18 weeks of diabetes, following 12 weeks of supplementary diets. Young *et al.*[10] observed no changes in plasma vitamin E after six weeks of STZ-induced diabetes, but ascorbate levels in diabetic animals were reduced. Higuchi *et al.*[42] found decreased hepatic and serum vitamin E concentrations after 20 weeks of STZ diabetes, but no changes after 14 days. In type 1 and type 2 diabetic patients, α-tocopherol levels in platelets and plasma were increased.[43] Other authors have reported that vitamin E levels were increased in diabetic patients with microalbuminuria[44] and with increased low and very low density lipoprotein.[45] However, since α-tocopherol is a lipophilic compound, plasma levels probably do not reflect local concentrations in different tissue membranes. Therefore, further studies need to be performed to clarify whether changes in vitamin E content

of cell membranes occur in diabetes and if they differ among tissues.

4. EXPERIMENTAL STUDIES WITH ANTIOXIDANT TREATMENT

A. Neuropathy

Nerve dysfunction in experimental diabetes has been extensively studied in the diabetic rat, mostly in the STZ model. Motor and sensory nerve conduction velocity (MNCV and SNCV) are the principal parameters used to study the therapeutic effect of different experimental drugs on nerve function. In the STZ-rat, Cameron *et al.*[46] showed a protective effect of the antioxidant butylated hydroxytoluene (BHT) on MNCV and SNCV in the sciatic and saphenous nerves after two months of treatment. Resistance to ischaemic conduction failure (RICF) in the sciatic trunk, which is increased in diabetes, was also tested. The increase in RICF was reduced approximately 40% by BHT treatment, without affecting nerve capillarisation, sorbitol levels or metabolic control. In a second study from the same group[47] antioxidant treatment with probucol prevented MNCV and SNCV slowing as well as reduced endoneurial blood flow in STZ-diabetic rats. Primaquine, a pro-oxidant drug, reduced conduction velocity as well as nerve blood flow and endoneurial oxygen tensions in non-diabetic rats. Probucol treatment protected against these changes.[47]

Recently, Karasu *et al.*[48] showed that both vitamin E and probucol had protective effects on the reduction of sciatic nerve blood flow in STZ-diabetic rats. They did not report data on nerve conduction velocity following antioxidant treatment. No additive effect of probucol on the protective capacity of evening primrose oil on MNCV and SNCV was observed. However, α-tocopherol supplementation had no protective effect on nerve dysfunction in another study. In contrast, the authors reported increased local vitamin E concentrations in diabetic nerve, even without supplementation.[49]

In a study from our own laboratory,[50] treatment with GSH twice weekly was found to protect against MNCV and SNCV reduction in STZ-induced diabetes. Preliminary results of studies with α-lipoic acid, another drug with antioxidant features, have reported beneficial effects on nerve conduction velocity.[51]

Little is know, however, about the mechanism of the antioxidant effect in oxidative stress-induced nerve dysfunction. The increase in nerve blood flow after probucol treatment suggests that antioxidants are mainly effective due to their capacity to increase tissue oxygenation and nutrition. However, others have claimed that reduced nerve blood flow is not the main cause of nerve dysfunction in diabetes.[52] The involvement of the polyol pathway has been suggested as changes in endoneurial GSH/NADPH balance occur consequent to elevated aldose reductase activity. Low *et al.*[30] showed that conjugated dienes were increased in the sciatic nerve of diabetic rats. This was associated with a reduction in nerve noradrenalin and hydroperoxides, while MDA was not changed. Nerve SOD was also reduced in diabetic animals and this was corrected by insulin treatment. In this study, a combined role of changes in microvascular function and nerve oxidative metabolism was suggested in the pathogenesis of nerve dysfunction.

B. Cataract and Retinopathy

Yeh *et al.*[53] showed that lipid peroxidation, measured as MDA, was increased in the diabetic lens, and that this increase was reversed by insulin and by aldose reductase inhibition. Different protein glycation parameters have been measured in the diabetic lens by Lyons *et al.*[54] They found no increase in lens CML (a glucose-induced protein autoxidation product), while other protein glycation products were increased by diabetes. They concluded that ROS had no major role in the pathogenesis of diabetic cataract. Jones *et al.*[55] showed that crystallin purified from lenses from diabetic animals was more susceptible to oxidative damage, and that this increased toxicity of ROS could be partly prevented by ascorbate supplementation. This suggests that oxidative stress may be important in the etiology of cataract formation. Finally, the antioxidant α-lipoic acid was found to

prevent both *in vivo* pro-oxidant induced cataract and *in vitro* cataractogenesis induced by hyperglycemia.[56]

In the pathogenesis and treatment of diabetic retinopathy, few data on the role of oxidative stress are available. Doly *et al.*[57] showed that a free radical scavenger, EGb 761, protected against electroretinographic changes found in isolated retina from diabetic rats. The same group showed that SOD and EGb 761 reverse changes in ionic flux in the retina caused by ischemia and reperfusion.[58] The authors suggested that ROS may play a role in the vascular changes in the diabetic retina, and that antioxidant treatment can be beneficial.[59]

C. Nephropathy

The role of angiotensin converting enzyme (ACE) inhibitors in the prevention and treatment of diabetic nephropathy is well-established. The ACE-inhibitor captopril has been used in most of these studies. However, due to its sulfhydryl group, captopril has not only ACE inhibiting properties, but can also function as an antioxidant drug.[60] Ha *et al.*[61] showed that STZ diabetic rats had increased urinary lipid peroxide levels; treatment with captopril reduced both albumin and lipid peroxide excretion. They suggested that oxidative stress may be responsible for renal endothelial damage, which may be reversed by antioxidant treatment. As no other antioxidants *without* ACE inhibiting capacities have been tested, further studies to confirm this relationship should be performed. Some authors have suggested that the kidney is probably well protected against increased oxidative stress, as glutathione[62] and SOD[63] were found to be increased in diabetic rats, suggesting an adaptive mechanism to combat increased ROS activity. However, as has been stated above (Table 1), many studies have shown a reduction of renal concentrations of SOD, catalase and glutathione.

D. Vascular Disease

In the pathogenesis of atherosclerosis, oxidised low density lipoprotein (Ox-LDL) may play a crucial role through different mechanisms. Due to its modification of chemotactic properties, it may stimulate smooth muscle cell proliferation and foam cell formation.[64,65] Furthermore, Ox-LDL interferes with the interaction of nitric oxide and the arterial vessel wall, and may therefore induce vasodilatation.[66]

The increase in free radical activity in diabetes leading to increased lipid peroxidation is probably a major causal factor in atherogenesis. Conjugated dienes are increased in diabetic patients with microangiopathy compared with those without vascular disease.[14] Furthermore, conjugated dienes and lipid peroxides in both non-diabetic and diabetic subjects with vascular disease are similarly increased while subjects without angiopathy show lower levels of lipid peroxidation, independent of their glucose levels.[13] As LDL is more susceptible to oxidation in diabetic subjects,[67] Ox-LDL may be an important factor in the increased atherogenesis in these patients.

Hyperglycemia and increased oxidative stress lead to endothelial dysfunction. SOD, glutathione, and GPx were decreased in endothelial cells from diabetic rabbits. These changes were reversed by insulin treatment.[29] Furthermore, the reduction in *in vitro* endothelial cell proliferation observed under hyperglycemic circumstances was reversed by incubation with SOD, catalase and glutathione.[68] In cultured endothelial cells, high glucose levels caused a decrease in GSH as well as NADPH release in the presence of H_2O_2, suggesting a perturbation of the glutathione redox cycle due to hyperglycemia.[69] Tesfamariam suggested that the NADPH depletion found in diabetes may be the cause of reduced nitric oxide formation, and may thus be important in the increased vascular resistance and reduced microvascular flow in diabetes.[70]

Treatment of non-diabetic patients with antioxidants is probably beneficial in reducing the prevalence of vascular disease, although few data from clinical trials are available. In epidemiological studies, the use of antioxidants has been associated with a reduction in coronary heart disease.[71,72] A definite answer as to the benefits of antioxidants in the prevention of angiopathy will have to await the results of prospective studies which are currently being performed. No data are available on the possible benefits of antioxidant

drugs to prevent vascular disease in diabetic patients, but the observed association between increased conjugated dienes, Ox-LDL and microangiopathy needs to be studied further.

5. CONCLUSION

Hyperglycemia leads to an increased production of ROS, either by glucose autooxidation or by reduced antioxidant activity. Changes in free radical scavengers have been observed in differed organs but the significance of these changes remains unclear, as they are not always consistent and may increase or decrease depending on the tissue studied. However, these changes may contribute to the specific damage in certain target organs, although other mechanisms such as tissue hypoxia, increased aldose reductase activity and changes in cell metabolism are probably also important causal factors in the pathogenesis of diabetic complications.

Oxidative stress may lead to functional changes by both lipid membrane, protein and DNA oxidation. Endothelial cells are easily damaged by ROS, possibly mainly due to higher levels of circulating Ox-LDL. As endothelial dysfunction and reduced microcirculation have been associated with the pathogenesis of all diabetic complications, the endothelium may be a possible target for the preventive effects of antioxidant drugs. Prevention of increased oxidative stress by intensive insulin treatment also leads to a reduction in ROS activity, which may explain the reduced incidence of diabetic complications after improvement of glycemic control. As antioxidant treatment has few drawbacks and is easily tolerated, it may be useful in the treatment of diabetic patients; prospective studies should be performed to evaluate this possibility.

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