New Insights on Oxidative Stress and Diabetic Complications May Lead to a "Causal" Antioxidant Therapy

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ABSTRACT—Evidence implicates hyperglycemia-derived oxygen free radicals as mediators of diabetic complications. However, intervention studies with classic antioxidants, such as vitamin E, failed to demonstrate any beneficial effect. Recent studies demonstrate that a single hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron-transport chain seems to be the first and key event in the activation of all other pathways involved in the pathogenesis of diabetic complications. These include increased polyol pathway flux, increased advanced glycosylation end product formation, activation of protein kinase C, and increased hexosamine pathway flux. Superoxide overproduction is accompanied by increased nitric oxide generation, due to an endothelial NOS and inducible NOS uncoupled state, a phenomenon favoring the formation of the strong oxidant peroxynitrite, which in turn damages DNA. DNA damage is an obligatory stimulus for the activation of the nuclear enzyme poly(ADP-ribose) polymerase. Poly(ADP-ribose) polymerase activation in turn depletes the intracellular concentration of its substrate NAD^+, slowing the rate of glycolysis, electron transport, and ATP formation, and produces an ADP-ribosylation of the GAPDH. These processes result in acute endothelial dysfunction in diabetic blood vessels that, convincingly, also contributes to the development of diabetic complications. These new findings may explain why classic antioxidants, such as vitamin E, which work by scavenging already-formed toxic oxidation products, have failed to show beneficial effects on diabetic complications and may suggest new and attractive "causal" antioxidant therapy. New low–molecular mass compounds that act as SOD or catalase mimetics or L-propionyl-carnitine and lipoic acid, which work as intracellular superoxide scavengers, improving mitochondrial function and reducing DNA damage, may be good candidates for such a strategy, and preliminary studies support this hypothesis. This "causal" therapy would also be associated with other promising tools such as LY 333531, PJ34, and FP15, which block the protein kinase β isoform, poly(ADP-ribose) polymerase, and peroxynitrite, respectively. While waiting for these focused tools, we may have other options: thiazolinediones, statins, ACE inhibitors, and angiotensin 1 inhibitors can reduce intracellular oxidative stress generation, and it has been suggested that many of their beneficial effects, even in diabetic patients, are due to this property.

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The relationship between diabetes and premature vascular disease is well established (1). Recent prospective studies indicate that long-term glycemic control is an important predictor not only of microvascular disease (2,3), but also of macrovascular complications (4).

Vascular endothelial cells are an important target of hyperglycemic damage, but the mechanisms underlying this damage are not fully understood (5).

It has been suggested that, in diabetes, oxidative stress plays a key role in the pathogenesis of vascular complications, both microvascular and macrovascular (6), and an early marker of such damage is the development of an endothelial dysfunction (6,7). However, the role of oxidative stress in diabetes is questioned by the results of intervention studies with antioxidants, which are elusive or unsuccessful (8).

Recent basic and clinical studies have uncovered new insights into the role of oxidative stress in diabetic complications, suggesting a different and innovative approach to a possible "causal" antioxidant therapy. The aim of this review is to give an update on this topic.

HYPERGLYCEMIA, OXIDATIVE STRESS, AND ENDOTHELIAL DYSFUNCTION

— Vascular function in diabetes has been studied extensively in both animal models and humans. Impaired endothelium-dependent vasodilation has been a consistent finding in animal models of diabetes induced by alloxan or streptozotocin (9,10). Similarly, studies in humans with type 1 or type 2 diabetes have found endothelial dysfunction when compared with vascular function in nondiabetic subjects (11,12).

In vitro, the direct role of hyperglycemia has been suggested by evidence that arteries isolated from normal animals, which are subsequently exposed to exogenous hyperglycemia, also exhibit attenuated endothelium-dependent relaxation (13). Consistently, in vivo studies have also demonstrated that hyperglycemia directly induces, both in diabetic and normal subjects, an endothelial dysfunction (14,15).

The role of free radicals generation in producing the hyperglycemia-dependent endothelial dysfunction is suggested by studies showing that both in vitro (16,17) and in vivo (18–21), the acute effects of hyperglycemia is counterbalanced by antioxidants.

Increased superoxide production in endothelial cells during hyperglycemia: the unifying hypothesis for the development of diabetic complications

Brownlee (22) recently pointed out the key role of superoxide production in endothelial cells at the mitochondrial level during hyperglycemia in the pathogenesis

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Abbreviations: AT1, angiotensin 1; NF, nuclear factor; NO, nitric oxide; NOS, nitric oxide synthase.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

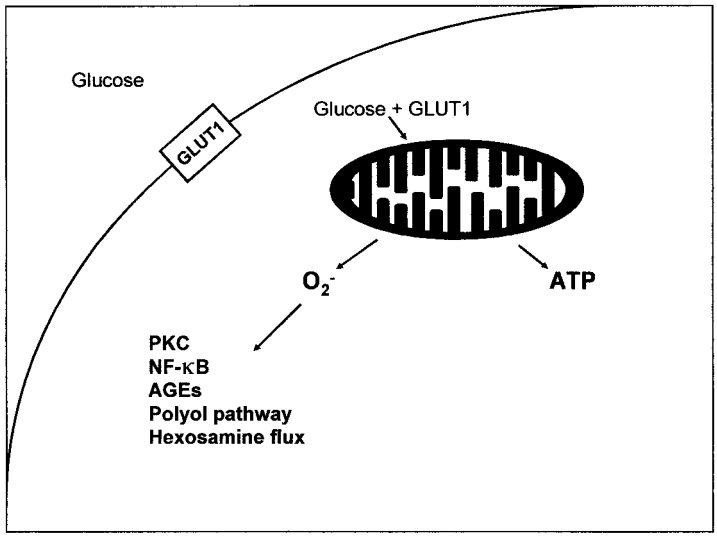

Figure 1—*In endothelial cells, glucose can pass freely through the cell membrane in an insulin-independent manner via GLUT1. Intracellular hyperglycemia induces overproduction of superoxide at the mitochondrial level. Overproduction of superoxide is the first and key event in the activation of all other pathways involved in the pathogenesis of diabetic complications, such as polyol pathway flux, increased advanced glycosylation end product (AGE) formation, activation of protein kinase C (PKC) and NF-κB, and increased hexosamine pathway flux.*

of diabetic complications. This new insight is consistent with the four pathways suggested to be involved in the development of diabetic complications (increased polyol pathway flux, increased advanced glycosylation end product formation, activation of protein kinase C, and increased hexosamine pathway flux) and with a unifying hypothesis regarding the effects of hyperglycemia on cellular dysfunction (23,24). The authors used endothelial cells subjected to physiologically relevant glucose concentrations as a model system for analyzing the vascular response to hyperglycemia because the non-insulin-dependent glucose transporter GLUT1 facilitates diffusion of high levels of glucose into the endothelium. In the presence of increased glucose, endothelial generation of reactive oxygen species, particularly superoxide anion, was shown to be enhanced (23). Several pathways can be considered as likely candidates for oxygen free radical formation in cells. These include NAD(P)H oxidase, the mitochondrial respiratory chain, xantine oxidase, the arachidonic cascade (lipoxygenase and cycloxygenase), and microsomal enzymes (25). Brownlee et al. (23) have determined that the source of free radicals in endothelial cells incubated in high glucose is the transport of glycolysis-derived pyruvate in mitochondria at the level of complex II (succinate:ubiquinone oxidoreductase), one of the four inner membrane–associated complexes central to oxidative phosphorylation. The data in the papers (23) indicate that, at least in the cell culture, endothelium in an environment mimicking physiological hyperglycemia cannot control its appetite for glucose. Accelerated flux of glucose through glycolysis and feeding of pyruvate (thus formed) to the tricarboxylic acid cycle overloads mitochondria, causing excessive generation of free radicals. Although oxygen free radicals have been shown to have a physiological role in signal transduction, their sustained generation at the levels shown in endothelial cells exposed to high glucose can be expected to have substantial effects on cellular properties. Each of the pathways implicated in secondary complications of diabetes has been shown to arise by a single unifying mechanism (22). A central contribution of the works of Brownlee et al. (23) is to demonstrate that suppression of intracellular free radicals, using low molecular inhibitors or by expression of the antioxidant enzyme manganese-superoxide dismutase, prevents each of these events (i.e., glucose-induced formation of oxidants is a proximal step in cell perturbation).

Furthermore, activation of nuclear factor (NF)-κB by this mechanism ties hyperglycemia to the expression of multiple genes related to vascular stress response (26). Figure 1 summarizes this finding.

SUPEROXIDE, NITRIC OXIDE, PEROXYNITRITE, AND NITROTYROSINE FORMATION

— Even increased superoxide generation in hyperglycemia is a key event in activating the other pathways involved in the pathogenesis of diabetic complications; it represents only a first step in the production of the endothelial dysfunction in diabetes.

Nitric oxide (NO) production plays a central role in modulating endothelial function (27). NO is generated from the metabolism of L-arginine by the enzyme nitric oxide synthase (NOS), of which there are three isoforms: the constitutive types, brain (bNOS) and endothelial (eNOS), and the inducible type, iNOS (28). iNOS is induced de novo by various stimuli, including hyperglycemia (29), while the mitochondrial-generated superoxide can inhibit eNOS, although enough NO is still produced (30).

The superoxide anion may quench NO, thereby reducing the efficacy of a potent endothelium-derived vasodilator system that participates in the general homeostasis of the vasculature (31), and evidence suggests that during hyperglycemia, reduced NO availability exists (14).

The activation of protein kinase C, due to superoxide overproduction, induces a de novo synthesis of the enzyme NAD(P)H oxidase, which significantly contributes to produce more superoxide anions (32).

Hyperglycemia also favors, through the activation of NF-κB, an increased expression of both NAD(P)H and iNOS (33). Overexpression of iNOS is accompanied by increased generation of NO (33).

Superoxide overproduction, when accompanied by increased NO generation, favors the formation of the strong oxidant peroxynitrite (34), which in turn avidly oxidizes tetrahydrobiopterin, an iNOS cofactor, to dihydrobiopterin (35). Under conditions of tetrahydrobiopterin deficiency, iNOS is in an uncoupled state, which means that electrons flowing from the iNOS reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine, resulting in production of superoxide rather than NO (36,37). Exposure to peroxynitrite during hyperglycemia also produces an

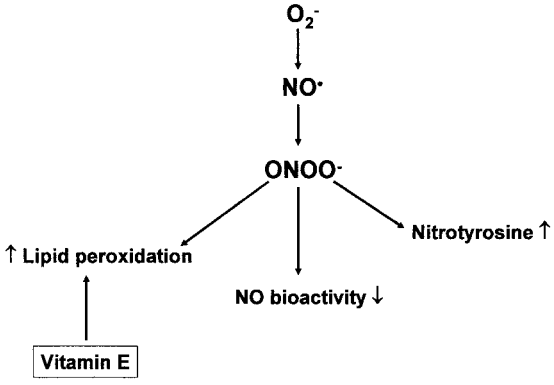

Figure 2—*O_2^- reacting with NO produces peroxynitrite ($ONOO^-$) (see Fig. 3 for details). The radical scavenging antioxidant vitamin E is active only against certain components of oxidative stress, particularly lipid peroxidation products, leaving other consequences of O_2^- untouched.*

uncoupling state of eNOS, presumably via a zinc depletion of the enzyme, favoring superoxide overproduction (38). Consistently, in hyperglycemic conditions, an overproduction of both superoxide and NO has been reported, with a threefold increase in superoxide generation (37). As previously reported, the simultaneous overgeneration of NO and superoxide favors the production of a toxic reaction product, the peroxynitrite anion (34). The peroxynitrite anion is cytotoxic because it oxidizes sulfydryl groups in proteins, initiates lipid peroxidation, and nitrates amino acids such as tyrosine, which affects many signal transduction pathways (34). The production of peroxynitrite can be indirectly inferred by the presence of nitrotyrosine (39).

The possibility that diabetes is associated with increased nitrotyrosine formation is supported by the recent detection of increased nitrotyrosine plasma levels in type 2 diabetic patients (40). Several pieces of evidence support a direct role of hyperglycemia in favoring this phenomenon. Nitrotyrosine formation is detected in the artery wall of monkeys during hyperglycemia (41) and also in the plasma of healthy subjects during a hyperglycemic clamp (42) and in diabetic patients during an increase of postprandial hyperglycemia (43). Hyperglycemia is also accompanied by nitrotyrosine deposition in a perfused working heart from rats, and it is reasonably related to unbalanced production of NO and superoxide through iNOS overexpression (44). Nitrotyrosine formation is followed by the development of an endothelial dysfunction in both healthy subjects (41) and in coronaries of perfused hearts (44), and this effect is not surprising because it has been shown that nitrotyrosine can also be directly harmful to endothelial cells (45).

The toxic action of nitrotyrosine, and therefore perhaps of peroxynitrite, is supported by study evidence. Increased apoptosis of myocytes, endothelial cells, and fibroblasts in heart biopsies from diabetic patients (46), in hearts from streptozotocin-induced diabetic rats (47), and in working hearts from rats during hyperglycemia (44) is selectively associated with levels of nitrotyrosine found in those cells.

PEROXYNITRITE, DNA DAMAGE, AND ENDOTHELIAL DYSFUNCTION: THE ROLE OF POLY(ADP-RIBOSE) POLYMERASE ACTIVATION

— Peroxynitrite is a potent initiator of DNA single-strand breakage, which is an obligatory stimulus for the activation of the nuclear enzyme poly(ADP-ribose) polymerase (48). As described above, when endothelial cells respond to high glucose, reactive nitrogen and oxygen species generation occurs (49). These reactive species trigger DNA single-strand breakage, which induces a rapid activation of poly(ADP-ribose) polymerase (49). The role of hyperglycemia and related oxidative stress in producing DNA damage is supported by the recent findings that increased amounts of 8-hydroxyguanine and 8-hydroxydeoxyguanosine (markers of oxidative damage to DNA) can be found in both the plasma and tissues of streptozotocin diabetic rats (50). These concentrations are correlated and can be reduced by the control of hyperglycemia and by the use of the antioxidants probucol and vitamin E (50).

Poly(ADP-ribose) polymerase activation in turn depletes the intracellular concentration of its substrate NAD^+, slowing the rate of glycolysis, electron transport, and ATP formation, and produces the ADP-rybosylation of GAPDH (51). This process results in acute endothelial dysfunction in diabetic blood vessels (48). The possibility of normalizing diabetic endothelial dysfunction using a specific of poly(ADP-ribose) polymerase inhibitor, PJ34, supports this evidence (52,53).

A NEW THERAPEUTIC APPROACH: THE "CAUSAL" ANTIOXIDANT THERAPY

— As previously stated, convincing evidence is now available about the possible role of oxidative stress in the development of diabetic complications (6). However, clinical trials with antioxidants, in particular with vitamin E, have failed to demonstrate any beneficial effect (8).

On this matter, it has recently been suggested that antioxidant therapy with vitamin E or other antioxidants is limited to scavenging already-formed oxidants and may therefore be considered a more "symptomatic" rather than a causal treatment for vascular oxidative stress (54). Figure 2 helps explain this concept.

According to the evidence discussed in this article, it is suggested that interrupting the overproduction of superoxide by the mitochondrial electron-transport chain would normalize the pathways involved in the development of diabetic complications. It might, however, be difficult to accomplish this using conventional antioxidants, because these scavenge reactive oxygen species in a stechiometric manner. New low–molecular mass compounds that act as SOD or catalase mimetics have the theoretical advantage of scavenging reactive oxygen species continuously by acting as catalysts with efficiencies approaching those of native enzymes (54). Such compounds normalize endothelial dysfunction in streptozotocin-induced diabetic rats (55) and improve diabetes-induced decreases in endoneurial blood flow and motor nerve conduction velocity (56). Another interesting compound is L-propionyl-carnitine. This substance has been shown to act as an intracellular superoxide scavenger, improving mitochondrial function and reducing DNA damage (57–60). These properties have been shown to have beneficial effects on diabetic heart function, peripheral nerve function, and vascular blood flow in experimental diabetes (58,60,61).

In the last years, another substance has received much attention: lipoic acid (62). It may have a unique self-regenerating capacity as a mitochondrial antioxidant (62), and the possibility that it restores endothelial dysfunction in both animal models of diabetes and in diabetic patients has been reported (63,64).

Other promising tools are LY 333531, PJ34, and FP15, which block the protein kinase β isoform, poly(ADP-ribose) polymerase, and peroxynitrite, respectively. Not surprisingly, they have been shown to ameliorate the endothelial dysfunction induced by hyperglycemia (52,53,65, 66). LY 333531 has been demonstrated to reduce oxidative stress generation in the retina: this is consistent with the evidence that protein kinase C activation may increase superoxide generation through NAD(P)H (67). PJ34 is not an antioxidant and does not directly interfere with the reactivity of peroxynitrite (48); however, poly(ADP-ribose) polymerase$^{-/-}$ mice show that after coronary occlusion and reperfusion, nitrotyrosine staining is markedly reduced by PJ34 (68). There is evidence that poly(ADP-ribose) polymerase inhibition can suppress the expression of iNOS (69,70) and that poly(ADP-ribose) polymerase can regulate the function of mitochondria in oxidatively challenged cells: poly(ADP-ribose) polymerase inhibitors or poly(ADP-ribose) polymerase deficiency can suppress mitochondrial permeability transition and mitochondrial oxidant generation (71). Because mitochondria represent a principal source of reactive oxidants in endothelial cells placed in high glucose (22) may be that poly(ADP-ribose) polymerase inhibition suppresses nitrotyrosine generation preserving mitochondrial integrity.

FP15 is a potent peroxynitrite decomposition catalyst (66). It inhibits tyrosine nitration and reduces the toxicity of peroxynitrite for β-cells and vascular endothelium during the development of diabetes in rats (66). Therefore, in the near future, a causal antioxidant therapy may include SOD and catalase mimetics, L-propionyl-carnitine, lipoic acid, protein kinase Cβ and poly(ADP-ribose) polymerase inhibitors, and peroxynitrite catalysts. This combination would aim to block the noxious cascade activated by hyperglycemia through the overproduction of superoxide and NO.

However, while waiting for these focused tools, we may already have other possibilities. It is now evident that thiazolinediones (72), statins (73), ACE inhibitors (74), and ATI inhibitors (75,76) have a strong intracellular antioxidant activity, and it has been suggested that many of their beneficial ancillary effects are due to this property. Thiazolinediones have an inhibiting effect on iNOS and significantly reduce peroxynitrite generation (72). An animal study supports the efficacy of thiazolidinediones in reducing the harmful effect of peroxynitrite (77). Indeed, statins increase NO bioavailability and decrease superoxide production (78), probably interfering with NAD(P)H

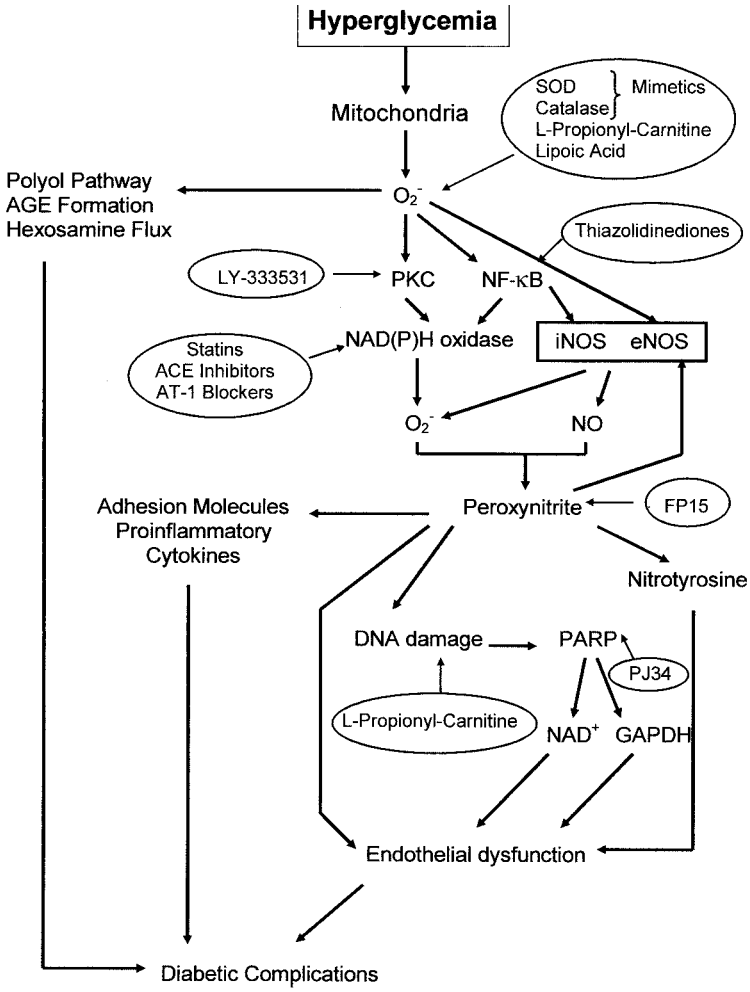

Figure 3—*Superoxide overproduction reduces eNOS activity, but through NF-κB and protein kinase C (PKC) activates NAD(P)H and increases iNOS expression: the final effect is an increased NO generation. This condition favors the formation of the strong oxidant peroxynitrite, which in turn produces, in iNOS and eNOS, an uncoupled state, resulting in the production of superoxide rather than NO, and damages DNA. DNA damage is an obligatory stimulus for the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP). Poly(ADP-ribose) polymerase activation in turn depletes the intracellular concentration of its substrate NAD^+, slowing the rate of glycolysis, electron transport, and ATP formation, and produces an ADP-ribosylation of the GAPDH. This process results in acute endothelial dysfunction in diabetic blood vessels, which contributes to the development of diabetic complications. NF-κB activation also induces a proinflammatory conditions and adhesion molecule overexpression. All these alterations produce the final picture of diabetic complications. SOD or catalase mimetics, L-propionyl-carnitine and lipoic acid, reducing mitochondrial overproduction of superoxide, and reducing DNA damage may be good candidates for causal intracellular antioxidants. This causal therapy would also be associated with LY 333531, PJ34, and FP15, which block protein kinase β isoform, poly(ADP-ribose) polymerase, and peroxynitrite, respectively. Other current options may be thiazolinediones, statins, ACE inhibitors, and ATI inhibitors, which also have, at different levels, intracellular causal antioxidant activity. AGE, advanced glycosylation end product.*

activity (79), and improve endothelial dysfunction (73), even in diabetic patients (80,81). This hypothesis is supported by the evidence that the action of statins is independent of the cholesterol-lowering effect (73,80,81) and that studies show that (at least) simvastatin ameliorates diabetic retinopathy (82) and reduces cardiovascular disease as primary prevention in diabetic patients (83). Moreover, statins reduce nitrotyrosine formation in diabetic patients (81) and attenuate angiotensin II–induced free radical production (84). This effect is of particular interest because it is now well recognized that angiotensin II and activated angiotensin 1 (AT-1) receptors produce intracellular oxidative stress (85,86), and evidence shows that hyperglycemia is able to directly modulate cellular angiotensin production (87). Therefore, it is not surprising that the possible use of both ACE inhibitors and ATI receptor blockers should be of particular interest in the prevention of hyperglycemia-derived oxidative stress.

Important trials have shown that these compounds are particularly efficacious in preventing diabetic complications such as retinopathy (88), nephropathy (89–93), and cardiovascular disease (91,93). ACE inhibitors and ATI blockers have been demonstrated to act as causal antioxidants (85,86), and it has recently been suggested that this property may account for their beneficial effect on diabetic complications (94). This hypothesis is strongly supported by the recent evidence that treatment with either ACE inhibitors or AT-1 blockers prevents nitrotyrosine deposition in the kidney and the development of proteinuria in diabetic rats (95). A summary of the various options proposed as causal antioxidant therapy is reported in Fig. 3.

In conclusion, a large body of evidence suggests a possible role of oxidative stress in the pathogenesis of diabetic complications. This raises the concept that an antioxidant therapy may be of great interest in these patients. It has recently been suggested that diabetic subjects with complications may have a defective cellular antioxidant response against the oxidative stress generated by hyperglycemia, which can predispose the patient to organ damage (96). New insights into the mechanisms leading to the generation of oxidative stress in diabetes are now available. Presumably these findings have led to the discovery and to the evaluation of new antioxidant molecules, such as SOD and catalase mimetics, that hopefully may inhibit, at an early stage, the mechanism leading to diabetic complications. While waiting for these new and specific compounds, it is reasonable to suggest that already-available substances, such as thiazolinediones, statins, ACE inhibitors, and ATI blockers, should also be used because they are effective causal antioxidants.

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