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Forum Review

Lipid Peroxidation in Diabetes Mellitus

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ABSTRACT

There is considerable evidence that hyperglycemia represents the main cause of complications of diabetes mellitus (DM), and oxidative stress resulting from increased generation of reactive oxygen species plays a crucial role in their pathogenesis. In fact, in the absence of an appropriate response from endogenous antioxidant mechanisms, the redox imbalance causes the activation of stress-sensitive intracellular signaling pathways. The latter play a key role in the development of late complications of DM, as well as in mediating insulin resistance (*i.e.,* resistance to insulin-mediated glucose uptake by some cells) and impaired insulin secretion. This review, focused on lipid peroxidation in DM, will examine the mechanisms and clinical readouts of oxidative stress in this setting, the relationship between lipid peroxidation and antioxidant status in type 1 and type 2 DM, the effects of hyperglycemia and metabolic control on *in vivo* markers of lipid peroxidation (*i.e.,* isoprostanes), and the association between isoprostane formation and platelet activation. Finally, possible targets of antioxidant therapy for diabetic vascular complications will be discussed. *Antioxid. Redox Signal*. 7, 256–268.

INTRODUCTION: DIABETES AND CARDIOVASCULAR DISEASE

The prevalence of diabetes mellitus (DM), a metabolic disorder characterized by high levels of blood glucose and insufficient secretion or action of endogenous insulin, is increasing worldwide (8, 9). It represents a major cause of morbidity in Western countries and, among its comorbid conditions, atherosclerosis is probably the most important. In fact, DM is commonly associated with both microvascular and macrovascular complications (46). Although insulin treatment, oral medications, diet, and physical exercise can delay the development of microangiopathy (*i.e.,* retinopathy and nephropathy) (92), the development of macroangiopathy, an accelerated form of atherosclerosis leading to early coronary artery disease (CAD), increased risk of cerebrovascular disease, and severe peripheral artery disease (PAD) (46), cannot be prevented uniquely by metabolic control (93). Both CAD and PAD represent the main causes of morbidity and mortality in DM (81). Diabetic patients have a two- to fourfold increase in the risk of dying from cardiovascular disease (2). Furthermore, a 7-year follow-up study showed that diabetic patients without previous myocardial infarction carry the same risk of such an event as nondiabetic patients with previous myocardial infarction (46).

Although there is a steady downward trend in cardiovascular mortality and morbidity in the general population, this has not been observed among diabetic patients and has led many to reevaluate current treatment goals and pharmacological regimens for patients with type 2 (T2DM). Mortality and comorbidities make DM costly to both individuals and society in terms of quality of life and cost of care. Thus, developing better strategies for the prevention and treatment of the disease represents the primary objective for the current basic and clinical research in this field.

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MECHANISMS AND CLINICAL READOUTS OF OXIDATIVE STRESS IN DIABETES

Islet redox stress as a mediator of insulin resistance and β-cell dysfunction

Hyperglycemia causes oxidative stress mainly due to enhanced production of mitochondrial reactive oxygen species (ROS) (12), nonenzymatic glycation of proteins (11), and glucose autoxidation (100). Increased levels of free fatty acids (FFAs) can contribute to oxidative stress by promoting mitochondrial uncoupling and β-oxidation (36). Furthermore, oxidative stress induced by hyperglycemia and FFAs leads to the activation of stress-sensitive signaling pathways, which worsen both insulin secretion and action and promote the development of overt T2DM (36).

In patients with insulin resistance, metabolic syndrome, and T2DM, a milieu of enhanced oxidative stress has been documented within the islet of the pancreas. In fact, the β cell is particularly susceptible to the damage mediated by ROS. Multiple toxicities, including angiotensin II (Ang II), advanced glycosylation/fructosylation end products (AGEs/AFEs), antioxidant reserve impairment, amylin/amyloid, aging, FFAs, insulin, glucose, hypertension, triglyceride, and homocysteine, contribute to elevated redox stress within the islet, resulting in the formation of damaging ROS (47).

There is evidence supporting the presence of a local renin-angiotensin-aldosterone system (RAAS) operating within the islet and producing an excess of Ang II. This pathway could be triggered by either insulin or amylin; Ang II is in turn able to potently stimulate the generation of superoxide (O_2^-) via the activation of vascular NAD(P)H oxidase (44, 76). Interference with this mechanism by angiotensin-converting enzyme (ACE) inhibitors may explain the 32% reduction in the risk of developing T2DM observed in the HOPE study (48).

AGEs are produced via a nonenzymatic protein glycation due to hyperglycemia. Within the islet, they play a direct role in promoting formation of amyloid. Moreover, AFEs are end products with great affinity for proteins and, as AGEs, induce production of ROS (66). The receptor for AGEs (RAGE) is up-regulated by their presence and causes activation of the nuclear factor-κB (NFκB) signal transduction system, resulting in a chronic inflammatory state. Moreover, RAGE also binds amyloid, indicating that it could represent a potential target for inhibiting the accumulation of amyloid and the associated cellular dysfunction (101).

Together with increased generation of ROS, impaired formation of endogenous antioxidants, namely, superoxide dismutase (SOD), reduced glutathione (GSH), and ascorbic acid, and reduction in the antioxidant capacity of uric acid and vitamin E, as well as in the activity of glutathione peroxidase (GPx) and catalase, have been documented in DM (47). Impairment of antioxidant reserve may be explained by both damage of antioxidant enzymes caused by protein glycation and consumption by an excess demand (*e.g.,* compromised GSH function due to depletion of NADH in the polyol pathway) (47). A decreased antioxidant reserve in DM may be related to gene polymorphisms, as shown for an association between coronary spastic angina, myocardial infarction, and the polymorphism Glu298Asp in exon 7 of the endothelial nitric oxide (NO) synthase gene (71). Probably other polymorphisms may be related to insulin resistance, metabolic syndrome, or also amylin-derived islet amyloid (ADIA) deposition.

Amylin is a polypeptide cosynthesized and cosecreted by the islet β cell with insulin. It constitutes the monomeric substrate of the polymer ADIA, which forms between β cells, and between β cells and endothelial cells within the islet. It is present on histological examination in at least 70% of T2DM patients (47) and creates a secretory and absorptive insulin defect. Thus, hyperinsulinemia and hyperamylinemia are closely linked phenomena strongly associated with insulin resistance, metabolic syndrome, and T2DM. It has been shown recently that increased oxidative stress, with concomitant reduced expression of SOD, is related to islet amyloid, decreased β-cell mass, and β-cell volume density (86).

Aging contributes to islet damage by promoting impairment in endothelial NO synthesis, enhanced endothelial apoptosis, and increased cellular levels of oxidized low-density lipoprotein (oxLDL), TNF-α and caspase-3 activity (47).

FFAs, in particular their metabolically active form, the cytosolic long-chain acyl-CoA esters, contribute to progressive β-cell dysfunction and development of insulin resistance by enhancing production of ROS by mitochondria via reduction in ADP availability (5). FFAs cause pancreatic damage also by a mechanism of lipoapoptosis mediated by nonmitochondrial metabolites, resulting in β-cell dysfunction and loss (47). Regarding their role in promoting insulin resistance, an increase in cytosolic triglyceride stores in adipose and nonadipose tissues characterizes central obesity: intramyocellular lipids have been found highly correlated with insulin resistance (5).

Hyperinsulinemia is associated with insulin resistance, metabolic syndrome, and early T2DM. As insulin can interact with angiotensin-receptors, hyperinsulinemia contributes to pancreatic islet damage by activation of the local RAAS described above (47).

Inflammatory cytokines, including TNF-α and interleukin-6 (IL-6), are closely linked with development of DM, as well as insulin resistance and metabolic syndrome (47). Moreover, the activation of the signaling pathway NFκB is associated with redox stress and inducible NO synthase in the apoptosis of β cells in both type 1 DM (T1DM) and T2DM. Both NFκB and TNF-α are induced by ROS (34).

Hyperglycemia contributes to β-cell damage via four main mechanisms: generation of AGEs; activation of the polyol/sorbitol pathway, leading to amplification of the redox stress within the islet milieu (98); moreover, monosaccharides and fructose-lysine can form ROS via autoxidative reactions, contributing to damaging lipids and proteins through cross-linking and fragmentation (40); finally, glucose-mediated NO direct scavenging, as well as decline in its lifetime, has been shown (10) in diabetes.

Among toxicities involved in determining islet redox stress, hypertension plays a role mediated by increased ROS activity, activation of Ang II, and elevated amylin levels (47, 53).

Hyperhomocysteinemia is not a direct result of diabetes unless there is an associated impairment of renal function.

Probably, the diabetic population has the same incidence of the 677 C→T polymorphism of methylene tetrahydrofolate reductase gene as the general population (*i.e.,* 10–15%), with subsequent mild to moderate hyperhomocysteinemia. Eleveted homocysteine levels induce formation of ROS, oxidative inactivation and reduced generation of endothelial NO, and the conversion of the latter into toxic peroxynitrite ($ONOO^-$) (75).

Hypertriglyceridemia plays a role in the development of redox stress, not limited to FFA and lipotoxicity, discussed above. Hypertriglyceridemia is closely associated with small dense LDL particles, which are more likely to be oxidized (70). A high fat diet is essential for the development of islet amyloid in the human islet amyloid polypeptide transgenic mouse model (52). Moreover, tryglycerides stored in ectopic nonadipose cells, including the islet β cells, are capable of causing cellular dysfunction or lipoapoptosis, as previously discussed.

Finally, elevated redox stress is associated with increased matrix metalloproteinase (MMP) activity, particularly the inducible MMP-9 (95). Activation of the latter may result in disconnection of the β cell and the surrounding extracellular matrix, with subsequent apoptosis. Moreover, MMP-9 may convert ADIA fibrils into toxic amyloid particles contributing to the apoptotic process (47).

Oxidant stress and diabetic neuropathy

ROS not only are involved in the development of T1DM and T2DM, but also play a pivotal role in long-term development of associated complications.

Animal and *in vitro* studies performed during the last three decades demonstrated the role of four major pathways of glucose metabolism in the development of microvascular complications (90): (a) enhanced polyol pathway activity causing sorbitol and fructose accumulation, NAD(P)H-redox imbalances, and changes in signal transduction; (b) nonenzymatic glycation of proteins producing AGEs; (c) activation of protein kinase C (PKC), initiating a cascade of stress responses; and (d) increased hexosamine pathway flux (12, 90). Recently, a link has been established among these pathways, providing a unified mechanism of tissue damage. Each pathway becomes perturbed as a consequence of hyperglycemia-mediated O_2^- superoxide overproduction by the mitochondrial electron transport chain. In diabetes, O_2^- accumulation and increased polyol pathway activity, AGE accumulation, PKC activity, and hexosamine flux are responsible for progressive cellular dysfunction. In nerve, it corresponds to impaired neural function and loss of neurotrophic support, as well as to long-term apoptosis of neuron and Schwann cells (84, 88). Moreover, reduction in nerve growth factor, neurotrophin 3, ciliary neurotrophic factor, and insulin-like growth factor-I has been documented in nerves from diabetic animals, correlating with the presence of neuropathy (3).

Interestingly, although chronic hyperglycemia has been postulated to be a cause of vascular diabetic complications (57, 92), hyperglycemic spikes, frequent in diabetic subjects, are probably the most important contributors to diabetic complications, including neuropathy (14). In fact, acute hyperglycemia in T1DM at onset or in rapid decompensation in chronic diabetes causes impairment of the motor and sensory nerve conduction velocity (38). Moreover, impairment of nerve function has been observed in healthy subjects undergoing acute hyperglycemic clamp (38). As acute hyperglycemia can lower the pain threshold in both animals and humans (59), this could contribute to the genesis of neuropathic pain affecting diabetic patients.

Oxidant stress and diabetic nephropathy

Diabetic nephropathy represents the most common cause of renal failure in Western society.

Hyperglycemia contributes to the genesis of glomerular hyperfiltration preceding the occurrence of nephropathy in DM (99). Moreover, acute hyperglycemia causes an increase in glomerular filtration rate in diabetic subjects, with a larger effect in patients with proteinuria than in those without proteinuria (80). *In vitro* experiments have shown that intermittent exposure of mesangial cells to high glucose concentrations represents a stimulus for collagen hyperproduction (91), an important event in the pathogenesis of diabetic nephropathy. These observations have been confirmed in human studies, showing a close relationship between postprandial hyperglycemia and the onset and development of nephropathy in both T1DM and T2DM (89).

The renin-angiotensin system (RAS) is a paracrine regulator of renal function and blood flow, playing a crucial role in the progression of chronic renal disease, also in DM. Indeed, the beneficial effects of blocking the RAS with ACE inhibitors on the delay or prevention of incipient to overt nephropathy and renal failure are widely recognized (83).

Vascular endothelial growth factor (VEGF), an endothelial-cell mitogen playing a crucial role in angiogenesis, has been implicated in the development of nephropathy, as well as in retinopathy and neuropathy in DM (1, 39).

Interestingly, PKCβ, a common intracellular signaling pathway, is enhanced, together with transforming growth factor-β (TGF-β) expression, in diabetic rats with nephropathy, and localized in the glomerular mesangium; PKCβ-specific inhibition led to reduction in albuminuria, structural damage, and TGF-β expression (55).

Oxidant stress and endothelial dysfunction

Endothelium synthesizes a number of vasoactive substances, including the vasodilators NO, prostacyclin, as well as the vasoconstrictors Ang II and endothelin-1 (7). Its physiological function is represented by the inhibition of vascular contraction, leucocyte adhesion, smooth muscle cell (SMC) growth, and platelet aggregation (20). However, exposure to physical, humoral, or chemical stimuli can cause changes in its characteristics, leading to a state of endothelial dysfunction.

In DM, altered endothelium-dependent vascular relaxation has been documented, in relation to hyperglycemia-derived oxygen free radicals. ROS enhance sensitivity of contractile

elements to Ca^{2+} and promote mobilization of cytosolic Ca^{2+} in vascular SMCs (7, 20). Moreover, ROS directly activate a number of transcription factors with consequent up-regulation of adhesion molecules toward platelets and leucocytes and decrease the bioavailability of NO (7). Finally, they enhance the formation of AGEs and the oxidation of LDLs. Among ROS, O_2^- is considered the main cause of the contraction of SMCs (7). Its formation is due to an increased activity of a number of enzymes including NO synthases, which generate O_2^- in a Ca^{2+}-dependent manner, in the absence of the substrate L-arginine and the cofactor tetrahydrobiopterin (103). Moreover, several data strongly indicate that NAD(P)H oxidase represents the major source of O_2^- in endothelial and vascular SMCs (7), contributing to endothelial dysfunction in other clinical settings, such as hypercholesterolemia and hypertension (7). Along these lines, endothelial dysfunction in the central retinas of obese and non–insulin-dependent diabetic BBZ/WOR rats has been linked to NADH oxidase-mediated oxidative-stress (35).

An increase in O_2^- levels can be the consequence of enhanced generation, decreased metabolism, or both. Deficiency or inactivation of SOD enzymes (intracellular Cu/Zn- or Mn- and extracellular Cu/Zn-containing isoforms) may play a crucial role in determining endothelial dysfunction in several clinical settings, including DM.

GPx H_2O_2-scavenger activity has been found to be reduced in endothelial cells exposed to high glucose concentrations (7), suggesting an impaired free-radical scavenger function. Similarly, catalase, another H_2O_2 scavenger, could be involved in this process, as its biosynthesis and activity are increased by hyperglycemia both *in vitro* and *in vivo*, in order to neutralize enhanced oxidant stress (7).

Hyperglycemia enhances the production of NO by both the constitutive and inducible isoforms of NO synthase (7). In diabetic vessels, NO is scavenged by O_2^- to form $OONO^-$, with two opposite consequences: on the one hand, O_2^- is rapidly neutralized, and on the other hand, the vasodilator NO is consumed and a potentially damaging molecule is formed. In fact, $OONO^-$ increases insulin secretion and causes DNA damage and cell death in pancreatic islets, probably playing a crucial role in the initiation of T1DM (7). Furthermore, it may mediate the apoptotic effect of hyperglycemia on endothelial cells via NFκB activation, nitrosylate proteins leading to organ malfunction, and cause lipid peroxidation, depletion of plasma antioxidants such as GSH and cysteine, endothelial dysfunction via an impairment of adrenoreceptors, and platelet activation through isoprostane formation (7, 15).

ROS cause endothelial dysfunction also by promoting growth. In particular, increased generation of NAD(P)H oxidase-mediated ROS seem to be responsible for Ang II-induced vascular SMC hyperthrophy (7). In DM, hyperglycemia stimulates the synthesis of a variety of growth factors, such as TGF-β, VEGF, as well as extracellular components such as collagen and fibronectin. TGF-β potently stimulates the accumulation of the aforementioned matrix proteins (7), with a reversion of this effect following insulin-mediated normalization of glycemic levels (7), thus suggesting a crucial role for this cytokine in the genesis of matrix alterations observed in diabetic microvessels.

LIPID PEROXIDATION AND ANTIOXIDANT STATUS IN TYPE 1 AND TYPE 2 DIABETES

Ex vivo *markers of lipid peroxidation*

Induction of diabetes in rats with streptozocin or alloxan uniformly results in an increase in thiobarbituric acid reactive substances (TBARS), indirect evidence of intensified free-radical production (64).

Free radical production has been reported to be increased in patients with DM, and it has been suggested that hyperglycemia may directly contribute to the generation of oxidative stress (64). Plasma from diabetic subjects contains increased levels of TBARS, lipid hydroperoxides, and lipoperoxides (65).

In addition, the formation of AGEs, such as pentosidine, acrolein, and carboxymethyl-lysine, may lead to oxidant damage of proteins, which in turn may accelerate AGE formation (6). Evidence of oxidatively modified proteins was provided both through the quantification of plasma protein carbonyl levels, which were significantly higher in type 1 diabetics, and immunoblot analysis of protein-bound carbonyls (65). Additionally, a marked increase in protein oxidation was observed in type 1 patients through assessment of advanced oxidation protein products considered to be an oxidized albumin index (65). The carbonyl content of plasma proteins is also increased in type 2 diabetics, and oxidant damage alters the structure and the function of coagulative proteins (32) with an unbalanced promotion of procoagulant reactions.

O_2^- plasma concentrations are increased in diabetic patients and correlate with the glycemic levels, providing strong support for this hypothesis (14). Postprandial hyperglycemia, together with a drop in the antioxidant activity, is linked with a greater LDL oxidation (14). The production of free radicals associated with chronic hyperglycemia may result from non-enzymatic glycation and glucose autoxidation (17). O_2^- may also be generated inside the cells during exposure to hyperglycemia (21). Furthermore, monocytes may be an important source of free radicals in hyperglycemia (22). The simultaneous increase of O_2^- and NO generated by hyperglycemia (21) produces $ONOO^-$, a potent oxidant that oxidizes sulfhydryl groups in protein and initiates lipid peroxidation. The production of $ONOO^-$ can be indirectly inferred by the presence of nitrotyrosine in plasma. Increased nitrotyrosine levels have been described in the plasma of diabetics, during acute hyperglycemia and in the postprandial state after a standard meal (18).

Extracellular fluids lack protection by the antioxidant enzymes, but contain several molecules that delay or inhibit the oxidative process (45). Important biological antioxidants in plasma appear to be vitamin C, vitamin E, uric acid, and protein sulfhydryl (SH) groups (45).

The relative contribution of these antioxidants *in vivo* is not yet well defined. However, these antioxidants act synergistically *in vivo* to protect against radical damage. Thus, the assay of the total radical-trapping antioxidant parameter (TRAP) has been proposed to evaluate plasma antioxidant capacity, taking into consideration the mutual cooperation of

known and unknown antioxidants present in plasma (16, 94). Reduced fasting TRAP value has been reported in type 1 (94) and type 2 (16) diabetic patients. Moreover, antioxidant defenses are reduced during the oral glucose tolerance test both in normal subjects and in type 2 diabetics (17). In fact, rapidly increasing glycemia is accompanied by a substantial decrease in plasma TRAP, supporting the hypothesis that during acute hyperglycemia an oxidative stress is produced, leading to consumption of antioxidant capacity. This is confirmed by decreased levels of most of the measurable circulating antioxidants during acute hyperglycemia (17).

Vitamins A, C, and E are derived from the diet and detoxify free radicals directly. Vitamin E reacts directly with peroxyl and O_2^- radicals and singlet oxygen and protects membrane from lipid peroxidation. The deficiency of vitamin E is concurrent with increased peroxides and aldehydes in many tissues. There have been conflicting reports about vitamin E levels in diabetic animals and human subjects. In fact, plasma levels of vitamin E have been reported to be unaltered, increased, or decreased by diabetes (64).

Isoprostanes as in vivo *markers of lipid peroxidation*

Streptozotocin-induced diabetic rats had marked increases in plasma levels and urinary excretion rates of F_2-isoprostanes, and dietary supplementation with vitamin E normalized (plasma) and reduced (urine) isoprostane levels (72).

Human T1DM at onset represents an interesting paradigm of the interrelationship between the immunoinflammatory reaction, lipid peroxidation, and platelet activation. We recently reported that enhanced lipid peroxidation and platelet activation represent early events in the development of this disease in children and adolescents (30). Patients with newly diagnosed diabetes had significantly increased urinary excretion of both 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) (Fig. 1) and 11-dehydrothromboxane B_2 (11-dehydro-TXB_2) as well as higher plasma levels of a number of inflammatory markers (30). In most of these patients, but not in all, oxidative stress and platelet activation were reduced after 1 year, coincidently with a fall in the systemic levels of IL-6 and TNF-α. Thus, it appears that biochemical signals of oxidative stress and platelet activation can be appreciated early at the onset of DM, and that their variable intensity is, at least in part, driven by IL-6 production and disease duration. These noninvasive indices may help in further examining the pathophysiology of T1DM and monitoring pharmacologic interventions aimed at interfering with disease development and progression.

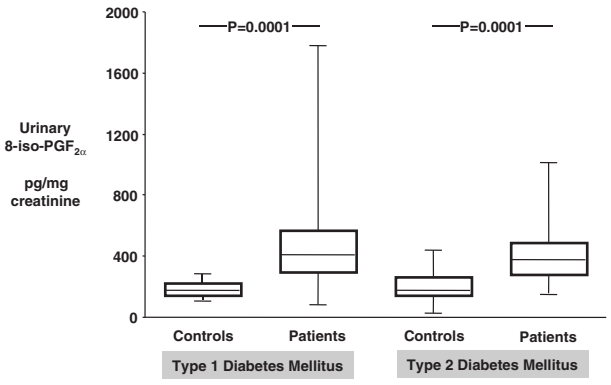

FIG. 1. Box and whisker plots of urinary 8-iso-$PGF_{2\alpha}$ excretion rates assessed in subjects with T1DM (n = 62) and T2DM (n = 62) and in age-matched controls.

Catella-Lawson and FitzGerald (13) have reported a trend toward increased urinary 8-*iso*-$PGF_{2\alpha}$ excretion in a group of 18 type 1 diabetics, with statistically significant elevations in patients presenting with diabetic ketoacidosis. Furthermore, increased LDL oxidation in diabetics was more readily reflected by 8-*iso*-$PGF_{2\alpha}$ than by conjugated diene formation (13).

In a 3-year longitudinal study of the effects of oxidative stress on the early natural history of type 1 diabetes, urinary excretion of 8-*iso*-$PGF_{2\alpha}$ was higher in the poorly controlled than in the well controlled patients and correlated with insulin requirements (51).

Although T2DM is a clearly established risk factor for cardiovascular disease, the mechanism(s) responsible for accelerated atherogenesis remains elusive. Altered lipoprotein levels; changes in lipoprotein composition, possibly affecting LDL binding to its receptors; and reduced LDL clearance resulting from impaired receptor recognition of glycated LDL have been described in diabetic patients (for review, see 52). Moreover, both high glucose levels and protein glycation enhance LDL oxidation by metal ions, and these reactions also yield AGE products (54, 64). In fact, LDL isolated from non–insulin-dependent diabetics contains higher levels of AGE products and conjugated dienes and is more easily oxidized by copper than is native LDL (19). In addition, plasma from patients whose insulin-dependent DM is poorly controlled has less antioxidant capacity (94).

In type 2 diabetic (db/db) mice, serum levels of F_2-isoprostanes and O_2^- production in carotid arteries were significantly elevated and were reduced by rosiglitazone (an agonist of peroxisome proliferator-activated receptor-γ) treatment (4). Consistent with the concept of enhanced lipid peroxidation in diabetes, Gopaul *et al.* (41) have reported that the average concentration of esterified 8-*iso*-$PGF_{2\alpha}$ in plasma from 39 patients with T2DM was approximately threefold higher than in healthy individuals. However, increased plasma levels of 8-*iso*-$PGF_{2\alpha}$ were not related to hyperglycemia or hyperlipidemia (41). A direct measure of *in vivo* lipid peroxidation appears to be superior to an indirect measure in this setting. In fact, a divergence between LDL oxidative susceptibility (not different from controls) and urinary 8-*iso*-$PGF_{2\alpha}$ levels (higher than in controls) has been described (33).

We found (27) that the formation and urinary excretion of 8-*iso*-$PGF_{2\alpha}$ were abnormally elevated in the vast majority of a relatively large group of type 2 diabetic patients (Fig. 1) carefully characterized for other variables potentially influencing lipid peroxidation (Table 1). Thus, we excluded the contribution of advanced age by adequate age-matching of individual patients and control subjects and of cigarette smoking by only recruiting nonsmokers into the study. Similarly, differences in 8-*iso*-$PGF_{2\alpha}$ formation between diabetic patients and healthy subjects could not be accounted for by the presence of macrovascular complications, arterial hypertension, or hypercholesterolemia. We found a highly signifi-

TABLE 1. URINARY EXCRETION RATES OF 8-ISO-$PGF_{2\alpha}$ IN DM

Clinical setting	*Patients*	*Controls*	*Reference*
T2DM (*n* = 72 vs. 72)	323 ± 179*	197 ± 69*	32
T2DM (*n* = 62 vs. 62)	419 ± 208*	208 ± 92*	27
T1DM (*n* = 23 vs. 23)	400 ± 146*	197 ± 69*	27
T2DM (*n* = 25 vs. 25)	2.03 ± 1.17†	0.71 ± 0.35†	33
T2DM (*n* = 14 vs. 44)	191 ± 136*	129 ± 50*	50
T1DM at onset (*n* = 23 vs. 23)	501 (386–746)‡	165 (140–210)‡	30

Values are expressed as means ± SD.
*pg/mg of creatinine.
†ng/mg of creatinine.
‡Values are expressed as median (range).

cant correlation between blood glucose and urinary 8-*iso*-$PGF_{2\alpha}$, suggesting that lipid peroxidation may be, at least in part, related to the determinants of glycemic control. This observation is consistent with the *in vitro* findings of Natarajan *et al.* (77), who demonstrated enhanced formation and release of 8-*iso*-$PGF_{2\alpha}$ by porcine vascular SMCs cultured under hyperglycemic conditions.

That impaired glycemic control rather than the attendant macrovascular complications is responsible for enhanced formation of F_2-isoprostanes in T2DM is also supported by the similar findings in T1DM (27). We further examined the relation between metabolic control and F_2-isoprostane formation by studying 21 T2DM patients with inadequate glycemic control, before and after intensive antidiabetic treatment, and closer monitoring. Reduced blood glucose levels were associated with a fall in urinary 8-*iso*-$PGF_{2\alpha}$ excretion rates, the average extent of which showed a remarkably good fitting, with the linear relation between blood glucose and urinary 8-*iso*-$PGF_{2\alpha}$, as established in the whole group of T2DM patients under baseline conditions (27).

Interestingly, oxidant stress (increase of plasma F_2-isoprostane levels) is an early event in the evolution of T2DM and could precede the development of endothelial dysfunction and insulin resistance (42). With regard to the relationship between hyperglycemia, oxidant stress, and insulin resistance, significantly higher plasma levels of 8-*iso*-$PGF_{2\alpha}$ have been documented in the obese Zucker rat, a widely used model of insulin resistance, than in insulin-sensitive controls (58). Vitamin E supplementation reduced plasma isoprostane levels and reversed glucose-induced hyperinsulinemia in this model (58). This could explain the beneficial effects of antioxidant therapy on insulin action previously described. However, it is not clear whether 8-*iso*-$PGF_{2\alpha}$ plays a crucial role in patients with insulin resistance and hyperinsulinemia without overt hyperglycemia (69).

Moreover, well controlled type 2 diabetic patients free of clinical macrovascular complications have elevated plasma levels of F_2-isoprostanes and C-reactive protein (CRP) (74).

Recently, Sampson *et al.* reported direct evidence of increased free radical damage during acute hyperglycemia in T2DM (87). In fact, acute hyperglycemia after a glucose load is associated with an acute increase in plasma concentrations of 8-*iso*-$PGF_{2\alpha}$, providing direct evidence for a link between abnormal glucose levels and increased free radical-mediated generation of these compounds from arachidonic acid in membrane and lipoprotein phospholipids (87).

Isoprostane formation and platelet activation

F_2-Isoprostanes, such as 8-*iso*-$PGF_{2\alpha}$, may modify aspects of platelet function, such as adhesive reactions and activation by low concentrations of other agonists. Concentrations of 8-*iso*-$PGF_{2\alpha}$ in the range of 1 nmol/L to 1 μmol/L induce a dose-dependent increase in platelet shape change, Ca^{2+} release from intracellular stores, and inositol phosphates (73, 79). Moreover, 8-*iso*-$PGF_{2\alpha}$ causes dose-dependent, irreversible platelet aggregation in the presence of concentrations of collagen, ADP, arachidonic acid, and prostaglandin H_2 (PGH_2)/thromboxane A_2 (TXA_2) analogues that, when acting alone, fail to aggregate platelets (79). Although these effects are prevented by the PGH_2/TXA_2 receptor (TP) antagonists and 8-*iso*-$PGF_{2\alpha}$ may cross-desensitize biochemical and functional responses to thromboxane mimetics, 8-*iso*-$PGF_{2\alpha}$ fails to activate either of the TP isoforms described in platelets at concentrations that typically circulate during syndromes of oxidant stress (79). The ability of 8-*iso*-$PGF_{2\alpha}$ to amplify the aggregation response to subthreshold concentrations of other platelet agonists may be relevant to settings where platelet activation and enhanced free-radical formation coincide (79).

We previously demonstrated enhanced thromboxane biosynthesis in T2DM and provided evidence for its platelet origin and its reduction in response to tight metabolic control (24). Moreover, we provided evidence that the metabolic disorder rather than the attendant vascular disease is responsible for persistent platelet activation in this setting (26). It was speculated that increased oxidant stress in diabetes could induce enhanced generation of 8-*iso*-$PGF_{2\alpha}$ and other biologically active isoeicosanoids and that these compounds could in turn contribute to platelet activation in this setting. Altered 8-*iso*-$PGF_{2\alpha}$ formation in T2DM correlated with the rate (Fig. 2) of TXA_2 biosynthesis (27). Moreover, improvement of metabolic control in these patients was accompanied by a statistically significant reduction in 11-dehydro-TXB_2 excretion. On the basis of these findings, we have suggested that changes in the rate of arachidonate peroxidation to form biologically active isoeicosanoids, such as 8-*iso*-$PGF_{2\alpha}$, may represent an important biochemical link between altered

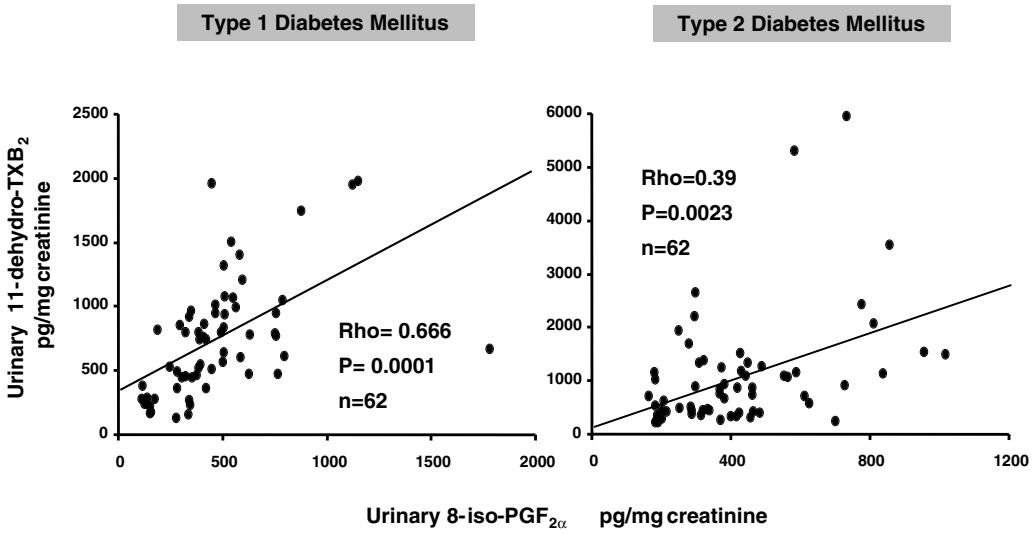

FIG. 2. Correlation between individual urinary 8-iso-$PGF_{2\alpha}$ and 11-dehydro-TXB_2 excretion rates assessed in subjects with T1DM (n = 62) and T2DM (n = 62). Dots represent individual measurements; regression plots are depicted by solid lines.

glycemic control, oxidant stress, and platelet activation in T2DM (27).

Thus, F_2-isoprostanes may transduce the effects of oxidant stress associated with complex metabolic disorders into specialized forms of cellular activation. The consistent linear relationship (Fig. 2) between the excretion rates of 8-*iso*-$PGF_{2\alpha}$ and 11-dehydro-TXB_2 also demonstrated in obese women (29), as well as in patients with hypercholesterolemia (25), and homozygous homocystinuria (28) suggests that a low-grade inflammatory state associated with these metabolic disorders may be the primary trigger of thromboxane-dependent platelet activation mediated, at least in part, through enhanced lipid peroxidation.

Interestingly, enhanced lipid peroxidation and platelet activation represent early events in the development of T1DM in children and adolescents (30). The finding that those patients with the shortest duration of disease and with the highest IL-6 levels had the highest rates of *in vivo* lipid peroxidation and platelet activation is consistent with the hypothesis that in children with T1DM the early increase in oxidative stress and platelet activation may be associated with inflammatory events that precede clinical manifestation of the disease. Once established, oxidative stress may sustain a vicious circle, because it has been shown that H_2O_2 induces the IL-6 promoter by activating NFκB through NFκB-inducing kinase (102).

Several feed-forward mechanisms are likely to amplify and sustain the relationship between systemic inflammation and platelet activation, such as a direct proinflammatory effect of CRP (78), the effects of F_2-isoprostanes on inflammatory gene expression (67), and the synthesis and release of inflammatory cytokines from activated platelets (61).

Antioxidant nutrients

Preventing the formation of hydroxyl radicals would be an efficient means to reduce hydroxyl-induced damage, and several compounds have been tested as antioxidants in diabetic animals with varying success. Even after diabetes is established, the buildup of TBARS may be reversed by treatment with vitamin E or combined vitamins C, E, and β-carotene (64).

However, short-term treatment (3 weeks) with vitamin C (1.5 g daily) in T2DM subjects did not significantly improve plasma concentration of 8-*iso*-$PGF_{2\alpha}$ (23). Moreover, plasma and urine F_2-isoprostanes, and urine levels of a major metabolite of F_2-isoprostanes, were unchanged by vitamin C at all doses, suggesting this vitamin does not alter endogenous lipid peroxidation in healthy young women (60).

To assess the reversibility of F_2-isoprostane increase in response to short-term vitamin E supplementation, we examined (27) the effects of vitamin E supplementation (600 mg daily for 2 weeks) on the urinary excretion of 8-*iso*-$PGF_{2\alpha}$ and 11-dehydro-TXB_2 to test the hypothesis of a cause-and-effect relation between enhanced lipid peroxidation and platelet activation in T2DM. Vitamin E supplementation was associated with statistically significant changes in plasma vitamin E levels, vitamin E content of LDL, and lag time for LDL oxidation. It also caused virtually complete normalization of 8-*iso*-$PGF_{2\alpha}$ excretion in T2DM. Moreover, changes in F_2-isoprostane formation were accompanied by similar reductions in thromboxane metabolite excretion, consistent with a cause-and-effect relation between enhanced lipid peroxidation and persistent platelet activation (Fig. 3) in this setting (27).

The strength of the association between antioxidant consumption and the prevention of coronary events is strongest in observational studies, which are confounded by self-selection of patients and coconsumption of other nutrients in whole foods (37, 56, 63, 68). Nutrition is a very complex research topic in CAD, and it is not clear whether an individual component of the diet (antioxidant vitamins, low intake of saturated fatty acids, high intake of unsaturated fatty acids) or a combination of nutrients and dietary habits may be responsible for cardioprotective effects.

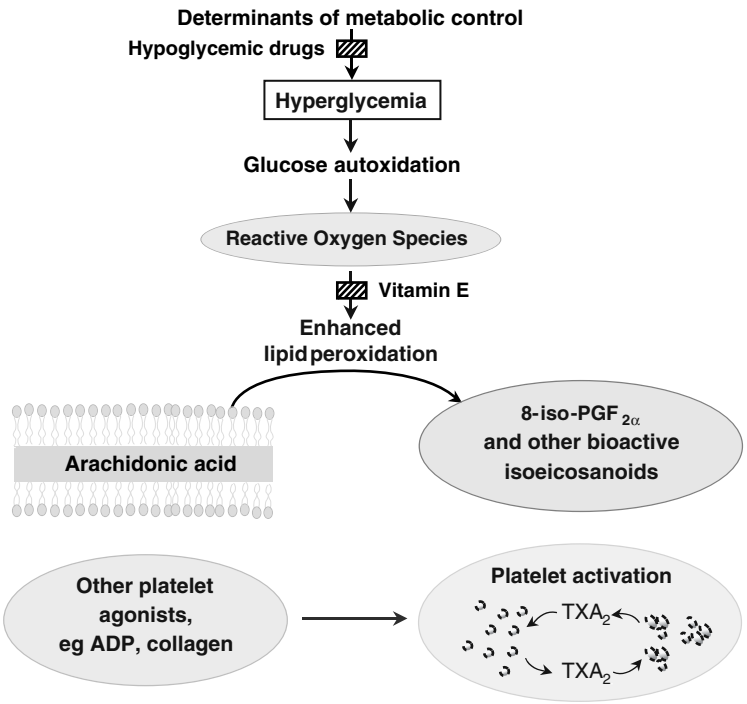

FIG. 3. The illustration depicts the role of glucose-induced oxidant stress in promoting nonenzymatic peroxidation of arachidonic acid, which in turn triggers persistent platelet activation in diabetes. It also illustrates the available pharmacological interventions potentially interrupting this mechanistic chain of events.

Animal studies and observational prospective human cohort studies are largely consistent with the concept that dietary supplementation with antioxidant vitamins reduces the progression of atherosclerosis (1, 83, 89, 91). However, firm recommendations to take antioxidant supplements in order to treat or prevent CAD require evidence derived from randomized controlled studies. Several large, well designed randomized placebo-controlled studies powered to detect differences in clinical events (ATBC, CARET, PHS, HOPE, GISSI, PPP, HPS) have recently failed to show a benefit of vitamin E supplementation in preventing cardiovascular events in different high-risk groups, including diabetic patients (37, 56, 63, 68). Moreover, the data indicate a null or adverse effect of β-carotene (37, 56, 63, 68). Two secondary prevention studies with relatively small numbers and short follow-up, CHAOS and SPACE, suggest that certain subpopulations may benefit from vitamin E supplements, but criteria for the identification of these subgroups require clear definition and validation (37, 56, 63, 68).

A striking feature of these and other trials of antioxidants is the absence of a biochemical basis for patient inclusion or, indeed, dose selection. Patients with high levels of oxidant stress or depletion of natural antioxidant defense systems may be the most likely to benefit from antioxidant therapy. If this is the case, then reliable, quantitative indices of *in vivo* oxidant stress, such as urinary isoprostane levels, should be considered as an inclusion criterion for patient selection. Future trials of antioxidant therapy in cardiovascular disease should then be targeted toward such patients with high levels of oxidant stress or patients with depletion of natural antioxidant defense systems. Furthermore, the dose of antioxidant should be chosen based on a surrogate readout that is a reliable, reproducible, and easily obtainable *in vivo* measure of oxidant stress (Fig. 4).

Despite evidence implicating hyperglycemia-derived oxygen free radicals as mediators of diabetic complications, intervention studies with vitamin E failed to demonstrate any beneficial effect in this setting (37, 56, 63, 68). The recent results relative to diabetic patients in the Primary Prevention Project trial (85) are highly consistent with the existing literature, showing a substantial lack of effect of antioxidant vitamin supplementation in preventing major cardiovascular events in patients at risk. The Heart Protection Study (49), involving ~6,000 individuals with diabetes, showed that vitamin E supplementation did not produce any significant reduction in the 5-year incidence of cardiovascular events in patients with or without prior cardiovascular events.

Similarly, in the Microalbuminuria Cardiovascular Renal Outcomes (MICRO)–Heart Outcomes Prevention Evaluation (HOPE) study, the daily administration of vitamin E for an average of 4.5 years to 3,654 people with diabetes had no effect on cardiovascular outcomes or nephropathy (62).

Failure of vitamin E may be explained by the fact that vitamin E works by scavenging already formed oxidation products, whereas the key event in the activation of all pathways (polyol pathway flux, AGE formation, activation of PKC, and hexosamine pathway flux) involved in the pathogenesis of diabetic complications is a hyperglycemia-induced process of overproduction of O_2^- by the mitochondrial electron-transport chain (15). O_2^- overproduction is accompanied by increased NO generation, with formation of $ONOO^-$, strong oxidant that in turn damages DNA, with activation of the nuclear enzyme poly(ADP-ribose) polymerase. Thus, new low-molecular mass compounds (15) that act as SOD or catalase mimetics, working as intracellular O_2^- scavengers, improving mitochondrial function, and reducing DNA damage, may be better candidates than vitamin E for such "causal" antioxidant therapy. We should also consider that drugs often used in

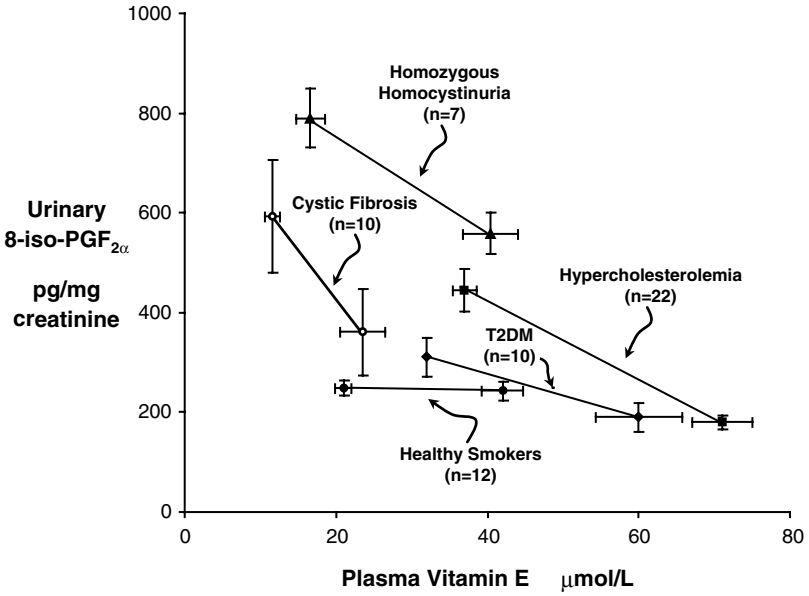

FIG. 4. Changes in plasma vitamin E levels and urinary 8-iso-$PGF_{2\alpha}$ excretion associated with vitamin E supplementation (600 mg/day for 2 weeks) in patients with hypercholesterolemia, T2DM, homozygous homocystinuria, and cystic fibrosis and in healthy cigarette smokers. Each solid line connects mean (± SEM) values measured before and after vitamin E supplementation.

diabetic patients, such as thiazolinediones or ACE inhibitors, have a strong intracellular antioxidant activity, explaining partially their beneficial ancillary effects (15).

Other pharmacological interventions

Lipid abnormalities in diabetes frequently consist of elevated triglyceride and LDL cholesterol levels and low high-density lipoprotein cholesterol levels. Subgroup analyses of primary and secondary prevention trials with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors (statins) indicate that lipid modification in diabetic patients is associated with significant CAD risk reduction (43). Diabetic subjects may receive greater benefit from statin treatment than nondiabetic individuals, because of a higher absolute risk. The Heart Protection Study has shown that statin therapy may significantly reduce the risk of nonfatal myocardial infarction or coronary heart disease death in diabetes (81).

Statins may reduce oxidative stress by reducing enhanced plasma levels of LDL, which are more susceptible to peroxidation in hypercholesterolemia, and change the LDL structure, making them more resistant to peroxidation (96). Statins may also inhibit NAD(P)H oxidase, thus decreasing the generation of ROS (97). In a recent randomized study in hypercholesterolemic subjects, simvastatin therapy reduced the elevated levels of urinary 8-*iso*-$PGF_{2\alpha}$ by approximately one third (31). These findings were paralleled by directionally similar changes in plasma oxLDL (31). Because oxLDL levels reflect oxidative events within a lipid pool that is highly relevant for atherosclerosis, these data complement data derived from peroxidative markers in the total lipid pool (F_2-isoprostanes).

Finally, it has been suggested that statins may add to or synergize with the biological effects of antioxidants. However, the addition of vitamin E (600 mg/day for 2 months) to simvastatin treatment did not reduce urinary 8-*iso*-$PGF_{2\alpha}$ excretion to a greater extent than simvastatin alone (31).

CONCLUSIONS

The availability of analytical tools for measuring F_2-isoprostane biosynthesis in man has greatly improved our understanding of the interplay between lipid peroxidation, low-grade inflammation, and platelet activation. A large body of clinical and experimental evidence supports the hypothesis that bioactive products of lipid peroxidation, including F_2-isoprostanes, are important transducers of the effects of metabolic and hemodynamic abnormalities into increased cardiovascular risk in diabetic subjects. Moreover, the use of F_2-isoprostane formation and excretion as a biochemical endpoint for dose-finding studies may allow reassessment of the adequacy of vitamin supplementation in diabetic patients. In fact, the science of vitamin supplementation for chronic disease prevention is not well developed, and much of the evidence comes from observational studies. However, suboptimal vitamin status in diabetics is not unusual in Western countries, due to persistent high levels of oxidant stress with increased depletion of antioxidants. Reliable assessment of F_2-isoprostane formation could be extremely valuable in the selection of the appropriate patient subgroups that may benefit from antioxidant interventions in this setting.

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ABBREVIATIONS

ACE, angiotensin-converting enzyme; ADIA, amylin-derived islet amyloid; AFEs, advanced fructosylation end prod-

ucts; AGEs, advanced glycosylation end products; Ang II, angiotensin II; CAD, coronary artery disease; CRP, C-reactive protein; 11-dehydro-TXB_2, 11-dehydrothromboxane B_2; DM, diabetes mellitus; FFA, free fatty acid; GPx, glutathione peroxidase; GSH, reduced glutathione; IL-6, interleukin-6; 8-*iso*-$PGF_{2\alpha}$, 8-*iso*-prostaglandin $F_{2\alpha}$; MMP, matrix metalloproteinase; NFκB, nuclear factor-κB; NO, nitric oxide; O_2^-, superoxide; $ONOO^-$, peroxynitrite; oxLDL, oxidized low-density lipoprotein; PAD, peripheral artery disease; PGH_2, prostaglandin H_2; PKC, protein kinase C; RAAS, renin-angiotensin-aldosterone system; RAGE, AGEs receptor; RAS, renin-angiotensin system; ROS, reactive oxygen species; SMC, smooth muscle cell; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TP, PGH_2/TXA_2 receptor; TRAP, total radical-trapping antioxidant parameter; TXA_2, thromboxane A_2; VEGF, vascular endothelial growth factor.

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