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Oxidative Stress and Stress-Activated Signaling Pathways: A Unifying Hypothesis of Type 2 Diabetes

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In both type 1 and type 2 diabetes, the late diabetic complications in nerve, vascular endothelium, and kidney arise from chronic elevations of glucose and possibly other metabolites including free fatty acids (FFA). Recent evidence suggests that common stress-activated signaling pathways such as nuclear factor-κB, p38 MAPK, and NH_2-terminal Jun kinases/stress-activated protein kinases underlie the development of these late diabetic complications. In addition, in type 2 diabetes, there is evidence that the activation of these same stress pathways by glucose and possibly FFA leads to both insulin resistance and impaired insulin secretion. Thus, we propose a unifying hypothesis whereby hyperglycemia and FFA-induced activation of the nuclear factor-κB, p38 MAPK, and NH_2-terminal Jun kinases/stress-activated protein kinases stress pathways, along with the activation of the advanced glycosylation end-products/receptor for advanced glycosylation end-products, protein kinase C, and sorbitol stress pathways, plays a key role in causing late complications in type 1 and type 2 diabetes, along with insulin resistance and impaired insulin secretion in type 2 diabetes. Studies with antioxidants such as vitamin E, α-lipoic acid, and *N*-acetylcysteine suggest that new strategies may become available to treat these conditions. (*Endocrine Reviews* 23: 599–622, 2002)

I. Introduction

THERE IS CONSIDERABLE evidence that hyperglycemia results in the generation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress in a variety of tissues. In the absence of an appropriate compensatory response from the endogenous antioxidant network, the system becomes overwhelmed (redox imbalance), leading to the activation of stress-sensitive intracellular signaling pathways. One major consequence is the production of gene products that cause cellular damage and are ultimately responsible for the late complications of diabetes.

In addition to playing a key role in late diabetic complications, activation of the same or similar signaling pathways also appears to play a role in mediating insulin resistance and impaired insulin secretion. The ability of antioxidants to

Abbreviations: AG, Aminoguanidine; AGE, advanced glycosylation end-products; AP, activator protein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CoA, coenzyme A; DHLA, dihydrolipoic acid; FFA, free fatty acids; GFAT, glutamine:fructose-6-phosphate amidotransferase; GSH, glutathione; IKK, IκB kinase; IR, insulin receptor; IRS, insulin receptor substrate; JNK/SAPK, NH_2-terminal Jun kinases/stress activated protein kinases; LA, α-lipoic acid; MCR, metabolic clearance rate; NAC, N-acetyl-L-cysteine; NAK, NF-κB-activating kinase; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; NO, nitric oxide; PBN, α-phenyl-tert-butylnitrone; PKC, protein kinase C; PPARγ, peroxisomal proliferator-activated receptor-γ; PTPase, protein tyrosine phosphatase; RAGE, receptor for AGE; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD2, manganese superoxide dismutase; UCP, uncoupling protein; VEGF, vascular endothelial growth factor.

protect against the effects of hyperglycemia and free fatty acids (FFA) *in vitro*, along with the clinical benefits often reported following antioxidant therapy, supports a causative role of oxidative stress in mediating and/or worsening these abnormalities. In this review, we propose the existence of common biochemical processes whereby oxidative stress induced by hyperglycemia and FFA causes insulin resistance, β-cell dysfunction, and late diabetic complications.

II. Overview of the Development of Type 2 Diabetes

Type 2 diabetes is characterized by excessive hepatic glucose production, decreased insulin secretion, and insulin resistance (1–5). Insulin resistance most often precedes the onset of type 2 diabetes by many years, is present in a large segment of the general population, and is multifactorial (1, 2). There are convincing data to indicate a genetic component associated with insulin resistance (1, 6–9). Insulin resistance is a feature of the offspring of parents with type 2 diabetes, and longitudinal studies of families indicate that it is a major risk factor for developing type 2 diabetes. In Pima Indians, a group with a very high prevalence of insulin resistance and type 2 diabetes, the insulin resistance has been suggested to have a codominant mode of inheritance (10)

Insulin resistance is also caused by acquired factors such as obesity, sedentary life style, pregnancy, and hormone excess (1, 3). During its early stage, insulin resistance is compensated for by hyperinsulinemia, thus preserving normal glucose tolerance. Reaven (2) and others (11–13) have obtained data indicating that approximately 25% of nondiabetic individuals exhibit insulin resistance within the range of that observed in patients with type 2 diabetes. Deterioration into impaired glucose tolerance occurs when either insulin resistance increases or the insulin secretory responses decrease, or both. Elevated glucose causes oxidative stress due to increased production of mitochondrial ROS (Table 1 and Ref. 14), nonenzymatic glycation of proteins (15, 16), and glucose autoxidation (17, 18). Elevated FFA can cause oxidative stress due to increased mitochondrial uncoupling (19, 20) and β-oxidation (21, 22), leading to the increased production of ROS. In addition, hyperglycemia- and FFA-induced oxidative stress leads to the activation of stress-sensitive signaling pathways. This, in turn, worsens both insulin secretion and action, leading to overt type 2 diabetes. Furthermore, insulin-resistant patients, with and without type 2 diabetes, are at increased risk for developing the metabolic syndrome, a major cause of heart disease, hypertension, and dyslipidemia (2, 23, 24). In this review, we now propose that oxidative stress induced by elevations in glucose and FFA plays a key role in causing insulin resistance and β-cell dysfunction. Thus, treatment aimed at reducing the degree of oxidative stress and activation of stress-sensitive signaling pathways would appear to warrant consideration for inclusion as part of the treatment program for patients with type 2 diabetes.

III. Oxidative Stress and Complications of Diabetes

There is considerable evidence that hyperglycemia causes many of the major complications of diabetes including nephropathy, retinopathy, neuropathy, and macro- and microvascular damage (1, 14, 25–27). Oxidative stress resulting from increased production of ROS (or their inadequate removal) plays a key role in the pathogenesis of late diabetic complications (Table 1, Fig. 1, and Refs. 14, 16, and 28–42). In uncontrolled diabetes, the level of superoxide dismutase, the enzyme responsible for inactivating the superoxide radical (43), along with the levels of the antioxidants vitamin E and α-lipoic acid [LA (Fig. 2)], are decreased (36, 44–48). There is also some evidence that a deficiency in erythrocyte catalase, an enzyme responsible for the removal of H_2O_2, is associated with increased frequency of diabetes (49, 50). Although our understanding of how hyperglycemia-induced oxidative stress ultimately leads to tissue damage has advanced considerably in recent years (14, 28, 51–53), effective therapeutic strategies to prevent or delay the development of this damage remain limited (54–57).

TABLE 1. Selected examples of biologically important reactive species

Type	Free radicals	Nonradicals
ROS	Superoxide, $^{\cdot}O_2^-$	Hydrogen peroxide, H_2O_2
	Hydroxyl, $^{\cdot}OH$	Hydrochlorous acid, HOCl
	Peroxyl, $^{\cdot}RO_2$	
	Hydroperoxyl, $^{\cdot}HO_2^-$	
RNS	Nitric oxide, $^{\cdot}NO$	Peroxynitrite, $OONO^-$
	Nitrogen dioxide, $^{\cdot}NO_2^-$	Nitrous oxide, HNO_2

ROS and RNS are defined as highly reactive molecules including charged species such as superoxide, hydroxyl radical, and nitric oxide and uncharged species such as hydrogen peroxide (31, 407). The formation of these species is discussed in the text. Oxidative stress is defined by Halliwell (407) as a serious imbalance between the production of reactive species and antioxidant defenses, leading to potential tissue damage. Table adapted from P. Rösen, P. P. Nawroth, G. King, W. Möller, H. J. Tritschler, and L. Packer (2001). The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association, and the German Diabetes Society. Diabet Metab Res Rev 17:189–202 (31). Copyright © 2001 John Wiley & Sons, Ltd. Reproduced with permission.

A. Hyperglycemia leads to mitochondrial dysfunction and activation of stress pathways both in vitro and in vivo

In vivo studies reveal that oxidative stress due to hyperglycemia occurs before late complications become clinically evident (30, 35, 36, 38–41, 58, 59), indicating that oxidative stress plays a crucial role in the pathogenesis of late diabetic complications (28–31, 33, 35, 41, 59–61). One area of intense study has been the regulation of stress-activated signaling pathways including nuclear factor-κB (NF-κB), p38 MAPK, NH_2-terminal Jun kinases/stress-activated protein kinases (JNK/SAPK), advanced glycosylation end-products (AGE)/receptor for AGE (RAGE), and protein kinase C (PKC).

Compelling evidence demonstrating the importance of ROS generation in mediating hyperglycemia-induced cellular damage was recently provided (62). In bovine endothelial cells, exposure to hyperglycemia initially increased the production of intracellular ROS and activated NF-κB. Subsequently, PKC activity, AGE, and sorbitol levels increased. Disruption of mitochondrial ROS production was achieved

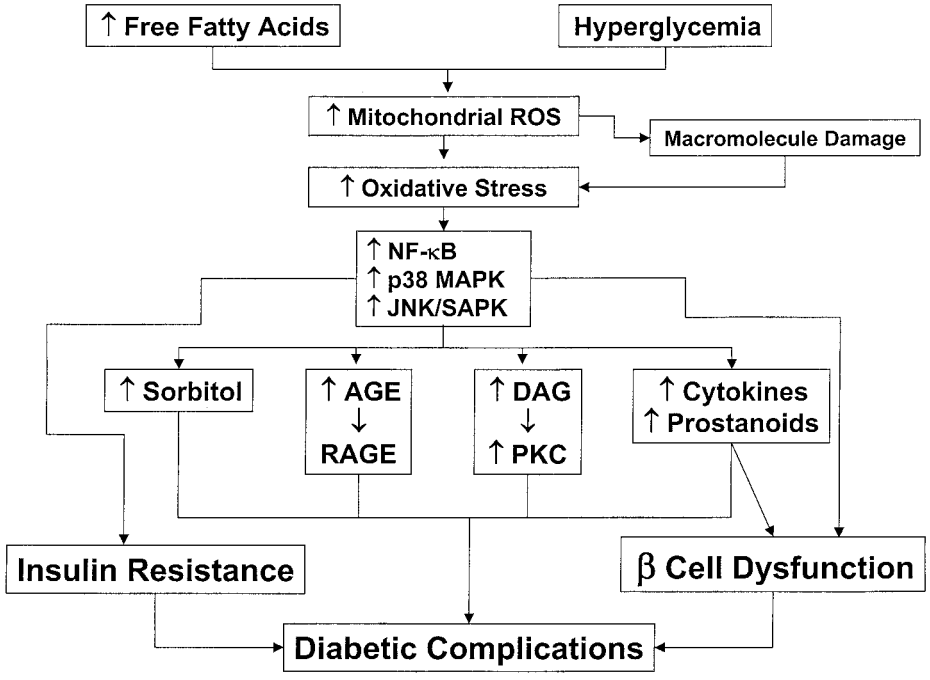

FIG. 1. Proposed general theory of how elevated FFA and hyperglycemia result in the pathophysiology of diabetes via the generation of ROS. This diagram shows the proposed causative link between hyperglycemia, elevated FFA, mitochondrial ROS generation (67, 408), oxidative stress, activation of stress-sensitive pathways (NF-κB, p38 MAPK, JNK/SAPK, and others), insulin resistance, β-cell dysfunction, and diabetic complications (51, 62). The proposed sequence of events reflects recent *in vitro* data that showed disruption of mitochondrial ROS production blocked the hyperglycemia-induced increase in ROS production along with hyperglycemia-induced effects on NF-κB, PKC, AGE, and sorbitol (62). Increased production of sorbitol (formed as a consequence of the hyperglycemia-mediated increase in aldose reductase activity), AGE, cytokines, prostanoids, along with PKC activation, can function as positive regulatory feedback loops to chronically stimulate stress-sensitive pathways. ROS (and RNS) can inflict damage directly upon cellular macromolecules that, in turn, result in oxidative stress.

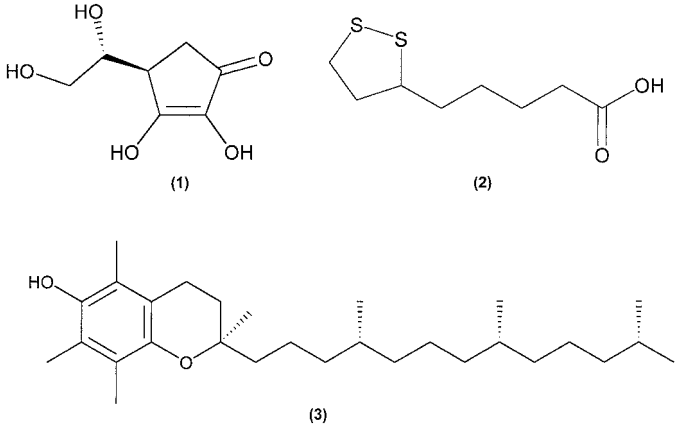

FIG. 2. Chemical structures of vitamin C (1), LA (2), and vitamin E (α-tocopherol; 3).

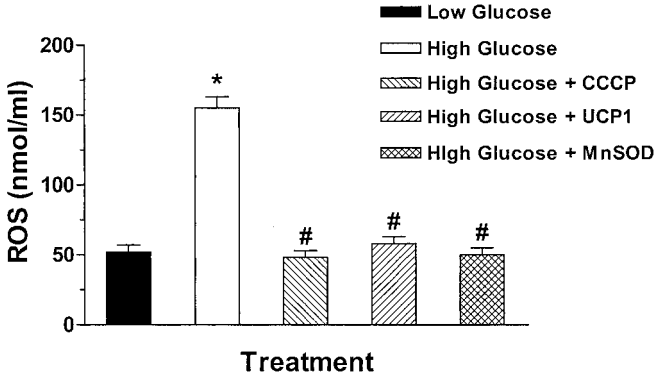

FIG. 3. Hyperglycemia-induced ROS formation and inhibitory effects of mitochondrial uncoupling agents and manganese superoxide. Bovine aortic endothelial cells were incubated for 2 h in 5 mM glucose (low) or 30 mM glucose (high) alone, and 30 mM glucose plus either 0.5 μM CCCP, UCP1, or SOD2 (MnSOD). cDNAs for UCP1 and SOD2 were cloned into plasmid pEB and used to prepare cationic liposomes. Intracellular ROS were measured using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). *, $P < 0.01$ (compared with 5 mM glucose); #, $P < 0.01$ (compared with 30 mM glucose). [Derived from Ref. 62.]

using several different approaches including: 1) treatment with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a small molecule uncoupler of mitochondrial oxidative phosphorylation; 2) overexpression of uncoupling protein (UCP)1, a protein uncoupler; or 3) overexpression of manganese superoxide dismutase (SOD2), the mitochondrial antioxidant enzyme. Each of these approaches blocked the hyperglycemia-induced increase in ROS production (Fig. 3). Consequently, the hyperglycemia-induced effects on NF-κB, PKC, AGE, and sorbitol were also suppressed.

Moreover, the effects of hyperglycemia on ROS formation and NF-κB activation preceded the stimulation of the other systems. Therefore, these data indicated that activation of NF-κB was an initial signaling event. If extended to other cell types and tissues, these studies would suggest that oxidative stress is the initial change induced by high glucose, followed

by activation of other pathways that lead to cellular dysfunction and damage (14) (Fig. 1).

B. ROS generation and oxidative stress

In the process of mitochondrial respiration, molecular oxygen is essential for the complete metabolism of glucose and other substrates during the production of ATP. During the course of normal oxidative phosphorylation, however, between 0.4 and 4% of all oxygen consumed is converted into the free radical superoxide ($^{\bullet}O_2^-$) (Refs. 63–68 and Table 1). Subsequently, $^{\bullet}O_2^-$ can be converted into other ROS and reactive nitrogen species (RNS). This $^{\bullet}O_2^-$ is normally eliminated by antioxidant defenses. $^{\bullet}O_2^-$ molecules within the mitochondria are quickly converted to H_2O_2 by the key mitochondrial enzyme, SOD2 (Refs. 63, 64, and 69, and Fig. 4). H_2O_2 is then either detoxified to H_2O and O_2 by glutathione peroxidase (in the mitochondria), or diffuses into the cytosol and is detoxified by catalase in peroxisomes. However, in the presence of reduced transition metals such as Cu or Fe, H_2O_2 can be converted to the highly reactive $^{\bullet}OH$ radical (Fenton reaction; Fig. 4).

Excessive levels of ROS lead to the damage of proteins, lipids, and DNA (70, 71). Thus, the aforementioned endogenous antioxidant systems exist within cells to neutralize ROS, and these systems are critical to maintaining proper cellular function. A major cellular antioxidant is reduced glutathione (GSH), which is regenerated most efficiently by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (Ref. 72 and Fig. 4). It can also be regenerated by LA in concert with other antioxidants (Refs. 73 and 74 and Figs. 4 and 5). When the aforementioned endogenous antioxidant network fails to provide a sufficient compensatory response to restore cellular redox balance, GSH levels fall and oxidative stress ensues. In addition to their ability to directly inflict damage upon cellular macromolecules, ROS play a significant role in activating stress-sensitive signaling pathways that regulate gene expression resulting in cellular damage (75–77).

C. NF-κB: a primary target for activation by hyperglycemia, ROS, oxidative stress, and inflammatory cytokines

One major intracellular target of hyperglycemia and oxidative stress is the transcription factor NF-κB (59, 78–81). NF-κB can be activated by a wide array of exogenous and endogenous stimuli including hyperglycemia, elevated FFA, ROS; TNF-α, IL-1β, and other proinflammatory cytokines; AGE-binding to RAGE; p38 MAPK; DNA damage; viral infection; and UV irradiation (79). NF-κB plays a critical role in mediating immune and inflammatory responses and apoptosis. The aberrant regulation of NF-κB is associated with a number of chronic diseases including diabetes and atherosclerosis.

NF-κB is activated through a common pathway, which involves the phosphorylation-induced proteasome-mediated degradation of the inhibitory subunit, IκB (82). A general overview of the sequence of events leading to NF-κB activation is shown (Fig. 6). In resting cells, NF-κB is present in the cytoplasm as an inactive heterodimer, consisting of the p50 and p65 subunits complexed with an inhibitor protein subunit, IκB. After stimulation, a serine kinase cascade is activated leading to the phosphorylation of IκB (83). This event primes IκB as a substrate for ubiquitination and subsequent degradation, freeing the NF-κB heterodimer to translocate to the nucleus. NF-κB regulates the expression of a large number of genes, including growth factors [*e.g.*, vascular endothelial growth factor (VEGF)], proinflammatory cytokines (*e.g.*, TNF-α and IL-1β), RAGE, adhesion molecules (*e.g.*, vascular cell adhesion molecule-1), and others. Many products of the genes regulated by NF-κB also, in turn, activate NF-κB (*e.g.*, VEGF, TNF-α, IL-1β, and RAGE).

Enzymes that catalyze the ubiquitination and degradation of phospho-IκB are constitutively active, indicating that the principal regulatory step in the activation of NF-κB is IκB phosphorylation (82, 83). The enzyme that phosphorylates IκB is IκB kinase (IKK), a heterotrimeric complex consisting of two catalytic subunits, IKKα (also called IKK1) and IKKβ (also called IKK2), and a regulatory subunit, IKKγ (84, 85). IKK is activated after serine phosphorylation catalyzed by

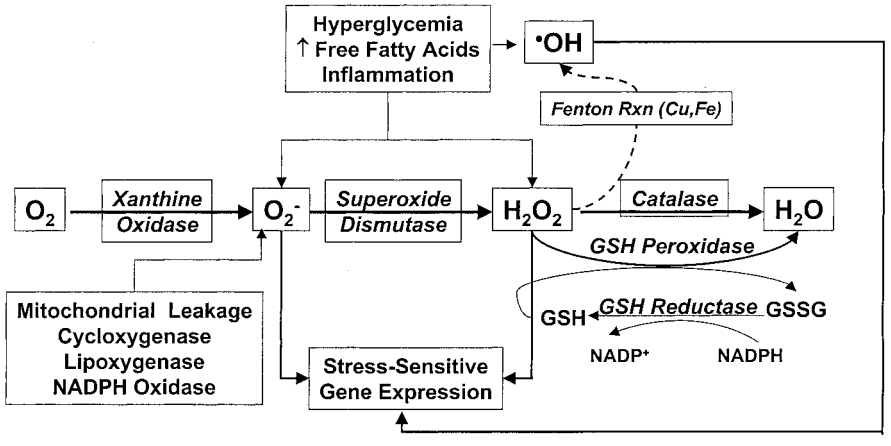

FIG. 4. Exogenous and endogenous stimuli leading to ROS generation and activation of stress-sensitive gene expression. The endogenous antioxidant enzymes including GSH, superoxide dismutase, GSH peroxidase, and catalase function to maintain redox equilibrium. However, in situations such as chronic hyperglycemia, the compensatory response is inadequate, leading to both ROS (and RNS) formation and activation of stress- and redox-sensitive gene expression (*e.g.*, via the redox-sensitive transcription factor NF-κB) (76, 77). Catalase is localized primarily in peroxisomes, whereas GSH peroxidase is the major peroxidase in mitochondria. [Derived from Ref. 78.]

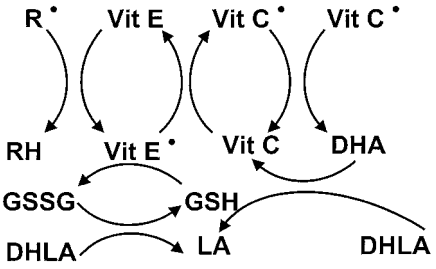

FIG. 5. Interaction and regeneration of endogenous antioxidants by LA and DHLA. Interaction and regeneration of endogenous antioxidants occurs through a cooperative set of reactions that can involve many substances (73, 74). Shown here is a highly simplified example of how LA and DHLA are capable of interacting with dihydroascorbate (DHA), vitamin C (Vit C), glutathione (oxidized, GSSG; reduced, GSH) to regenerate vitamin E (Vit E). LA after reduction to DHLA is able to facilitate the nonenzymatic regeneration of vitamin C and GSH, both of which are able to regenerate vitamin E. Reducing equivalents for the conversion of LA to DHLA are provided by reduced nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate (not shown). R•, Vit C•, Vit E•, charged species. [Reprinted with permission from J. L. Evans and I. D. Goldfine: *Diabetes Technol Ther* 2:401–413, 2000 (106).]

upstream serine kinases, including NF-κB-inducing kinase (NIK) (86) and NF-κB-activating kinase (NAK) (87). Although both IKKα- and IKKβ-subunits are subject to serine phosphorylation, only substitution of these sites in IKKβ completely prevents the activation of total IKK activity (85, 88).

Interestingly, IKKβ is directly inhibited by aspirin and salicylate (89), along with several antiinflammatory cyclopentenone prostaglandins including 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (90, 91), making these agents important tools with which to study the NF-κB pathway. The latter compound along with its metabolites are of particular interest because 1) they are naturally occurring derivatives of prostaglandin D_2; 2) they are thought to exert antiinflammatory activity *in vivo* (92, 93); and 3) they are natural high-affinity ligands for the peroxisomal proliferator-activated receptor-γ (PPARγ) (94), the molecular target for insulin sensitizing drugs (95–97). The recent discoveries and characterization of IKKβ, NIK, and NAK provide a unique opportunity to investigate and potentially identify novel molecular targets of antioxidant action, which have the demonstrated ability to block activation of the NF-κB pathway.

D. Hyperglycemia-dependent NF-κB activation in patients with diabetes mellitus

When patients with diabetes mellitus were studied, a positive correlation of NF-κB activation in peripheral blood mononuclear cells was found with the quality of glycemic control (indicated by hemoglobin A_{1C}) (98, 99). Moreover, a significant correlation between mononuclear NF-κB binding activity and the severity of albuminuria was observed in diabetic patients with renal complications (99). When patients with diabetes were treated with the antioxidant LA, a significant suppression of NF-κB activation, as well as of plasma markers for lipid oxidation, was observed (98, 99). These observations further support the idea that hyperglycemia-induced late diabetic complications result from a cycle

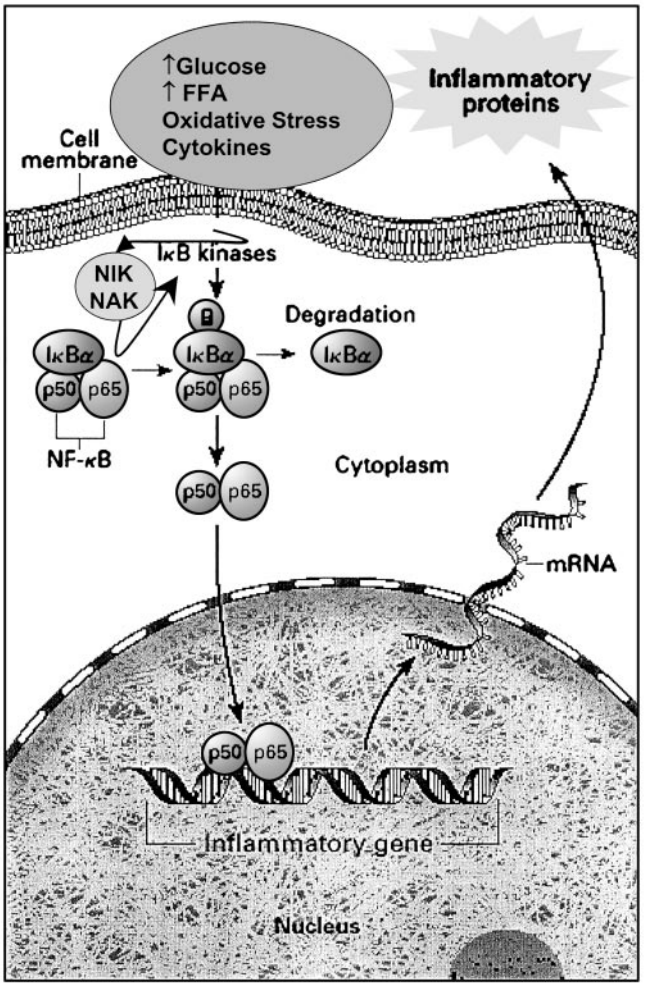

FIG. 6. Model of NF-κB by hyperglycemia, FFA, and cytokines. See text for details of events leading to NF-κB activation. NIK (83) and NAK (87) are serine kinases that function as IKKβ kinases. [Adapted and updated with permission from P. J. Barnes and M. Karin: *N Engl J Med* 336:1066–1071, 1997 (79). © Massachusetts Medical Society, 2001. All rights reserved.]

of oxidative stress-mediated cellular damage, which further exacerbates the condition of increased oxidative stress.

E. Decreased levels of antioxidants in diabetes and prevention of NF-κB activation by antioxidants

In addition to an increase in ROS, a decrease in antioxidant capacity occurs in diabetes mellitus (36, 46–48, 100). A decline in important cellular antioxidant defense mechanisms, including the glutathione redox system, vitamin C-vitamin E cycle, and the LA/dihydrolipoic acid (DHLA) redox pair (Figs. 2 and 5), significantly increases susceptibility to oxidative stress. Thus, attempts have been made to reduce oxidative stress-dependent cellular changes in patients with diabetes by supplementation with naturally occurring antioxidants, especially vitamin E (54, 101, 102), vitamin C, and LA. Oral vitamin E treatment appears to be effective in normalizing abnormalities in retinal hemodynamics and improving renal function in patients with type 1 diabetes of short disease duration (Ref. 54 and Fig. 7). Vitamin E was

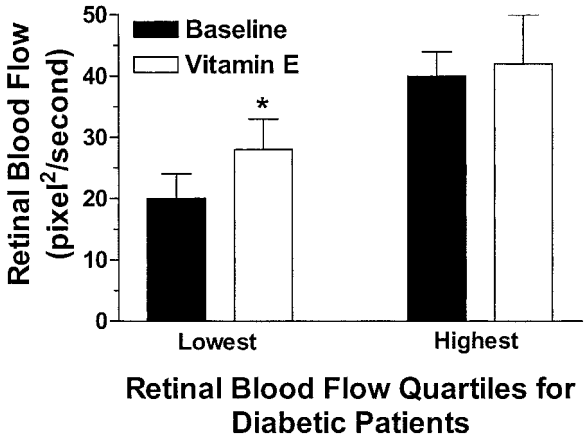

FIG. 7. Vitamin E treatment increases retinal blood flow patients with diabetes. An 8-month, randomized, double-blind, placebo-controlled crossover trial evaluated 36 patients with type 1 diabetes and 9 nondiabetic subjects. Subjects were randomly assigned to either 1800 IU vitamin E/d or placebo for 4 months and followed, after treatment crossover, for an additional 4 months. Retinal blood flow was measured at baseline and at months 4 and 8 using video fluorescein angiography. *, $P < 0.003$ (compared with baseline). [Derived from Ref. 54.]

beneficial in those individuals with the poorest glycemic control and the most impaired retinal blood flow (Ref. 54 and Fig. 7). These data suggest that vitamin E and perhaps supplementation with other antioxidants may provide an additional benefit in the treatment of either diabetic retinopathy or nephropathy.

In patients with diabetes, LA levels are reduced (48, 74, 103). LA has long been used for the treatment of diabetic neuropathy in Germany (56), and recent evidence indicates that it increases insulin sensitivity in patients with type 2 diabetes (104–106). LA is a naturally occurring antioxidant and cofactor in the pyruvate dehydrogenase complex and participates in establishing a cellular antioxidant network by raising intracellular glutathione levels (Ref. 107 and Fig. 5). LA has been shown to 1) quench free radicals, 2) prevent singlet oxygen-induced DNA damage, 3) exhibit chelating activity, 4) reduce lipid peroxidation, 5) increase intracellular glutathione levels, and 6) prevent glycation of serum albumin (73, 74). LA is able to reduce oxidative stress-mediated NF-κB activation *in vitro* (74, 108, 109) and in patients with type 2 diabetes (98, 99).

Activation of NF-κB can also be blocked by several other thiol-containing antioxidants including *N*-acetyl-L-cysteine (NAC) (110–112), a positively charged analog of LA with increased potency (113), and the glutathione precursor L-2-oxothiazolidine-4-carboxylic acid (114). Other clinically available antioxidants reported to have antiinflammatory, antioncogenic, and/or antiatherogenic properties that have been shown to block the activation of NF-κB include resveratrol (115, 116), (-)-epicatechin-3-gallate (117), pycnogenol (118), silymarin (119), and curcumin (120). IRFI-042, a novel vitamin E analog, inhibited the activation of NF-κB and reduced the inflammatory response in myocardial ischemia-reperfusion injury (121). α-Phenyl-tert-butylnitrone (PBN), a "spin-trapping" agent that reacts with and stabilizes free radical species (122–125), significantly reduced the severity of hyperglycemia in both alloxan- and streptozotocin-induced diabetes coincident with inhibiting both alloxan- and streptozotocin-induced activation of NF-κB (126). Inhibiting the activation of NF-κB prevents the activation and the transcription of genes under NF-κB control, including VEGF and others (127–129). An important goal of future studies in this area will be the determination of which antioxidants are the most effective at preventing NF-κB activation, along with the identification of the molecular site(s) of their action.

F. VEGF: an initiator of diabetic complications?

VEGF is an endothelial-cell-specific mitogen that plays a specific and critical role in the process of blood vessel formation (angiogenesis) (130–133). The development of a vascular supply is essential for organogenesis *in utero*, and for wound healing and reproductive functions in adults (130). Angiogenesis is also implicated in the pathogenesis of a variety of disorders including the growth and metastasis of solid tumors, retinopathy, age-related macular degeneration, and others (131, 132). Although the process of angiogenesis is complex and dependent upon a variety of growth factors and other components, the critical importance of VEGF and its interaction with its cognate tyrosine kinase receptor (VEGFR-2, KDR/Flk-1) in regulating vessel formation has been well established (130–133).

VEGF has been identified as a primary initiator of proliferative diabetic retinopathy and as a potential mediator of nonproliferative retinopathy (134–138). VEGF has also been implicated in the development of nephropathy and neuropathy in patients with diabetes (134, 139). VEGF serum concentrations were significantly higher in children with type 1 diabetes and markedly increased in adolescents and young adults with microvascular complications compared with healthy controls and diabetic patients without retinopathy or nephropathy (140). In adults with type 1 diabetes, plasma VEGF was significantly higher in patients with nephropathy compared with normoalbuminoric diabetics (141). Plasma VEGF was significantly increased in patients with type 1 diabetes exhibiting no clinical signs of vascular disease, suggesting that increased circulating VEGF might serve as an early indicator for the eventual development of microvascular complications (142). In light of the important role played by VEGF in the etiology of several complications of diabetes, the identification of safe and effective approaches to mitigate its production and/or action potentially would have significant therapeutic importance.

G. Antioxidants inhibit VEGF production

VEGF production is stimulated by hypoxia, hyperglycemia, AGE, and activation of stress-sensitive pathways including NF-κB, p38 MAPK, and JNK/SAPK (143–152). However, only a limited number of studies have evaluated whether antioxidants provide protection against hyperglycemia- or stress-induced VEGF production. Antioxidants inhibited VEGF expression induced by AGE in retinal vascular endothelial cells (146), and the thiol-containing antioxidant NAC inhibited VEGF production stimulated by H_2O_2 in endothelial cells (148) and in three human melanoma cell lines

(129). Several groups (153, 154) have shown that hypoxia stimulates the activation of NF-κB (a positive regulator of VEGF expression), and that mitochondrial ROS are required for this effect. Rotenone (an inhibitor of mitochondrial complex I), NAC, and pyrrolidinedithiocarbamic acid (an antioxidant) abolished the hypoxia-stimulated increase in ROS production, activation of NF-κB, and VEGF production (153). In light of the ability of VEGF to be induced by hyperglycemia and stress, it is likely that this area of research will receive increasing attention.

H. JNK/SAPK and p38 MAPK pathways: other primary targets for activation by hyperglycemia, ROS, and inflammatory cytokines

The JNK (also referred to as SAPK) and p38 MAPKs are members of the complex superfamily of MAP serine/threonine protein kinases. This superfamily also includes the ERKs (155). In contrast to ERKs (also referred to as MAPKs), which are typically activated by mitogens, JNK/SAPK and p38 MAPK are known as stress-activated kinases. This can be attributed to the fact that the activities of these enzymes are stimulated by a variety of exogenous and endogenous stress-inducing stimuli including hyperglycemia, ROS, oxidative stress, osmotic stress, proinflammatory cytokines, heat shock, and UV irradiation (Ref. 156 and Fig. 8).

Activated JNK/SAPKs bind to and phosphorylate the transcription factor *cJun*, which is one component of the activator protein (AP)-1 transcription factor complex (along with other members of the *cFos* and *cJun* families). Transactivation of *cJun* by JNK/SAPKs enhances the expression of genes with AP-1 recognition sites including *cJun*, thereby initiating a positive feedback loop (76). The redox regulation of AP-1 has been studied extensively and serves as a model for the redox regulation of other transcription factors including NF-κB and activating transcription factor-2. A closely related member of this family of transcription factors is AP-2. This transcription factor is activated by inflammatory cytokines and prostaglandins in cultured mesangial cells (157), and its DNA-binding activity *in vitro* is redox sensitive (158). Activation of AP-2 is associated with decreased expression of SOD2, a major antioxidant enzyme (159).

The most familiar function attributed to the JNK/SAPK pathway is its role as a mediator of apoptosis (160). Blockade of the JNK/SAPK pathway by expression of dominant negative *cJun* increases cell survival, an effect that can also be achieved by treatment with the thiol antioxidant and redox regulator, NAC (161, 162). JNK/SAPK is activated by hyperglycemia-induced oxidative stress and is likely involved in apoptosis mediated by hyperglycemia in human endothelial cells (163). Interestingly, H_2O_2 generation, JNK/SAPK

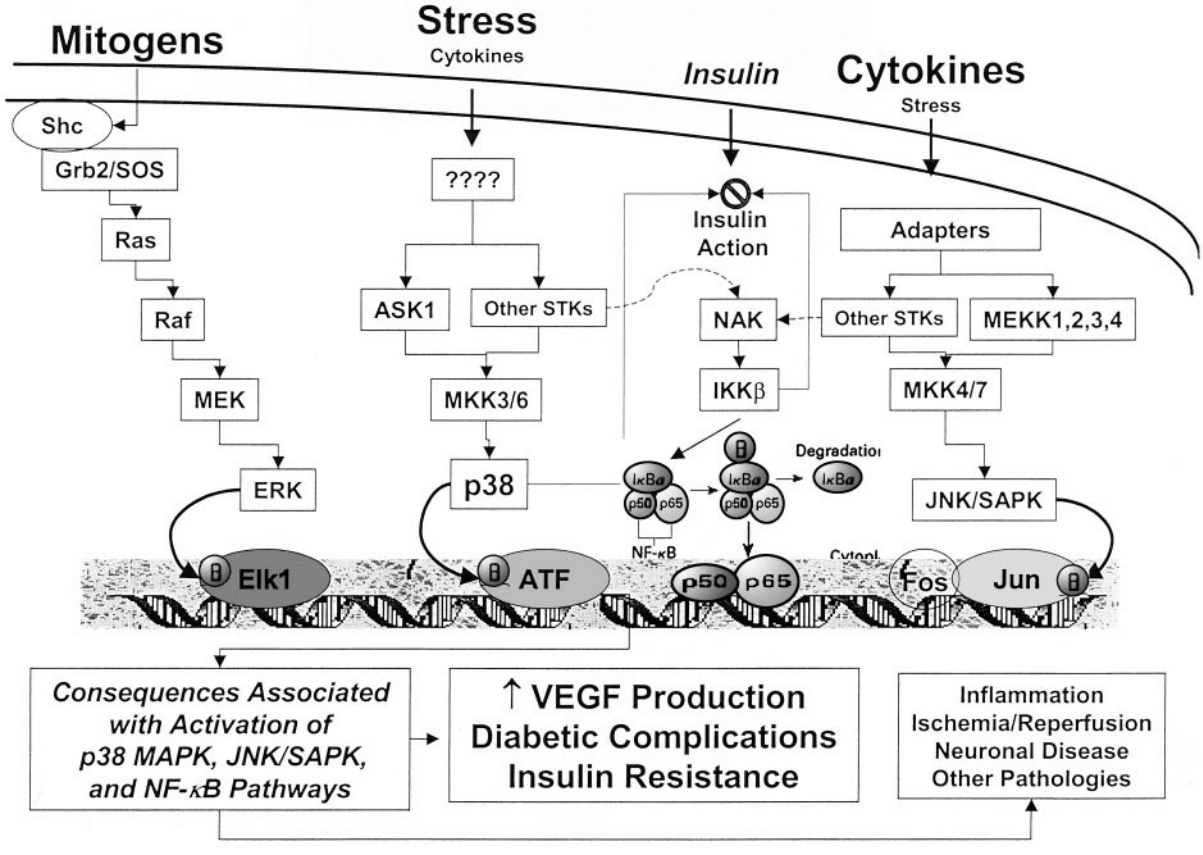

FIG. 8. Overview of the MAPK superfamily. The p38 MAPK and JNK/SAPK are contained within the larger MAPK (also called ERK) superfamily (155, 156, 167, 409). Activation of the p38 MAPK pathway results in a variety of cellular changes in transcription, many of which are mediated through the activation of activating transcription factor (ATF)-2. Significant cross-talk and synergism exist especially between the p38 MAPK and JNK/SAPK pathways. In addition, recent data indicate a negative impact on the insulin signaling pathway by p38 MAPK (249) and JNK/SAPK (237).

activity, and subsequent apoptosis induced by hyperglycemia could be suppressed by the antioxidant vitamin C (163). Another study confirmed the activation of JNK/SAPK by hyperglycemia and reported that this effect was enhanced by angiotensin II (164) and by the products of the lipoxygenase pathway in RIN m5F cells (165). A recent study (166) has found that the induction of gene 33/Mig-6, a transcriptionally inducible adaptor protein frequently associated with pathological conditions of chronic stress including diabetic nephropathy, requires JNK/SAPK. Furthermore, transient expression of this stress protein results in the selective activation of JNK/SAPK, suggesting the existence of a reciprocal positive feedback loop. Thus, induction of this protein by JNK/SAPK could serve as a potential marker for pathologies associated with chronic oxidative stress.

Activation of p38 MAPK also influences a large number of cellular processes including inflammation and immunity, cell growth and apoptosis, and tissue-specific responses to stress by regulating gene expression, other signaling pathways (*e.g.*, NF-κB, insulin, cytokine, arachidonate, and others), and cytoskeletal rearrangement. In addition, p38 MAPK rapidly regulates other serine kinases (155). Chronic activation of the p38 MAPK pathway is often associated with disease pathology, including inflammation, ischemia/reperfusion injury, infectious disease, and neuronal disease (167). In this regard, selective p38 MAPK inhibitors are in clinical development as antiinflammatory agents (168–170).

p38 MAPK is activated in response to hyperglycemia and in diabetes. In vascular smooth muscle cells, treatment with insulin (100 nM) and hyperglycemia (25 mM) for 12–24 h induced the activation of p38 MAPK. This was associated with a marked impairment in inducible nitric oxide (NO) synthase induction upon subsequent acute exposure to insulin (171). In rat aortic smooth muscle cells, glucose (16.5 mM) caused a 4-fold increase in p38 MAPK (172). In glomeruli of rats made diabetic by streptozotocin, p38 MAPK activity was increased compared with controls, followed by increased phosphorylation of heat shock protein 25, a downstream substrate of p38 MAPK (173). These effects appeared to be the result of increased ROS production. Taken together, these recent data suggest that the NF-κB, JNK/SAPK, and p38 MAPK pathways are candidate stress-sensitive signaling systems that can chronically lead to the late complications of diabetes.

I. Additional important hyperglycemia-activated pathways

In addition to the stress-sensitive pathways discussed above, hyperglycemia activates several other well-characterized biochemical pathways that play a significant role in the development of diabetic complications. In each case, activation of these pathways appears to be linked to a hyperglycemia-mediated rise in ROS production and consequent increase in oxidative stress (51, 62).

a. AGE/RAGE pathway. AGE describes a heterogeneous group of proteins, lipids, and nucleic acids that are formed nonenzymatically (174, 175). AGE formation is enhanced in the presence of hyperglycemia and oxidative stress (176, 177). AGE bind to their cognate cell-surface receptor, RAGE, resulting in the activation of postreceptor signaling, generation of intracellular oxygen free radicals, and the activation of gene expression (175, 178–184). Retinal expression of VEGF, a mediator of the late complications of diabetes (134, 139), is increased by AGE-RAGE interaction (146). Thus, AGE are not only markers, but act also as mediators of late diabetic complications and chronic vascular diseases.

b. PKC pathway. In tissues in which diabetic complications develop, the concentration of diacylglycerol, an allosteric activator of PKC, is increased (52). As a consequence of the increase in diacylglycerol, several isoforms of PKC are activated. PKC-β is the major isoform that is induced in the vasculature, kidney, and retina (52). Increased PKC activity arises from chronic hyperglycemia and is associated with many processes involved in the pathology of diabetic complications including the regulation of vascular permeability, blood flow, and neovascularization. The significance of the activation of the PKC pathway as a major cause of diabetic complications is strongly supported by the ability of a specific synthetic inhibitor of PKC-β to ameliorate abnormal retina and renal hemodynamics in diabetic rats (55). Furthermore, activation of the PKC pathway by hyperglycemia synergizes with other kinase pathways. For example, in mesangial cells, hyperglycemia led to a PKC-dependent enhancement of the activation of MAPK by the vasoactive peptide endothelial-1 (185). Interactions between these pathways and perhaps other stress-activated pathways are likely to play an important role in determining the long-term effects of hyperglycemia.

c. Polyol pathway. When intracellular glucose rises, aldose reductase activity is stimulated and catalyzes the formation of sorbitol, which can be oxidized to fructose by sorbitol dehydrogenase (186). Sorbitol accumulates intracellularly, causing cell damage. Furthermore, stress-sensitive signaling pathways including p38 MAPK and JNK are strongly activated by sorbitol. The significance of the activation of the polyol pathway as a cause of diabetic complications has been demonstrated in transgenic mice that overexpress the aldose reductase gene (187–190), and by the observations that inhibitors of this enzyme prevent the development of neuropathy, nephropathy, retinopathy, and cataract formation in these animals (191).

d. Hexosamine pathway. Several lines of evidence have established that the excessive flux of glucose or FFA into a variety of cell types results in the activation of the hexosamine biosynthetic pathway (192–196). It has been proposed that the activation of this pathway leads to insulin resistance and the development of late complications of diabetes (192–197). Transgenic mice that overexpress glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme of hexosamine biosynthesis, are insulin resistant (194, 198). Overexpression of GFAT in the liver of transgenic mice shifts their phenotype toward energy storage, resulting in hyperlipidemia and obesity (199). In mesangial cells, overexpression of GFAT increased NF-κB-dependent promoter activation (200). The hexosamine pathway also functions as a cellular "sensor" of energy availability and mediates the effects of glucose on the expression of several gene products

including leptin (201–203). Recent data have implicated the activation of the hexosamine pathway by hyperglycemia-induced increase in ROS formation. In bovine endothelial cells, hyperglycemia induced a significant increase in the hexosamine pathway (204), which was blocked by an inhibitor of electron transport, a mitochondrial uncoupling agent (CCCP), and the expression of either UCP1 or SOD2 (204).

J. ROS generation by enzymatic pathways of arachidonic/linoleic acid metabolism

The formation of superoxide and other ROS is not only a consequence of hyperglycemia, but is also a product of certain enzymes that utilize molecular oxygen for catalysis including cyclooxygenases and lipoxygenases (Fig. 4). Studies have established that the leukocyte type 12-lipoxygenase is activated by growth factors, inflammatory cytokines, and hyperglycemia (reviewed in Ref. 53). Several oxygenated products of this important enzyme are able to activate growth and stress-sensitive kinases (205) and signaling pathways linked to increased vascular and renal disease, including PKC, vascular smooth muscle cell hypertrophy, increased matrix production, and oncogene activation (206–208). Furthermore, 12(R)-hydroxyeicosatetraenoic acid, a product of the 12-lipoxygenase enzyme, is an extremely potent angiogenic agent (209) and is able to activate NF-κB and increase the expression on VEGF (144, 209). In addition, the superoxide anion can interact with NO, forming toxic free radicals called peroxynitrites (Table 1). These RNS impair the ability of NO to maintain vascular tone and could promote or accelerate the atherosclerotic process (210–212). In this context, numerous studies have reported the clinical benefit of antioxidants in improving vascular tone (213–217).

IV. Oxidative Stress and Insulin Resistance

Oxidative stress is not only associated with complications of diabetes, but has been linked to insulin resistance *in vivo* (defined as a subnormal response to a given amount of insulin) (33, 218–221). *In vivo*, studies in animal models of diabetes indicate that antioxidants, especially LA, improve insulin sensitivity. Several clinical trials have also demonstrated improved insulin sensitivity in insulin-resistant and/or diabetic patients treated with the antioxidants vitamin C, LA, vitamin E, and glutathione (104, 222–225).

In patients with type 2 diabetes, both acute and chronic administration of LA improves insulin resistance as measured by both the euglycemic-hyperinsulinemic clamp and the Bergman minimal model (Refs. 104, 105, 226, and 227 and Fig. 9). In addition, the short-term (6 wk) oral administration of a novel controlled release formulation of LA lowered plasma fructosamine levels in patients with type 2 diabetes (228).

A. Activation of stress-kinases, IRS phosphorylation, and insulin resistance

Oxidative stress leads to the activation of multiple serine kinase cascades (229–231). There are a number of potential targets of these kinases in the insulin signaling pathway,

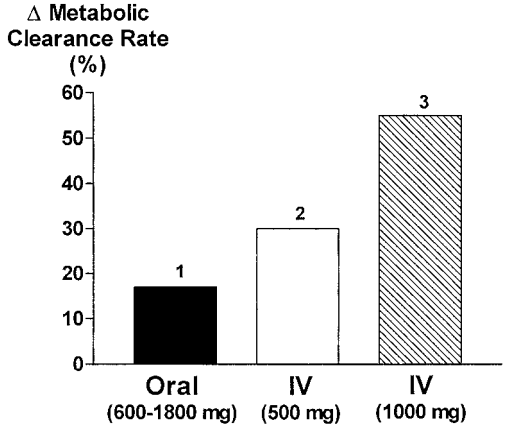

FIG. 9. LA increases insulin-stimulated glucose metabolism in patients with type 2 diabetes. Intravenous (IV) administration of LA is able to significantly increase insulin sensitivity [as judged by percent change (Δ) in metabolic clearance rate (MCR)] in patients with type 2 diabetes, whereas oral administration exerts a lesser effect. 1) Seventeen-percent increase in MCR ($P < 0.05$, data replotted from Ref. 104); 2) 30% increase in MCR ($P < 0.05$, data replotted from Ref. 227); 3) 55% increase in MCR ($P < 0.05$, data replotted from Ref. 226). [Reprinted with permission from J. L. Evans and I. D. Goldfine: *Diabetes Technol Ther* 2:401–413, 2000 (106).]

including the insulin receptor (IR) and the insulin receptor substrate (IRS) family of proteins. Increased phosphorylation of the IR or IRS on discrete serine or threonine sites decreases the extent of their tyrosine phosphorylation, and is consistent with impaired insulin action (232–237). The serine/threonine phosphorylated forms of IRS molecules are less able to associate with the IR and downstream target molecules, especially phosphatidylinositol 3-kinase (232, 238), resulting in impaired insulin action including protein kinase B activation, and glucose transport (239–241).

In 3T3-L1 adipocytes, induction of oxidative stress with H_2O_2 inhibits insulin-stimulated glucose transport (242–244). This effect is selective for insulin-stimulated signaling compared with platelet-derived growth factor-stimulated signaling (245) and was reversed by preincubation with the antioxidant LA (243). We have made similar observations using rat L6GLUT4 muscle cells (246) and have found that the protective effects of LA were associated with its ability to prevent the H_2O_2-induced decrease in the intracellular level of glutathione (247). Others (248) have recently reported the direct protective effect of glutathione on insulin action in HTC rat hepatoma cells transfected with the IR. After acute exposure to H_2O_2, we find that the NF-κB and p38 MAPK pathways are markedly activated and that their activation can be blocked by pretreatment with LA (Fig. 10).

To determine whether the protective effects of LA could also be observed under more physiological conditions, we have used hyperglycemia to induce oxidative stress and blunt the effects of insulin. Incubation of L6GLUT4-IR cells (L6 cells in which both GLUT4 and the IR were transfected) with 20 mM glucose caused a marked decrease in insulin-stimulated glucose transport ($P < 0.001$; Fig. 11). Coincubation with LA (100 μM) completely protected against the hyperglycemia-induced insulin resistance (Fig. 11).

In L6 muscle cells, activation of p38 MAPK by oxidative stress (H_2O_2) is linked to H_2O_2-mediated inhibition of

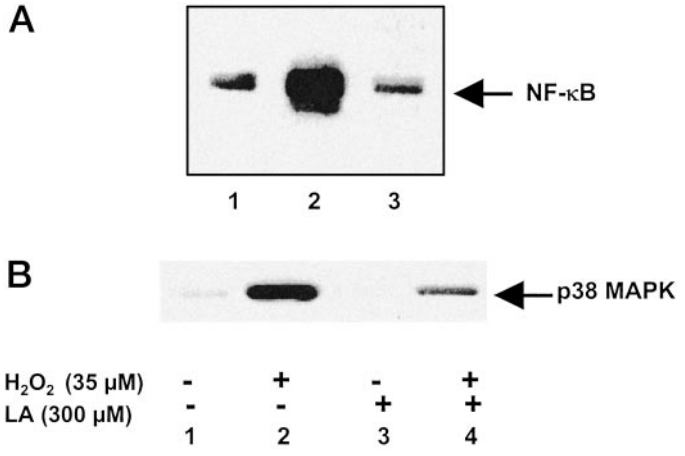

FIG. 10. Activation of NF-κB and p38 MAPK in L6 cells is blocked by LA. A, L6 muscle cells were incubated with the H_2O_2-generating system followed by measurement of NF-κB activation. Cells were treated for 30 min in the absence (lane 1) and presence of glucose oxidase (100 mU/ml) and glucose (5 mM, lanes 2 and 3). In lane 3, cells were preincubated for 18 h with LA (100 μM). NF-κB (p50 subunit) was measured by gel shift analysis. H_2O_2 treatment increased the binding of the p50 subunit of NF-κB (lane 2). This effect was blocked by preincubation with LA (lane 3). B, Cells were preincubated (as described above) in the absence (lanes 1 and 2) and presence of LA (lanes 3 and 4). Next, cells were washed and glucose oxidase (100 mU/ml) and glucose (5 mM) were added (lanes 2 and 4). Cells were solubilized, loaded on Tris-glycine gels, and filters were probed with anti-phospho-p38 MAPK antibody. H_2O_2 caused a marked activation of p38 MAPK, as judged by the increase in p38 MAPK phosphorylation (compare lanes 1 and 2). In the absence of H_2O_2, LA had no discernible effect on p38 MAPK phosphorylation (compare lanes 1 and 3). However, preincubation of cells with LA produced a substantial decrease in H_2O_2-induced p38 MAPK phosphorylation (compare lanes 2 and 4). Similar results have been obtained in other cells types, including nerve and endothelial cells, and in response to hyperglycemia-induced oxidative stress (data not shown).

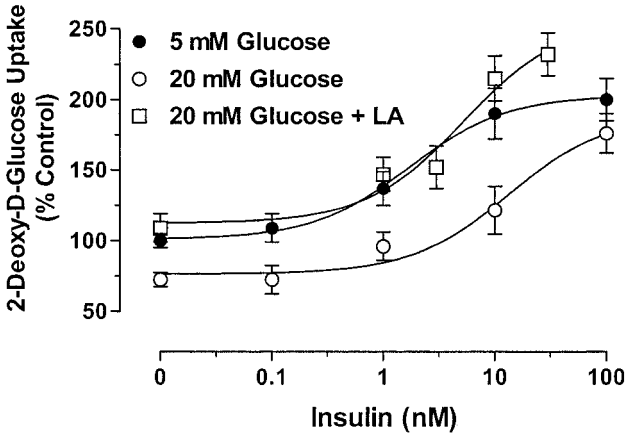

FIG. 11. Protective effect of LA on hyperglycemia-induced suppression of insulin-stimulated glucose transport. L6GLUT4 muscle cells [obtained from Dr. John Lawrence, Jr., University of Virginia, Charlottesville, VA (246)] were stably transfected to express the human IR (designated as L6GLUT4-IR cells). L6GLUT4-IR cells were cultured for 5 d in DMEM containing 5 mM glucose, 20 mM glucose, or 20 mM glucose plus LA (100 μM; LA). Cells were washed, incubated with increasing concentrations of insulin for 30 min, and 2-deoxy-D-glucose uptake was measured as described previously (247). Data points represent means ± SEM for three separate experiments using triplicate incubations ($P < 0.001$, 5 mM *vs.* 20 mM; ANOVA followed by Dunnett's post test; 5 mM *vs.* 20 mM + LA, not significant).

insulin-stimulated glucose transport (249). Inhibition of insulin signaling was reversed by a specific inhibitor of p38 MAPK (249). Interestingly, p38 MAPK has been suggested as an activator of the glucose transporter (250, 251). Due to the existence of multiple isoforms of this enzyme (156, 167), it is possible that this latter effect is mediated by a different isoform. In addition, both TNF-α and anisomycin (strong activators of JNK/SAPK) stimulate IRS-1-associated JNK/SAPK activity, resulting in increased serine phosphorylation of IRS-1 catalyzed by JNK/SAPK (237, 252). Consequently, insulin-stimulated tyrosine phosphorylation of IRS-1 was substantially reduced and insulin action was impaired.

B. IKKβ, IRS proteins, and insulin resistance

Recently, it has been reported that IKKβ, which activates NF-κB, is increased in insulin-resistant muscle from a variety of sources (253). Activation of IKKβ inhibits insulin action; salicylates and ligands for PPARγ, both of which inhibit IKKβ activity (90, 91), restore insulin sensitivity both *in vitro* and *in vivo* (254, 255). Treatment with aspirin and salicylates alters the phosphorylation patterns of the IRS proteins, resulting in decreased serine phosphorylation and increased tyrosine phosphorylation (254, 255). Recent evidence suggests that the potent insulin sensitizing activity of adiponectin (Acrp30), the circulating protein secreted from adipocytes, may be also associated with inhibition of NF-κB activation (256–258).

Support for the importance of IKKβ in insulin resistance *in vivo* is provided by results of recent gene knockout experiments in mice. IKKβ (+/−) heterozygotes were more insulin sensitive (as judged by increased glucose infusion rate during hyperinsulinemic-euglycemic clamp) compared with their normal (+/+) littermates (254, 255). This improvement in insulin sensitivity was even more dramatic when IKKβ (+/−) mice were crossbred with insulin-resistant *ob/ob* mice. Preliminary clinical evidence implicating IKKβ in insulin resistance has also been recently provided. Treatment of nine patients with type 2 diabetes for 2 wk with high-dose aspirin (7 g/d) resulted in reduced hepatic glucose production and fasting hyperglycemia and increased insulin sensitivity (259). Taken together, these data support a role for activation of IKKβ in the pathogenesis of insulin resistance and suggest that it might be an attractive pharmacological target to increase insulin sensitivity.

Additional evidence derived from cellular models, transgenic animals, and humans demonstrates the importance of IRS proteins in the regulation of β-cell function (260–264). Accordingly, enhanced serine/threonine phosphorylation on the IR or its substrates due to increased stress-sensitive kinase activity [*e.g.*, NF-κB-activating kinases, p38 MAPK, JNK/SAPK, PKCθ, or other serine/threonine kinase(s)] could provide a mechanistic explanation to link activation of the stress pathways to multiple cellular pathologies.

C. Oxidative stress, protein tyrosine phosphatases, and insulin resistance

In conjunction with the stress-induced activation of serine kinase cascades, alteration of the intracellular redox balance

can also result in the oxidation and inactivation of protein tyrosine phosphatases (PTPases) (75, 265–267). This class of enzymes, along with dual-function phosphatases, plays a major role in regulating a variety of signaling pathways including the stress-activated pathways (268–273). It has been known for quite some time that phosphotyrosyl turnover is essential for insulin-stimulated glucose transport in adipocytes and muscle (274, 275). Although the selective and reversible inhibition of certain PTPases such as PTP-1B improves insulin action and is antidiabetogenic (276–281), oxidation of the cysteine residues required for catalytic activity inactivates PTPases and can result in insulin resistance *in vitro* (274, 275).

Thus, the activation of each pathway (NF-κB, p38 MAPK, and JNK/SAPK) is sensitive to oxidative stress. Furthermore, activation of these pathways is linked to impaired insulin action, suggesting that they might play a role in oxidative stress-induced insulin resistance. Because these same systems are also important in the development of the late diabetic complications, these data suggest a unifying hypothesis of hyperglycemia-induced oxidative stress causing both insulin resistance and late diabetic complications.

D. Obesity, fatty acids, and insulin resistance

Insulin resistance in obesity is evident before the development of chronic hyperglycemia (1, 23). Therefore, it is unlikely that insulin resistance, at the prediabetic stage, results from oxidative stress triggered by hyperglycemia *per se*. However, the strong association of obesity and insulin resistance (282–284) suggests that a major mediator of oxidative stress-induced insulin resistance at the prediabetic stage might be a circulating factor secreted by adipocytes. In this regard, several possible candidate molecules have been suggested including TNF-α (285–287), leptin (288, 289), FFA (290–295), and most recently, resistin (296). However, the evidence is strongest that FFA are the most likely link between obesity and insulin resistance (292, 297–299).

Plasma FFA content is increased in many states of insulin resistance including obesity and type 2 diabetes (291, 293, 300–302). There is an inverse relationship between fasting plasma FFA concentrations and insulin sensitivity (303). There is an even stronger relationship between the accumulation of intramyocellular triglyceride and insulin resistance (304–312). Although the cause for this overaccumulation of lipid is unknown, McGarry and Dobbins (298) have postulated the importance of malonyl-coenzyme A (CoA) metabolism. Malonyl-CoA, the first committed intermediate in fatty acid biosynthesis and an inhibitor of carnitine palmitoyl transferase 1, plays a major role in regulating fatty acid synthesis and oxidation (313). Thus, dysregulation of malonyl-CoA production, if it leads to sustained increases in intracellular concentrations of malonyl-CoA and FFA, would result in reduced capacity to oxidize fat, leading to increased tissue stores, and could play a key role in the pathogenesis of insulin resistance and impaired β-cell function. Taken together, these data implicate FFA as a causative link between obesity, insulin resistance, and development of type 2 diabetes (298, 314, 315).

E. Fatty acids and insulin resistance

Several explanations have been offered to account for how elevated FFA could result in insulin resistance. The glucose-fatty acid cycle (Randle hypothesis) was the first to be widely accepted (290, 316, 317). Randle reasoned that the increased availability of FFA would cause an increase in the ratios of mitochondrial acetyl-CoA:CoA and reduced nicotinamide adenine dinucleotide:nicotinamide adenine dinucleotide$^+$, resulting in: 1) inactivation of the pyruvate dehydrogenase complex, 2) reduced glucose oxidation and increased intracellular citrate, 3) inhibition of phosphofructokinase, 4) accumulation of glucose-6-phosphate, and ultimately 5) inhibition of hexokinase II activity. The net result would be an accumulation of intracellular glucose and the concomitant decrease in muscle glucose uptake.

However, in contrast to the Randle hypothesis, which predicts that increased FFA availability would lead to an increase in im glucose-6-phosphate, recent studies have indicated that the decrease in muscle glycogen synthesis in healthy subjects caused by fat infusion was preceded by a reduction in im glucose-6-phosphate levels (318). FFA leads to a decrease in the intracellular concentration of glucose. These results provide the basis for implicating the glucose transport system (as opposed to hexokinase II or other intracellular sites) as the rate-controlling step for fatty acid-induced insulin resistance (297).

At the molecular level, FFA infusion resulted in decreased insulin-stimulated IRS-1 tyrosine phosphorylation along with decreased IRS-1-associated phosphatidylinositol 3-kinase activity in muscle biopsy samples (Refs. 318, 319, and reviewed in Ref. 320). In rats, infusion of FFA was associated with the activation of PKCθ (236), a Ca^+-independent isoform of the PKC family that is selectively expressed in skeletal muscle and T lymphocytes (321, 322). Thus, one characteristic of FFA-induced insulin resistance is that FFA or their metabolites (ceramides, diacylglycerol, fatty acyl-CoAs) activate PKCθ, NF-κB-activating kinases, p38 MAPK, JNK/SAPK, or other serine/threonine kinase(s), leading to enhanced serine/threonine phosphorylation on the IR or its substrates. As discussed above, increased serine phosphorylation of IRS impairs insulin action.

F. Fatty acids, redox balance, and activation of stress pathways

In addition to the ability of FFA or their metabolites to impair insulin action by stimulating inhibitory protein kinase activity, FFA could impair insulin action by increasing the level of oxidative stress. Indeed, increased oxidative stress might provide a mechanistic basis for the observed FFA (or metabolite)-induced increase in serine kinase activity discussed above (230, 231).

In support of this idea, evidence *in vitro* indicates that elevated FFA have numerous adverse effects on mitochondrial function including the uncoupling of oxidative phosphorylation (19, 20), and the generation of reactive oxygen species including $^{\bullet}O_2^-$ (315). This latter situation is exacerbated because FFA not only induce a state of oxidative stress, but also impair the endogenous antioxidant defenses by decreasing intracellular glutathione (323, 324). As a likely con-

sequence of their ability to increase ROS formation and deplete glutathione, FFA are able to activate NF-κB (324–330). This latter effect might be linked to FFA-mediated activation of PKCθ (236), which has the unique ability among the PKC isoforms to activate NF-κB (331). As discussed above, activation of this stress-sensitive pathway results in the expression of genes known to be associated with impaired insulin action along with the complications of diabetes. FFA-induced activation of NF-κB can be prevented by pretreatment with vitamin E (324) and other antioxidants (332), suggesting that the alteration in cellular redox status is a contributory component of the proinflammatory effects of FFA. It should also be noted that FFAs and many of their derivatives interact directly with transcription factors to regulate gene expression (333).

In patients with type 2 diabetes, there is a significant inverse correlation between fasting plasma FFA concentration and the ratio of reduced/oxidized glutathione (a major endogenous antioxidant) (219). In healthy subjects, infusion of FFA (as 10% Intralipid) causes increased oxidative stress as judged by increased malondialdehyde levels and a decline in the plasma reduced/oxidized glutathione ratio (219). Malondialdehyde, a highly toxic by-product generated in part by lipid oxidation and ROS, is increased in diabetes mellitus (334). Similarly, infusion of FFA in healthy subjects caused a time- and dose-dependent increase in plasma thiobarbituric acid-reactive substance, coincident with an inhibition of insulin-stimulated glucose disposal (335). In both healthy individuals and in subjects with type 2 diabetes, restoration of redox balance by infusing glutathione improves insulin sensitivity along with β-cell function (225, 335).

Taken together, these studies suggest that activation of the NF-κB signaling pathway, and perhaps other stress-sensitive pathways, plays a role in FFA-induced insulin resistance. Because this same signaling pathway also plays a role in diabetic complications, these studies suggest a unifying hypothesis of FFA- and hyperglycemia-induced oxidative stress causing both insulin resistance and late diabetic complications. Moreover, the induction of insulin resistance by FFA-induced oxidative stress may serve as an early marker of late diabetic complications.

V. Oxidative Stress and β-Cell Dysfunction

The β-cell is particularly susceptible to the damages inflicted by oxidative stress. Through the concerted efforts of GLUT2 (the high K_m glucose transporter) (336–339), glucokinase (the glucose sensor) (340–343), and glucose metabolism, β-cells are responsible for sensing and secreting the appropriate amount of insulin in response to a glucose stimulus (344). Although this process involves a complex series of events, mitochondrial metabolism is crucial in linking stimulus to secretion (344–347). As discussed earlier, mitochondria are both free radical generators and their unwitting targets. Therefore, the ability of ROS and RNS to damage mitochondria and significantly attenuate insulin secretion is not surprising (348, 349). The following sections discuss the impact of physiological inducers of oxidative stress including hyperglycemia, FFA, and their combination on β-cell function.

Many studies have reported that β-cell dysfunction is the result of 1) chronic exposure to hyperglycemia, 2) chronic exposure to FFA, and 3) a combination of chronic hyperglycemia and FFA. Furthermore, these effects appear to be dependent upon the oxidative stress induction of the NF-κB and additional stress-sensitive targets (350–352). There is some evidence that activation of NF-κB is mostly a proapoptotic event in β-cells (353). There is considerable evidence that chronic hyperglycemia in patients with type 2 diabetes contributes to impaired β-cell function (5, 354). However, evidence for a direct toxic effect of glucose *in vitro* has been conflicting. This conflicting evidence is due, in large part, to the definition of toxicity along with differences, sometimes subtle, in experimental design. Moreover, recent data suggest that the combined effects of elevations in glucose and FFA, acting by the generation of ROS, may be particularly toxic (reviewed in Ref. 355).

A. β-Cell glucose-induced toxicity

In humans with type 2 diabetes, reducing hyperglycemia with either diet, insulin, sulfonylureas, or pioglitazone results in improved insulin secretion (reviewed in Ref. 5; also see Refs. 356 and 357). Conversely, in healthy subjects, glucose infusion reduces insulin release, an effect that requires 3 d of treatment with very high glucose (12.6 mM) (356). *In vivo*, β-cell exhaustion and/or toxicity caused by chronic, elevated glucose levels has been studied in both animal models of diabetes in which hyperglycemia resulted from genetic abnormalities (reviewed in Ref. 354; also see Ref. 358) and from manipulation of normal animals, *e.g.*, glucose infusion, partial pancreatectomy, or neonatal streptozotocin (reviewed in Ref. 354; also see Refs. 358 and 359). In these *in vivo* studies, dissociation of the unique effects of hyperglycemia from those caused by concurrent neurological, endocrinological, and nutritional factors (especially lipids) has been complicated. Moreover, high glucose *in vivo* also reduces hepatic insulin removal, so that insulin, normally measured in the circulation might have been unchanged, despite decreased insulin secretion (356).

In vitro, a deleterious effect of chronic high glucose on β-cell function is difficult to demonstrate in normal cells from animals with no genetic susceptibility to diabetes (5, 358–360). However, six-month culture of either HIT-T15 or βTC-6 cells with elevated glucose did decrease insulin release, insulin mRNA, and binding of insulin mRNA transcription factors (361, 362). As can be seen from the data in Fig. 12, chronic culture of HIT-T15 cells in medium containing 11.1 mM glucose in the presence of the antioxidants, NAC, or aminoguanidine (AG) markedly prevents glucotoxic effects on insulin gene activity (Ref. 363 and Fig. 12). In this same study, antioxidants partially prevented glucose-induced decreases in insulin mRNA, DNA binding of pancreas/duodenum homeobox-1, insulin content, and glucose-stimulated insulin secretion.

Attempts to demonstrate a direct inhibitory effect of chronic hyperglycemia on the actual insulin secretory mechanism of normal pancreas or in islets have been difficult (5, 359). It is possible that exposure to high glucose alone for limited periods is only weakly toxic and, with time, could

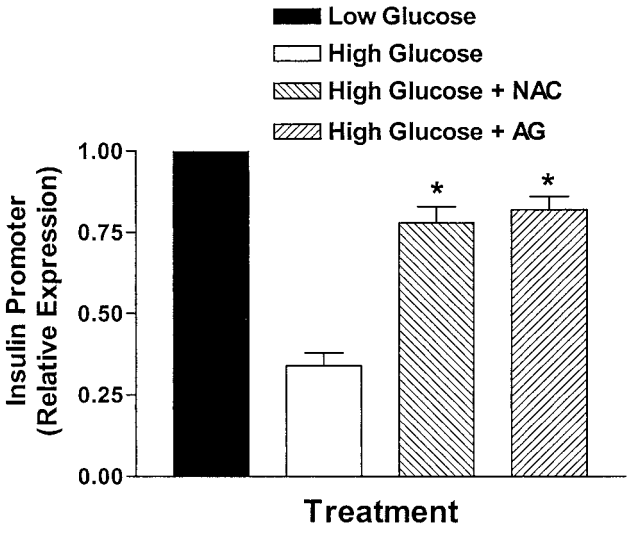

FIG. 12. Hyperglycemia-induced suppression of insulin gene activity and inhibitory effects of antioxidants. HIT-T15 cells [a clonal line of pancreatic islet β-cells (410] were cultured in low glucose as described previously (411). Beginning at passage 74, cells were split weekly and continuously cultured in RPMI 1640 medium containing 11.1 mM glucose in the absence or presence of 500 μM NAC or 10 μM AG. At passages 106–112, a plasmid containing the human insulin gene nucleotide sequences −326 to +30 linked to the chloramphenicol acetyl-transferase reporter gene was transfected into cells chronically treated with NAC or AG, and chloramphenicol acetyl-transferase activity was measured. The relative expression of the insulin promoter in cells cultured in low glucose (passages 73–76) was normalized (set to 1) to luciferase activity obtained using pGL3LUC, a plasmid containing the pGL3 promoter, which was cotransfected. *, $P <$ 0.01 (compared with high glucose). [Derived from Ref. 363.]

stimulate compensatory mechanisms (364). Part of the inconsistency may also arise from subtle but important differences in the definition of glucose-induced exhaustion *vs.* true toxicity (365). In addition, differences in experimental designs used to examine glucose toxicity can vary in the mechanisms they actually measure (5). Thus, 1) the spontaneous decrease in insulin release from pancreas or islets occurring after 2- to 3-h glucose stimulation (third-phase secretion) (366) may reflect, at least in part, the gradual decline of endogenous potentiating factors; 2) declining release during multiple acute stimulations (367, 368) is the normal damping of factors causing first-phase release (5, 369, 370); and 3) culture in high glucose followed by a washout period and subsequent test stimulus, a common procedure (5, 359), is affected by priming (memory, time-dependent potentiation) (5, 369, 370).

Many studies have not established that a reduction in insulin secretion occurs simply because releasable stored insulin was depleted by prior exposure to high glucose. Indeed, "desensitization" in animal models with mild hyperglycemia, or islets exposed to glucose, is often characterized by an increased sensitivity to low glucose, decreased stored insulin, and a subsequent decreased response to a glucose challenge (359, 366). We believe that these data indicate a chronic hypersensitization/depletion phenomenon, which is to be distinguished from a pathological impairment of β-cell function.

The results discussed above also emphasize the absolute requirement of relating islet insulin secretion to the concurrent insulin content. However, one caveat in relating insulin secretion to total insulin content is that all stored insulin is not equally available for release (reviewed in Ref. 5). Thus, insulin is stored in spatially distinct compartments within the β-cell that differ in their availability, with granules proximal to the plasma membrane being particularly labile. In contrast, proinsulin and insulin still in the endoplasmic reticulum and Golgi, and "old" insulin in granules destined for degradation, are not available for secretion. It is also emphasized that the demonstration of a decrease in insulin mRNA might not reflect overall insulin synthetic activity, because translational synthesis is often not measured and can change in a direction opposite to the mRNA.

B. β-Cell lipid-induced toxicity

Similar to the effects of glucose, the effects of lipids on endocrine β-cells are also complex. Increased FFA concentrations enhance insulin secretion both *in vitro* and *in vivo* (reviewed in Ref. 298; also see Ref. 371), and it is speculated that accumulation of long-chain acyl-CoA esters in the cytoplasm is responsible (298, 372). *In vitro*, long-term exposure to FFA reportedly inhibits insulin mRNA and synthesis (373, 374) and partially inhibits postculture, glucose-stimulated insulin release (373, 375). However, examination of the data showing decreased secretion during a test stimulus can often be entirely accounted for by the reduced insulin content. Presumably, this was the result of unmeasured positive effects on secretion during the previous culture period (373, 375).

Increased sensitivity to low glucose after prolonged high FFA (20, 376–378) and coculture of normal islets with high FFA and moderate glucose for 1 wk causes increased secretory response during a test stimulus (Ref. 378 and reviewed in Ref. 314). Thus, culture of normal islets with FFA tends to decrease insulin mRNA and content but increases β-cell sensitivity to low glucose and has little effect on fractional secretion at high glucose (379, 380). These results suggest that, in normal tissue, the insulin-synthetic machinery is more sensitive to down-regulation than the secretory mechanism. In contrast, in other experiments, prolonged culture of β-cell preparations with FFA causes decreased mitochondrial membrane potential, increased UCP leading to the opening of K^+-sensitive ATP channels, and selective impairment of glucose-, but not K^+-, stimulated insulin secretion (381, 382). Impaired insulin secretion has been associated with an FFA-induced increase in ROS (20).

In contrast, prolonged culture of β-cell preparations from animals with a predilection for type 2 diabetes, particularly those with impaired leptin production or its receptors, clearly results in consistently demonstrable impaired secretion as well as other deleterious effects on β-cell function (reviewed in Ref. 383). Therefore, genetic defects may amplify the toxic effects of FFA that are not evident with normal insulin-secreting cells. The probability that long-term FFA may damage diabetes-prone β-cells by progressively increasing total islet triglyceride deposition is strongly suggested (298, 314, 372, 383). This, in turn, produces mitochondrial changes, impaired glucose-induced β-cell proliferation, impaired insulin secretion, and β-cell apoptosis, with the latter possibly mediated by increased islet ceramide (375, 383), subsequent activation of JNK/SAPK and other pathways,

and increased NO production (Ref. 314 and Fig. 13). Inhibitors of NO production block apoptosis *in vitro*. PPARγ, a transcription factor that regulates several enzymes catalyzing lipogenesis, may also precipitate lipotoxicity. It is elevated in islets of diabetic Zucker rats, and glucose homeostasis in these animals can be prevented or ameliorated with troglitazone, an exogenous ligand of PPARγ (383). Recent studies show that saturated, long-chain fatty acids are the most toxic (375, 384).

C. β-Cell combined glucose/lipid toxicity

Because type 2 diabetes is characterized by elevations in both glucose and FFA, it is possible that their combined presence is required to maximize β-cell toxicity. This possibility is supported by recent studies showing that when either isolated islets or HIT cells were exposed to chronic elevated FFA and glucose, there was a clear decrease in both insulin mRNA and activation of an insulin reporter-gene construct (385). In these studies, secretion was assessed only as accumulated insulin in the culture media, and changes in insulin secretion rate and fractional release were not assessed. In other studies, coculture of islets with high glucose and palmitate resulted in almost complete impairment of glucose-stimulated insulin secretion, despite partially sustained stored insulin (20). Data from Poitout, Robertson, and colleagues (355, 386, 387) have indicated that β-cell lipotoxicity is an amplifying effect that is manifested only in the context of concurrent hyperglycemia.

D. Role of oxidative stress in β-cell dysfunction

Oxidative stress has been implicated in β-cell dysfunction caused by autoimmune attack, actions of cytokines, and alloxan. It is also a very important factor in type 2 diabetes, aging, production of glycation products, and glucose- and FFA-generated toxicity (reviewed in Refs. 349, 363, and 388–391). ROS and RNS ($\dot{O}_2^-$, H_2O_2, and NO; Table 1) have all been implicated, and their negative effects on islet-cell nuclear and mitochondrial DNA, as well as GSH reductive state, have been described (363, 392).

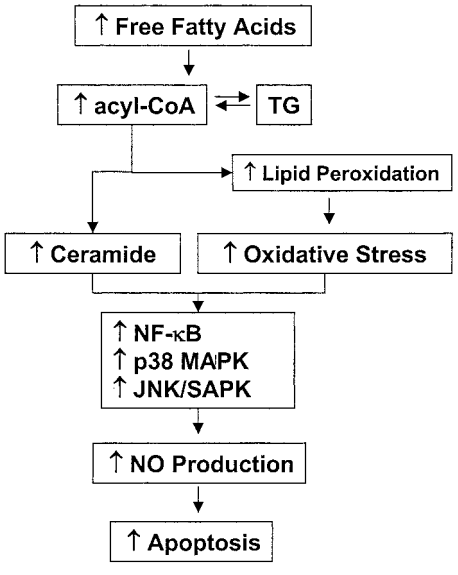

FIG. 13. Lipotoxic-mediated apoptosis. FFA overaccumulation leads to increased *de novo* production of ceramide (314), increased lipid peroxidation, and oxidative stress (383, 412, 413). Ceramide is an important signaling molecule that activates stress-sensitive signaling pathways leading to increased production of NO and eventually apoptosis (160, 414–416). NO production can be blocked by the antioxidants AG and nicotinamide (412, 413). [Derived from Ref. 413.]

β-Cells are sensitive to ROS and RNS, because they are low in free-radical quenching (antioxidant) enzymes such as superoxide dismutase, glutathione peroxidase, and catalase (393), as well as ROS-scavenging proteins such as thioredoxin (394). During chronic hyperglycemia, increased expression of several antioxidant genes and antiapoptotic genes appears to be involved in the compensatory response of β-cells, presumably contributing to their ability to survive under conditions of oxidative stress (350). Overexpression of the antioxidant enzymes in islets or transgenic mice prevents many of the deleterious effects noted above (388, 395, 396). Oxygen stress, generated by acute exposure of β-cells to H_2O_2, increases the production of p21 (an inhibitor of cyclin-dependent kinase), decreases insulin mRNA, cytosolic ATP, and calcium flux in the cytosol and mitochondria, along with causing apoptosis (reviewed in Ref. 349). Insulin secretion, stimulated by glucose or methyl succinate, is inhibited within 30 min, whereas the response to K^+ remains normal (349). These results indicate that mitochondrial events involved in glucose-mediated insulin secretion are particularly affected by oxidative stress.

Inhibition of glucose oxidation and insulin secretion also occurs when islets are exposed to lipid peroxidation products (397). Conversely, antioxidants such as NAC, AG, zinc, and the spin-trapping agent PBN can protect against β-cell toxicity and the generation of glycation end-products and can inhibit the activation of NF-κB (110, 126, 363, 391, 398–401). Recently, β-cell function was evaluated in islets after overexpression of GFAT, the rate-limiting enzyme of hexosamine biosynthesis (402). Activation of the hexosamine pathway resulted in significant deterioration of glucose-stimulated insulin secretion along with other indices of β-cell function, coincident with an increase in H_2O_2 (402). These effects were counteracted by treatment with the antioxidant NAC.

It is intriguing to consider the possibility that a direct target of ROS in β-cells might be the low-affinity glucose phosphorylating enzyme glucokinase, the glucose sensor (341). In intact islets and in partially purified enzyme preparations, glucokinase is inhibited by the diabetogenic agent alloxan (403). Alloxan-induced glucokinase inactivation is antagonized by glucose and several thiol-containing compounds (403, 404). Additional mechanistic studies using alloxan have revealed that the cysteine residues in the vicinity of the glucose-binding site of glucokinase are critical for the enzyme activity, and that oxidation of these residues or the formation of disulfide bridges (*e.g.*, after alloxan treatment) results in enzyme inactivation (404–406). Generation of ROS in HIT-T15 cells transfected with the human glucokinase gene caused a significant reduction in glucokinase mRNA and protein expression, along with glucokinase V_{max} (maximum rate of enzyme-catalyzed reaction) (352). The effects of ROS were counteracted by the antioxidants NAC and aminoguanidine.

VI. Conclusions and Implications

The molecular mechanisms whereby oxidative stress causes diabetic complications are undefined. In a variety of tissues, hyperglycemia and elevated FFA result in the generation of ROS and RNS, leading to increased oxidative stress. In the absence of an appropriate compensatory response from the endogenous antioxidant network, the system becomes overwhelmed (redox imbalance), leading to the activation of stress-sensitive signaling pathways, such as NF-κB, p38 MAPK, JNK/SAPK, PKC, AGE/RAGE, sorbitol, and others. The consequence is the production of gene products, such as VEGF and others, which cause cellular damage and are ultimately responsible for the long-term complications of diabetes. In addition, activation of the same or similar pathways appears to mediate insulin resistance and impaired insulin secretion. It is our view that there appears to be a common biochemical basis that involves oxidative-stress-induced activation of stress-sensitive signaling pathways. Thus, the use of antioxidants may be very important in preventing activation of these pathways. Moreover, identification of the molecular basis for the protection afforded by a variety of antioxidants against oxidative-induced damage might lead to the discovery of pharmacological targets for novel therapies to prevent, reverse, or delay the onset of the resultant pathologies.

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