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Forum Review

Proposed Mechanisms for the Induction of Insulin Resistance by Oxidative Stress

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ABSTRACT

In diabetes (type 1 and type 2), increased flux of free fatty acids and glucose is associated with increased mitochondrial reactive oxygen species (ROS) production and, as a consequence, increased oxidative stress. ROS have been shown to activate various cellular stress-sensitive pathways, which can interfere with cellular signaling pathways. Exposure of different cell lines to micromolar concentrations of hydrogen peroxide leads to the activation of stress kinases such as c-Jun N-terminal kinase, p38, IκB kinase, and extracellular receptor kinase 1/2. This activation is accompanied by a down-regulation of the cellular response to insulin, leading to a reduced ability of insulin to promote glucose uptake, and glycogen and protein synthesis. The mechanisms leading to this down-regulation in oxidized cells are complicated, involving increased serine/threonine phosphorylation of insulin receptor substrate-1 (IRS1), impaired insulin-stimulated redistribution of IRS1 and phosphatidylinositol-kinase between cytosol and low-density microsomal fraction, followed by a reduced protein kinase-B phosphorylation and GLUT4 translocation to the plasma membrane. In addition, prolonged exposure to ROS affects transcription of glucose transporters: whereas the level of GLUT1 is increased, GLUT4 level is reduced. As can be expected, administration of antioxidants such as lipoic acid in oxidized cells, in animal models of diabetes, and in type 2 diabetes shows improved insulin sensitivity. Thus, oxidative stress is presently accepted as a likely causative factor in the development of insulin resistance. *Antioxid. Redox Signal.* 7, 1553–1567.

DIABETES MELLITUS AND THE CELLULAR RESPONSE TO INSULIN

TYPE 2 DIABETES MELLITUS (T2DM) represents the final stage of a chronic and progressive syndrome representing a heterogenous disorder caused by various combinations of insulin resistance and decreased pancreatic ß-cell functions. Insulin increases glucose uptake in muscle and fat and inhibits hepatic glucose production, thus serving as the primary regulator of blood glucose concentrations. Insulin also stimulates cell growth and differentiation, promotes the storage of substrates in fat, liver, and muscle by stimulating lipogenesis, and glycogen and protein synthesis, and inhibits lipolysis, glycogenolysis, and protein breakdown (73). The factors leading to the development of T2DM are both genetic and environmental. However, a search of candidate genes responsible for the insulin resistance can demonstrate that only 5–10% of patients exhibit specific mutations (30). Therefore, study in recent years has focused on different potential factors that may decrease insulin sensitivity. These include the effect of high glucose (120), chronic insulin (20), fatty acids (30), oxidative stress (122), and cytokines (115). Although the mechanisms for the development of insulin resistance are still not fully understood, it is generally associated with obesity. At the cellular level, insulin resistance is associated with the down-regulation of the insulin-signaling cascade and/or the insulin-stimulated glucose transporter GLUT4 (134).

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Insulin-signaling pathway

In the normal state, the binding of insulin to the α subunit of the insulin receptor (IR) triggers the autophosphorylation of the β subunit at distinct tyrosine residues (Fig. 1A). This autophosphorylation in turn triggers the binding and the activation of different intracellular substrate molecules to the IR. The main docking proteins to bind to the IR are a family of proteins called IR Substrates (IRSs), of which IRS1 and IRS2 are the main isoforms in insulin-sensitive tissue (for review, see 157). The binding of IRSs to the IR triggers phosphorylation on multiple tyrosine residues within the C-terminal region of IRS, leading to the generation of highly specific binding sites of a number of SH2 domain-containing signaling molecules. Those molecules include phosphatidylinositol 3-kinase (PI3K), Nck, and Grb-2, of which PI3K seems to be a central insulin-signaling molecule in mediating the metabolic effect of insulin. PI3K is comprised of a catalytic and a regulatory subunit (p110 and p85, respectively). As a result of IRS tyrosine phosphorylation, the p85 subunit of PI3K binds to the PH domain of IRS1/2, leading to an increase in the catalytic activity of p110. This activation results in a subsequent rise in intracellular phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate content (PIP3) (132), which is essential for the translocation of GLUT4 from an intracellular pool to the plasma membrane and for the increase in glucose uptake by muscle and adipocytes (150, 162). A wide range of downstream targets of PI3K have been identified. Among them are serine/ threonine kinases such as phosphoinositide-dependent protein kinase (PDK1) (152), protein kinase B (PKB) (77), protein kinase C (PKC) γ and ζ (5), p70 S6 kinase (20), and glycogen synthase kinase 3 (GSK3) (25).

Research in recent years has focused on several isolated factors leading to the down-regulation of the insulin signal. Among them are tumor necrosis factor-α (TNFα) (74), chronic insulin (23), activation of the hexosamine pathway (108), and elevated flow of free fatty acids (FFA) and glucose (30). Recent evidence shows that increased flux of FFA, glucose, or hexosamine could raise mitochondrial reactive oxygen species (ROS) production, leading to increased intracellular oxidative stress (14, 93). Concomitantly, an emerging new line of studies shows that increased oxidative stress as an isolated factor can indeed induce insulin resistance (85, 123).

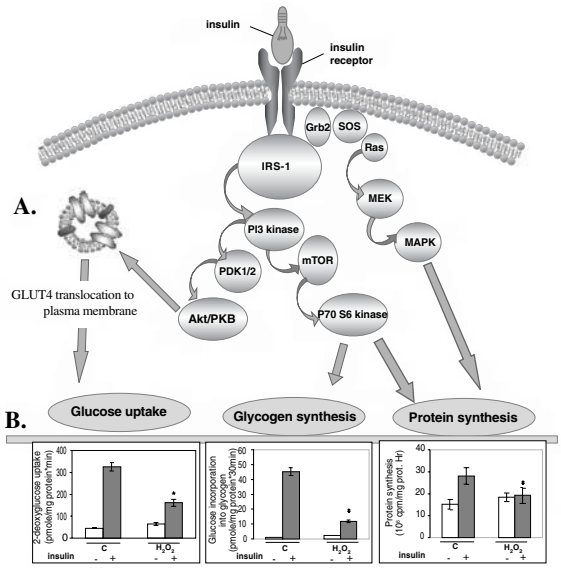

FIG. 1. The effect of H_2O_2 on insulin-stimulated metabolism. (**A**) Schematic presentation of the insulin signaling cascade. (**B**) 3T3-L1 adipocytes were serum-starved overnight followed by incubation for 2 h in the absence or presence of 50 μ*M* H_2O_2 generated by the addition of glucose oxidase (100 mU/ml) to glucose-containing medium. Cells were rinsed, and stimulated without or with 100 n*M* insulin for 20 min (glucose uptake and glycogen synthesis) or for 3 h (protein synthesis), after which 2-deoxyglucose uptake, the incorporation of glucose to glycogen, and (50, 72, 116, 118, 128, 133) methionine incorporation into protein were measured as previously described (76).

DIABETES AND INCREASED OXIDATIVE STRESS

ROS such as superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$), and hydrogen peroxide (H_2O_2) are an obligatory consequence of an aerobic environment and of aerobic metabolism (38, 55). ROS are present in cells and tissues at low yet measurable concentrations, depending on the balance between their rate of production and their rate of clearance by various cellular enzymatic and nonenzymatic antioxidant compounds. The first group in the antioxidant defense system comprises mainly superoxide dismutase (SOD), gluthathione peroxidase, and catalase, which convert different ROS into less offensive products (59), and the second group comprises molecules able to inhibit or delay the oxidation of cellular substrates (99). Until recently, several mechanisms have been offered to explain the increased oxidative stress in both type 1 diabetes mellitus and T2DM. These studies suggest that diabetes is a bipolar process in which, on the one hand, there is an increased generation of ROS (46, 127), and, on the other hand a decrease in plasma antioxidant levels like vitamin E, vitamin C, lipoic acid, and glutathione (GSH) (37, 90, 101, 142, 159). Table 1 summarizes literature of the past 17 years in which various markers for oxidative stress were assessed in plasma and tissues obtained from human diabetic subjects (type 1 and type 2) as compared with healthy controls. Notably, numerous, although not all, studies reached the conclusion of increased oxidative stress in human diabetes, despite the use of various parameters and techniques. Most consistent are observations of increased lipid peroxidation products, decreased plasma vitamin C and erythrocyte GSH, as well as reduced SOD activity. Taken together, the overall picture that emerges from Table 1 and from studies in animal models of diabetes is that the diabetic state is associated with increased oxidative stress. The high chemical reactivity of ROS underlies their interaction with various cellular components, including lipids, proteins, and DNA, potentially resulting in altered function of membranes and enzymes, and could lead to tissue injury (7).

TABLE 1. EVIDENCE FOR INCREASED OXIDATIVE STRESS IN HUMAN DIABETIC SUBJECTS

	Antioxidants	*Antioxidant enzymes*	*ROS*	*Oxidation products: lipid/DNA/protein*
Plasma/serum	Vitamin C: ↓ (56, 90, 135, 142, 165) ↑ (71) Vitamin E: ↓ (71, 94, 142) ↔ (22, 27, 75, 90, 151, 160) GSH: ↓ (71) Lipoic acid: ↓ (101) Urate ↓ (90) Total antioxidant capacity: ↓ (90, 96, 112, 127, 149) ↔ (89, 160)	GPx: ↑ (71)	Lipid hydroperoxides: ↑ (94, 127)	TBARS: ↑ (2, 40, 49, 82, 137, 142) ↔ (46) Lipid peroxides: ↑ (53, 127, 153) ↔ (140) Oxidized LDL or antibodies: ↑ (9, 15, 149) ↔ (2, 79, 89, 160)
Urine	Vitamin C ↓ (165)			8-Isoprostanes: ↑ (29)
Cells/tissues	Vitamin E: Platelets, RBC/blood ↓ (22, 75, 144) GSH: Platelets ↓ (144) RBC/blood ↓ (126, 142, 145, 153, 164) ↑ (82) ↔ (83, 91) PMN ↓ (19)	GPx: PMN ↓ (19, 92) RBC/blood ↓ (83) ↑ (142) ↔ (6) GR: PMN ↓ (92) ↔ (19) SOD: PMN: ↓ (19, 92) RBC/blood ↓ (6, 142) ↔ (153) Catalase: PMN ↔ (92) RBC/blood ↓ (142) ↔ (6)	Superoxide anion: PMN ↑ (19, 28) Platelets ↑ (129)	TBARS: PMN ↑ (92) RBC ↑ (69, 142) Lens ↑ (151) Lipid peroxides: RBC ↑ (27) 8-OHdG: PMN ↑ (19) Modified proteins: skin, heart, lung, kidney, artery ↑ (130)

Selected articles dealing with various oxidative stress markers in human diabetic subjects were traced using Medline (years 1984–2003). Arrows represent the findings as compared with nondiabetic humans. RBC, red blood cells; PMN, peripheral blood polymorphonuclear cells; GPx, glutathione peroxidase; GR, glutathione reductase; TBARS, thiobarbituric acid reactive substances (malondialdehyde); 8-OHdG, 8-hydroxydeoxyguanosine.

Mechanism for the increased oxidative stress in diabetes

Oxidative stress, resulting from increased production of ROS, plays a key role in the pathogenesis of late diabetic complications such as the pathophysiology of atherosclerosis in diabetes (42, 43), β-cell dysfunction (117), nephropathy (8), and increased occurrence of embryonal malformations in diabetic pregnancies (35). Several mechanisms have been offered to explain the increased oxidative stress in both type 1 and type 2 diabetic patients:

(a) The hyperglycemia associated with diabetes may lead to increased flux of glucose to various, but not all, tissues (93). In the process of mitochondrial respiration, 0.4–4% of all oxygen consumed is converted into the free radical superoxide (14). Subsequently, superoxide can be converted into other ROS and reactive nitrogen species by specific enzymatic reactions, including SOD, which generates H_2O_2 both in the mitochondria and in the cytosol.
(b) The increased circulating FFA in diabetes lead to elevation in fatty acid oxidation, which, as mentioned above, may also contribute to the increase in mitochondria respiration (107).
(c) Increased autooxidation of glucose is associated with the formation of advanced glycation end products (AGEs) (51). The interaction of AGEs with corresponding cell-surface receptors stimulates ROS production and consequently decreases intracellular glutathione levels (10).
(d) Increased NAD(P)H oxidase activity in phagocytic and nonphagocytic cells (87, 158).
(e) Lately, it was suggested that fat tissue of obese individuals exhibits increased macrophage infiltration and activation (156, 161). To what extent this could lead to a local increase in ROS is yet to be discovered.
(f) Increased expression of TNFα can induce elevation in H_2O_2 upon interaction with various cell types (115).

The relative contribution of each of these mechanisms in the occurrence of oxidative stress in diabetes may differ according to metabolic state, tissue specificity, and nutritional factors.

ROS and insulin action

ROS in general, and H_2O_2 in particular, may affect the insulin signling cascade and the insulin-dependent metabolic activities in a bimodal fashion, depending on the dose and time course.

Short-term exposure to millimolar ROS concentrations results in insulin-like (insulinomimetic) effects, such as the activation of glucose transport by nitric oxide (39) and H_2O_2 (45, 61) in muscle, which are accompanied by increased cellular basal tyrosine phosphorylation. This has been shown to be due to a direct inhibition of tyrosine phosphatases, leading to increased tyrosine phosphorylation of both the IR and IRS proteins (32, 88). The recent finding by Mahadev *et al*. showing an NAD(P)H oxidase-dependent insulin-stimulated glucose transport in adipocytes reinforces the concept of a physiological role for ROS in the cellular response to insulin (86). On the other hand, several studies have raised the possibility that oxidative stress may play a role in the induction of peripheral insulin resistance. These mainly include clinical studies that correlated oxidative stress parameters with the metabolic control of patients (103, 105, 136). Other studies reported improved insulin sensitivity and metabolic control by pharmacological doses of vitamin E, vitamin C, GSH, or lipoic acid, administered to healthy volunteers or to diabetic subjects (16, 66, 102, 104). Moreover, some evidence suggests that impaired oxidant–antioxidant balance may precede the occurrence of diabetes (125), suggesting the potential role of ROS as a risk factor and not only as a consequence of diabetes. These studies provide evidence to suggest an association between oxidative stress and abnormal glucose homeostasis, but fail to conclusively establish a cause and effect relationship between the two.

Thus, this review focuses on the possible involvement of oxidative stress and altered redox state in the development of insulin-resistance in insulin responsive cells. The primary aim of our lab, as well as others, was to characterize the effects of oxidative stress on the cellular response to insulin, and to identify oxidation-sensitive steps within the insulin-signaling cascade, to evaluate the specificity of these cellular responses, as well as the ability of antioxidants to prevent this damage.

In multiple studies utilizing L6 myotubes and 3T3-L1 adipocytes, we and others reported that exposure to oxidative stress inhibits the metabolic pathways induced by insulin (85, 122). For example, as can be seen in Fig. 1B, exposure for 2 h to 50 μ*M* H_2O_2 inhibited insulin-stimulated glucose uptake, glycogen synthesis, and protein synthesis. As will be discused herein, this reduction in the metabolic effects of insulin is accompanied by the activation of cellular stress kinases, impaired insulin-signaling cascade, as well as changes in gene regulation and protein stability (76, 110, 146).

ACTIVATION OF STRESS KINASE IN DIABETES AND IN OXIDIZED CELLS IN CULTURE

Studies in muscle and adipose tissue of diabetic patients reveal alterations in protein expression and in the activation of several kinases, which in turn were implicated in the development of adipocyte insulin resistance. An important group of kinases that are up-regulated in diabetic patients, as well as animal and cell line models of diabetes, are the cellular stress kinases. Particularly, the mitogen-activated protein kinases (MAPKs) have been shown to participate in the induction of insulin resistance. This family of kinases is comprised of a group of related serine-threonine protein kinases, among them the extracellular regulated kinase 1/2 (ERK1/2), c-Jun NH2-terminal kinase (JNK), and p38 MAPK. Their activity is induced by cellular stress, inflammatory cytokines, and G protein-coupled receptor agonists. These stress-activated kinases have been implicated in apoptosis, oncogenic transformation, and inflammatory responses in various cell types (for review, see 54). Chronic activated p38 MAPK is associated with several diseases, including diabetes, ischemia/reperfusion injury, and infectious disease (17, 24). Activated JNK binds

to and phosphorylates c-jun, an integral component of the transcription factor activation complex of activator protein-1 (AP1), the involvement of which has been abundantly demonstrated in redox regulation (131). Another important stress related transcription factor is nuclear factor-κB (NF-κB), which has been shown to mediate immune and inflammatory responses. Furthermore, its altered regulation has been shown to be associated with diabetes and atherosclerosis (4). The activation of NF-κB involves a proteasome-regulated signaling pathway in which IκB, an inhibitory subunit of NF-κB, is phosphorylated by the IκB kinase (IKK) complex and subsequently degraded by the proteasome machinery (21). The IKK proteins, particularly IKKβ, have been shown by several investigators to participate in the induction of insulin resistance in insulin-responsive cell lines (65, 70). Increased basal levels of phosphorylated ERK1/2, JNK1, and p38 MAPK were observed in adipocytes (17) or skeletal muscle (11) of obese, insulin-resistant, and/or type 2 diabetic subjects. In addition, mononuclear cells of diabetic patients show elevated binding of the transcription factor NF-κB (64), suggesting activation of IKKβ, a kinase known to be activated by various stresses (155). The particular involvement of p38 MAPK pathway has been shown to contribute to the reduction of GLUT4 expression observed in adipose tissue from type 2 diabetic patients (17). Studies performed in cell cultures exposed to various inducers of insulin resistance provide a link between chronically increased activity of these kinases with insulin resistance. The stress kinases (such as ERK, JNK, p38 MAPK, IKKβ) (1, 70, 113), as well as "signaling kinases" (Akt/PKB, atypical PKC-like PKCζ, and PI3K) (31, 84, 98), and kinases controlling metabolic pathways further downstream (GSK3, p70 S6 kinase) (34, 147), have all been suggested to provide negative input on acute insulin responses. In agreement with this concept is the finding that mice deficient in JNK1 show improved systemic insulin sensitivity (63). As these kinases are mostly serine/threonine kinases, the mechanism of interference with normal insulin signaling is believed to involve increased serine/threonine phosphorylation of key elements in the insulin-signaling cascade.

Recently, the direct effect of ROS on the serine kinases was studied in FAO rat hepatome cells. When these cells were exposed to 50 ± 5 μ*M* H_2O_2 for 1–2 h, an increase in the phosphorylation of JNK, p38, ERK1/2, and IKKβ is shown (Fig. 2A). Furthermore, exposure for 24 h to H_2O_2 or superoxide resulted in increased DNA-binding activity of NF-κB (Fig. 2B).

INVOLVEMENT OF OXIDATIVE STRESS IN THE INDUCTION OF INSULIN RESISTANCE

Oxidative stress induces IRS1 serine phosphorylation and degradation

A major negative regulatory role for insulin's action is the serine/threonine phosphorylation of IRS1 and IRS2 (157). A growing body of evidence demonstrates the involvement of some of the above-mentioned serine kinases, like PKC (12, 13), PKB (148), mTOR (mammalian target of rapamycin) (48), and GSK3 (34), in the phosphorylation of IRS molecules on serine/threonine residues. Moreover, the serine phosphorylation of IRS1 could determine its rate of degradation (60). The serine kinases, although attenuating the insulin signal in the physiological response, could lead in a pathological state to hyper-serine phosphorylation of IRS1. This serine phosphorylation may reduce the ability of IRS proteins to interact with the cytosolic domain of the IR (109), concomitantly with a reduced ability of SH-containing proteins, such as PI3K, to attach to IRS1 and transfer the insulin signal (1).

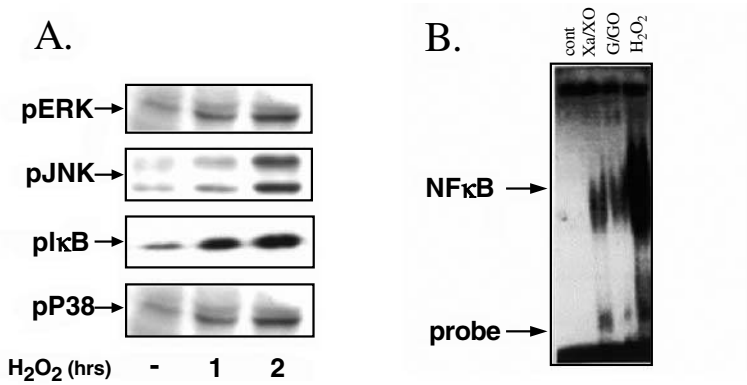

FIG. 2. Oxidative stress induces activation of cellular stress kinases. (**A**) FAO cells were exposed for 1–2 h to 50 μ*M* H_2O_2. Cell lysates were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by western blot analysis by exposing the membrane to the appropriate antibodies against ERK1/2, JNK, p38 MAPK, and IKKβ (**B**) increased DNA-binding activity of NF-κB was measured by preparing nuclear extracts from L6 myotubes exposed for 8 h to 50 μ*M* xanthine, 20 mU/ml xanthine oxidase (Xa/XO), and 50 mU/ml glucose oxidase (G/GO). Extracts were incubated with ^{32}P-labeled NF-κB oligonucleotide. Electophoretic mobility shift assay was performed as previously described (80). Autoradiograph of a native gel is shown.

Under normal conditions, this is an important counterbalancing mechanism that stops insulin's action. However, in a pathological response, the hyper-serine phosphorylation of the IRS proteins may lead to a chronic cellular desensitization to insulin. Thus, the effect of H_2O_2 on IRS1 was evaluated in FAO cells. As can be seen in Fig. 3A, exposure of cells for 2 h to H_2O_2 results in a reduction in IRS1 protein content as compared with control cells, which is accompanied by a retarded migration on the gel, indicating hyper-serine phosphorylation. The oxidation-induced reduction of the IRS1 protein could not be prevented by specific proteasome inhibitors, suggesting degradation of IRS1 via a proteasome-independent mechanism (114). Interestingly, insulin-stimulated PI3K activation in whole-cell lysates was not affected by oxidative stress (Fig. 3B).

Oxidative stress impairs cellular redistribution of insulin-signaling component

Recently, the concept that the activation of PI3K in the low-density microsomal fraction (LDM) or in GLUT4-containing vesicles is necessary for the specific ability of insulin to promote GLUT4 translocation has been suggested (62, 143). As can be seen in Fig. 4A, in the basal state, PI3K is most abundant in the cytosolic fraction. Upon insulin stimulation, autophosphorylation of the IR increases the binding of IRS molecules to the receptor, accompanied by the translocation of PI3K from the cytosol fraction to the LDM (Fig. 4B). The p85 subunit of PI3K binds to the IRS, leading to an increased generation of PIPs (Fig. 4C). This in turn leads to the translocation of several proteins downstream of the insulin-signaling cascade, like PDK1, PKB, and PKCζ, to the plasma membrane (PM) and to their activation (Fig. 4C). The exact mechanism(s) through which activation of those proteins leads to GLUT4 translocation from LDM to the PM (Fig. 4D) is not fully understood (121).

The reduction in insulin-stimulated glucose transport observed in oxidized cells (Fig. 1B) could not be attributed to a reduction in PI3K activity in whole-cell lysates (Fig. 3B). Thus, the ability of insulin to activate PI3K in various cellular fractions was studied in 3T3-L1 adipocytes. As can be seen in Fig. 4E, in unoxidized cells, insulin-increased IRS1 tyrosine phosphorylation in the LDM fraction (middle panel) was accompanied by a sevenfold increase in the activity of IRS1-associated PI3K (bottom panel). Exposure of cells for 2 h to H_2O_2 significantly reduced the ability of insulin to increase p85 content in the LDM (data not shown). This was associated with inhibition of insulin-stimulated PI3K activation in the LDM (bottom panel). Under those conditions, insulin-stimulated Ser^{473} PKB phosphorylation and the translocation of GLUT4 from internal pool to the PM were dramatically reduced (Fig. 4F). These findings were recently reinforced by the work of Ogihara *et al*., which shows the same compartment-specific down-regulation of IRS1 in muscle and adipose tissue of glutathione-depleted rats and 3T3-L1 adipocytes, both of which exhibit insulin resistance (95). Taken together, these data suggest that both the insulin-stimulated IRS1 tyrosine phosphorylation and the translocation and activation of PI3K to the LDM are impaired following oxidative stress and are associated with a reduction in the insulin-stimulated PKB phosphorylation and GLUT4 translocation.

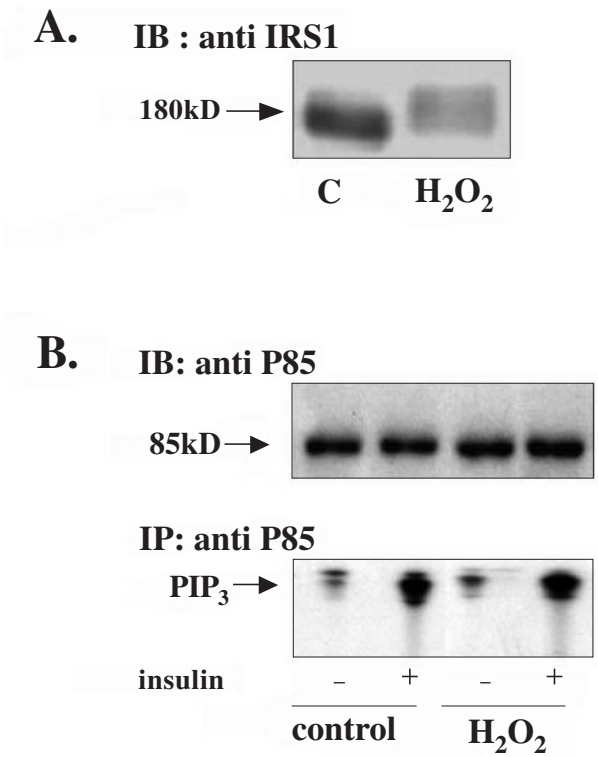

FIG. 3. Oxidative stress-induced alteration in IRS1 content and serine phosphorylation is not accompanied by alteration in PI3K activation in whole cell lysates. Lysates of 3T3 adipocytes exposed to H_2O_2 were subject to western blotting using (**A**) anti-IRS1 and (**B**) anti-p85 subunit of PI3K (**top panel**) as described (114). In addition, PI3K activity was measured as described (146) (**lower panel**).

OXIDATIVE STRESS INCREASES GLUT1 BUT DECREASES GLUT4 GENE TRANSCRIPTION

The notion that oxidative stress can affect DNA binding of transcription-regulating factors is well established, and could be caused by diverse mechanisms (110). Direct oxidation of specific moieties such as SH groups in domains critical for transcription factor function (like the DNA-binding domain), decreased expression, and alteration in the phosphorylation state have all been documented (3, 18, 81). Several transcription factors are known to be activated in response to oxidative stress, mainly NF-κB, and AP1 (3, 111). Exposing L6 myotubes and 3T3-L1 adipocytes to prolonged oxidative stress (18 h) resulted in an increase in the mRNA and protein content of GLUT1. This was accompanied by increased binding activity of AP1 to DNA. This increased activation could mediate GLUT1 transcriptional activation in response to oxidative stress. Such elevation in GLUT1 content may in turn increase

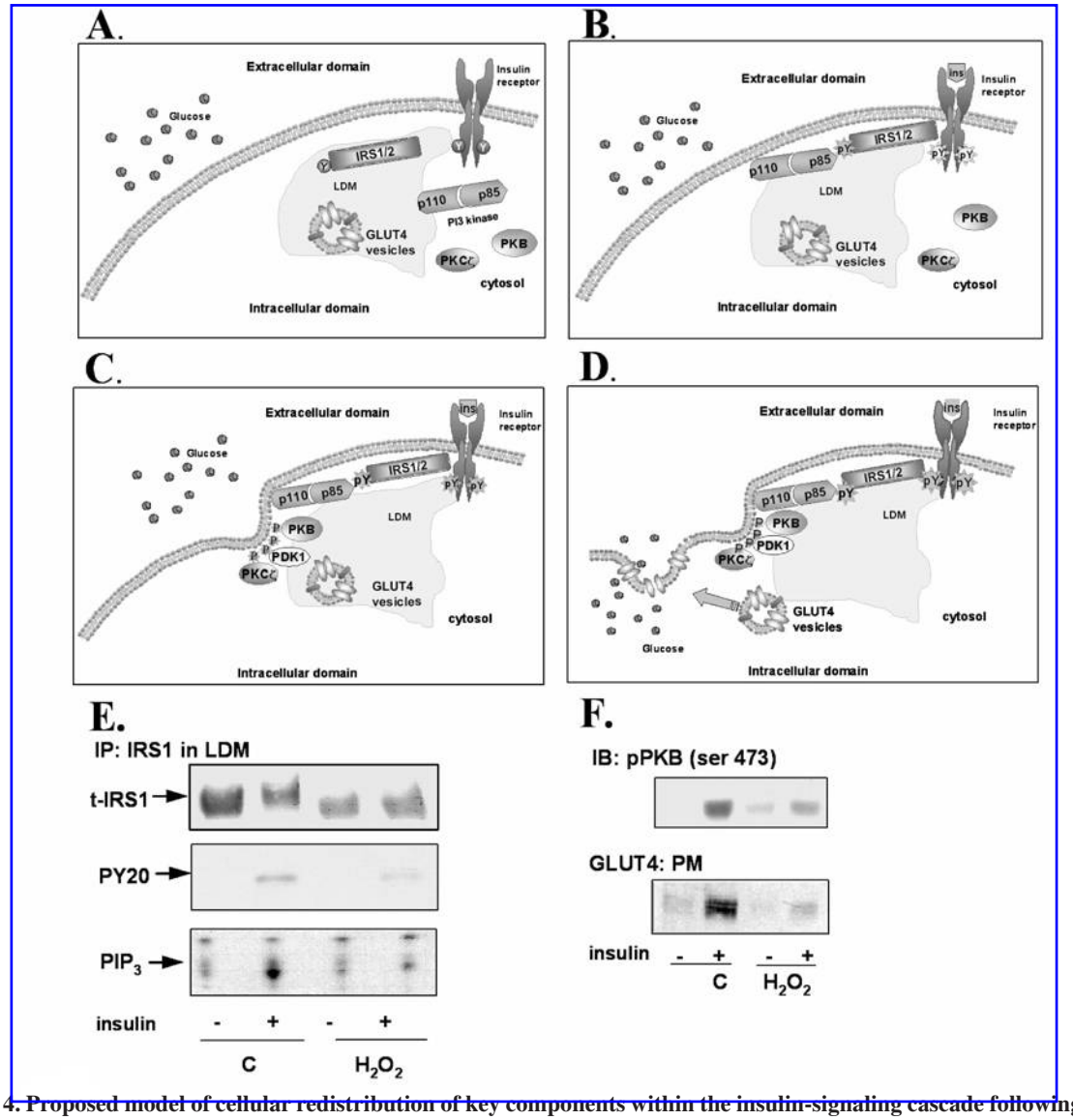

FIG. 4. Proposed model of cellular redistribution of key components within the insulin-signaling cascade following insulin stimulation. (**A**) Basal state. (**B**) Insulin stimulates PI3K translocation to the LDM fraction. (**C**) PIPs stimulate recruitment of PDK1, PKB, and PKCζ to the PM. (**D**) Effect of oxidative stress on insulin-stimulated (**E**) IRS1 content in LDM (**upper panel**), its tyrosine phosphorylation (**middle panel**), and IRS-1-associated PI3K activity in the LDM (**lower panel**). (**F**) Ser473 PKB phosphorylation (**upper panel**) and GLUT4 translocation to the PM (**lower panel**) as previously described (146).

basal glucose uptake and metabolism in various cell types, which in turn may increase mitochondrial ROS production.

Accumulating experimental evidence suggests that GLUT4 could be central in determining peripheral insulin sensitivity and thus could be an important factor in the pathogenesis of T2DM. Various groups have shown that GLUT4 expression is reduced in obese, nondiabetic subjects, and more so in type 2 diabetic patients (41). Moreover, disruption of a single allele of the GLUT4 gene resulted in a T2DM-like phenotype (138), whereas overexpression of GLUT4 protected against the development of diabetes in an animal model (58).

As exposure of 3T3-L1 adipocytes and L6 myotubes to H_2O_2 results in a marked decrease in both mRNA and protein expression of GLUT4 (76, 81), we evaluated the effect of oxidative stress on the gene regulation of GLUT4. A decreased DNA binding of nuclear proteins in 3T3-L1 adipocytes to the insulin-responsive element (IRE) sequence in the GLUT4 promoter was found following exposure to oxidative stress (110). Which transcription factor(s) bind to the IRE sequence of GLUT4 is still unknown. Another site in the GLUT4 promoter that is affected by oxidation is the CCAAT enhancer binding protein (C/EBP) site. Reduction in C/EBPα protein content and elevation in C/EBPδ protein content were found in the nucleus of adipocytes after 4 h of exposure to H_2O_2 (unpublished observations). This alteration in potential C/EBP dimer partners may lead to changes in the occupation of the C/EBP-

binding site in the GLUT4 promoter, and may play a role in the GLUT4 down-regulation in the oxidized cells.

CAN ANTIOXIDANTS PREVENT INSULIN RESISTANCE?

Epidemiological study demonstrates a close correlation between low ascorbate or α-tocopherol levels and the risk of developing insulin resistance (94, 154). This, together with the data presented herein, suggests that ROS may play an important role in the development and progression of insulin resistance in type 2 diabetic patients. If this hypothesis is true, then reversal of the imbalance between ROS and antioxidant capacity should improve insulin resistance. This has been demonstrated with the antioxidant α-lipoic acid (LA). LA, a cofactor of α-ketoacid dehydrogenases such as pyruvate dehydrogenase, is increasingly recognized as a powerful antioxidant (99). Its antioxidant mode of action includes iron chelation and direct scavenging of different ROS, rendering it a "preventative antioxidant" (26, 97). In addition, its lipid and water solubility capacitates it to recycle vitamin C, vitamin E, and GSH (57, 100). As LA levels are reduced in diabetes (Table 1), its effect on glucose homeostasis was studied in both cells in culture and animal models of diabetes (for review, see 119).

3T3-L1 adipocytes and FAO cells pretreated with micromolar concentrations of LA followed by oxidative stress exhibit increased intracellular GSH levels and an improved insulin-stimulated PKB activation compared with oxidized cells. In accordance, in 3T3-L1 adipocyte LA pretreatment prevented the oxidation induced reduction in insulin-stimulated glucose transport and GLUT4 translocation (124). Exposure of 3T3-L1 adipocytes to high glucose inhibited insulin-stimulated glucose transport by 50% compared with cells incubated in low glucose. This down-regulation was prevented by LA (47). Interestingly, LA in the millimolar range was shown to activate IRS1 and PI3K in L6 muscle cells and 3T3-L1 adipocytes, thus leading to a translocation of the GLUT4 transporter from the intracellular compartment to the cell surface (163). This antidiabetic effect of LA on glucose uptake and glucose oxidation has been further studied in several models of diabetes, such as in the muscle epitrochelaris of the obese Zucker rat, the *Psammomus obesus,* in isolated rat diaphragm, and in rat brain (36, 68, 76, 139, 141). Administration of LA resulted in an increase in cellular glucose uptake in all those models, accompanied by a decline in blood glucose levels and elevation in muscle GLUT4 content in both streptozotocin diabetic rats and *Psammomus obesus* (Table 2) (76). In the epitrochelaris muscle of Zucker rat, LA treatment improved insulin-mediated glucose uptake and increased both insulin-stimulated glucose oxidation and glycogen synthesis (36). In nondiabetic rats, high glucose was demonstrated by hyperinsulinemic–euglycemic clamp study to induce a decrease in insulin-stimulated glucose uptake, which was prevented by the antioxidant *N*-acetylcysteine (52).

In type 2 diabetic patients, acute and chronic treatment with LA in various doses ranging from 500 to 1,000 mg i.v. improved insulin-stimulated glucose disposal (66, 68, 78). A statistical 27% improvement in glucose utilization after oral administration of LA was also observed in a placebo-controlled double-blind study (78).

LA is not the only antioxidant that has been shown to improve glucose utilization in diabetic patients. Antioxidants such as α-tocopherol, ascorbate, and GSH have also been found to increase glucose utilization in diabetic patients (103, 104, 106). Yet in the case of α-tocopherol, the question whether the beneficial effect on glycemic control is related to improved β-cell function or peripheral insulin sensitivity is still open (44).

In conclusion, those experimental and clinical data suggest an inverse association between insulin sensitivity and levels of ROS. Whether antioxidants indeed represent an attractive therapeutic strategy for the teatment of insulin resistance in T2DM patients has to be investigated in large clinical trials.

Table 2. Effect of Lipoic Acid on Rodent Models of Diabetes Melitus

Animals	*Glucose (mg%)*		*Fold of control GLUT4 content in total membrane of muscle (treated)*
	Nontreated	*LA-treated*	
Psam fed	219.5 ± 5.0 (6)	106 ± 22.2* (10)	1.3 ± 1.2*
Psam 12 h of fasting	68.6 ± 4.7 (7)	55.5 ± 3.1* (10)	
STZ fed	641 ± 125 (16)	495 ± 131* (19)	2.9 ± 0.8*
STZ, 12 h of fasting	341 ± 36 (8)	189 ± 48* (8)	

Psammomus obesus (Psam) were fed a high carbohydrate diet for 3 weeks. After 1 week of diet, LA (30 mg/kg/day) was injected daily intraperitoneally for 14 days. Diabetes was induced in 10-weeks male Sprague–Dawley rats by a single injection of 65 mg/kg streptozotocin (STZ) in 100 m*M* citrate buffer (pH 4.5). Seven days after the induction of diabetes, animals were injected daily for 10 consecutive days with 30 mg/kg/day LA. Blood glucose levels were measured using a glucometer. Muscle GLUT4 content was measured using western blot analysis. Results are means ± SE of number of animals in parentheses.

*$p < 0.05$ compared with untreated animals.

CONCLUSIONS

Based on the literature cited above and on our results, we suggest the following model, which may explain the vicious cycle between insulin resistance and increased oxidative stress in diabetes: In uncontrolled diabetes, elevated glucose, AGEs, and FFA metabolism increase ROS production, which in turn increases intracellular damage due to activation of various stress kinases, protein glycosylation, lipid peroxidation, and changes in DNA. Those changes may disrupt the insulin-signaling cascade and interfere with the normal transcription of specific proteins, leading to insulin resistance. The ability of antioxidants to interfere with this vicious cycle *in vivo* is still under debate.

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ABBREVIATIONS

AGE, advanced glycation end product; AP1, activator protein-1; C/EBP, CCAAT enhancer binding protein; ERK1/2, extracellular receptor kinase; FFA, free fatty acids; GLUT, glucose transporter; GSH, glutathione; GSK3, glycogen synthase kinase 3; H_2O_2, hyodrogen peroxide; IKK, IκB kinase; IR, insulin receptor; IRE, insulin-responsive element; IRS, insulin receptor substrate; JNK, c-Jun NH_2-terminal kinase; LA, α-lipoic acid; LDM, low-density microsomal fraction; MAPK, mitogen-activated protein kinase; NF-κB, factor-κB; PDK1, phosphoinositide-dependent protein kinase-1; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PKC, protein kinase C; PM, plasma membrane; ROS, reactive oxygen species; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus; TNFα, tumor necrosis factor-α.

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