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Forum Review

Nitrosative Stress and Pathogenesis of Insulin Resistance

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ABSTRACT

Insulin resistance is a major causative factor for type 2 diabetes and is associated with increased risk of cardiovascular disease. Despite intense investigation for a number of years, molecular mechanisms underlying insulin resistance remain to be determined. Recently, chronic inflammation has been highlighted as a culprit for obesity-induced insulin resistance. Nonetheless, upstream regulators and downstream effectors of chronic inflammation in insulin resistance remain unclarified. Inducible nitric oxide synthase (iNOS), a mediator of inflammation, has emerged as an important player in insulin resistance. Obesity is associated with increased iNOS expression in insulin-sensitive tissues in rodents and humans. Inhibition of iNOS ameliorates obesity-induced insulin resistance. However, molecular mechanisms by which iNOS mediates insulin resistance remain largely unknown. Protein *S*-nitrosylation, a covalent attachment of NO moiety to thiol sulfhydryls, has emerged as a major mediator of a broad array of NO actions. S-nitrosylation is elevated in patients with type 2 diabetes, and increased *S*-nitrosylation of insulin signaling molecules, including insulin receptor, insulin receptor substrate-1, and Akt/PKB, has been shown in skeletal muscle of obese, diabetic mice. Akt/PKB is reversibly inactivated by *S*-nitrosylation. Based on these findings, *S*-nitrosylation has recently been proposed to play an important role in the pathogenesis of insulin resistance. *Antioxid. Redox Signal.* 9, 319–329.

INTRODUCTION

Insulin resistance is a major causative factor for type 2 diabetes, which is a polygenic disease accounting for >90% of patients with diabetes, and is associated with increased risk of cardiovascular disease. Obesity is pandemic worldwide. Therefore, the pathogenesis of obesity-related insulin resistance has been an issue of intense investigation for decades. Nevertheless, molecular mechanisms underlying obesity-related insulin resistance are not fully understood, although significant progress has been made by a number of investigators.

Impaired intracellular insulin signal transduction has been considered a major component of insulin resistance. Binding of insulin to its receptor results in activation of insulin receptor (IR) tyrosine kinase with dimerization and autophosphorylation, which in turn phosphorylates insulin receptor substrates (IRSs) at tyrosine residues. IRSs transduce signal from IR to phosphatidylinositol-3 kinase (PI3K), leading to activation of Akt/PKB. Among the signaling cascades activated by insulin, the PI3K–Akt/PKB pathway plays a central role in metabolic actions of insulin, including glucose transport, protein synthesis, glycogen synthesis, and inhibition of hepatic gluconeogenesis. Hence, defects in the signaling cascade of the IR-IRSs- PI3K-Akt/PKB, which are observed in insulin-resistant and/or obese diabetic animals and humans, have been postulated as a major contributor to insulin resistance.

INFLAMMATORY RESPONSE/STRESS SIGNALING AND INSULIN RESISTANCE

Recent studies highlighted the pathogenic role of inflammatory response and stress signaling pathways in obesity-related insulin resistance and type 2 diabetes. Chronic, low-grade inflammation is associated with obesity-induced insulin resistance and type 2 diabetes. The circulating level of

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high-sensitivity C-reactive protein (CRP), an acute phase protein and a marker of inflammation, is elevated in obese and/or insulin-resistant subjects (32). Reduction of body weight was correlated with a reduction in markers of inflammation, including CRP, tumor necrosis factor-α, and interleukin-6 and -8, in parallel with improved insulin sensitivity (28, 62).

From a point of view of intracellular signaling, the inhibitory κB kinase (IKK)–nuclear factor-κB (NF-κB) pathway, c-Jun amino-terminal kinase (JNK/SAPK, also known as stress-activated protein kinase), and endoplasmic reticulum (ER) stress have been shown to be involved in the pathogenesis of insulin resistance. The IKK–NF-κB pathway is a key mediator of inflammatory response, and activated in skeletal muscle of patients with type 2 diabetes (100). Heterozygous knockout of IKKβ ameliorates insulin resistance in high-fat diet–fed mice and in *ob/ob* mice (118) that are genetically obese and diabetic due to leptin deficiency. Salicylates and aspirin, which also inhibit IKKβ, increase insulin sensitivity in *ob/ob* mice and obese, diabetic Zucker (*fa/fa*) rats, and in patients with type 2 diabetes (50, 58). Recent studies with tissue-specific gene targeting showed that activation of IKKβ in liver (16), but not in skeletal muscle (91), causes insulin resistance, and that ablation of IKKβ in liver or myeloid cells (1), but not in skeletal muscle, ameliorates obesity-related insulin resistance.

The activity of JNK/SAPK is elevated in insulin-responsive tissues, including skeletal muscle, liver, and adipose tissue, in obesity-related diabetes (47). Gene targeting of JNK1 alleviates obesity and insulin resistance induced by high-fat diet and leptin deficiency (47). Adenovirus-mediated overexpression of dominant-negative JNK in the liver mitigated insulin resistance in genetically obese, diabetic (*db/db*) mice and in mice fed a high-fat, high-sucrose diet (76). Conversely, overexpression of wild-type JNK/SAPK in the liver of normal mice resulted in insulin resistance. Moreover, cell-permeable JNK inhibitor ameliorated insulin resistance and glucose intolerance in *db/db* mice (55).

Recently, a critical role of ER stress in obesity-induced insulin resistance has been demonstrated. Expression of 78-kDa glucose-regulated protein (Grp78, also termed Bip), an ER chaperone, phosphorylation of eukaryotic initiation factor-2α (eIF2α) and PKR-like kinase (PERK), indicators of ER stress response, are increased in the liver and adipose tissue, but not in skeletal muscle, of *ob/ob* mice and high-fat diet–fed mice (82). Heterozygous deficiency of X-box–binding protein 1 (XBP1), which regulates the transcription of ER chaperones, enhanced insulin resistance in association with aggravated ER stress by high-fat diet in mice (82). Moreover, adenoviral gene transfer of ORP150, an ER chaperone that protects cells from ER stress (107), to the liver significantly ameliorated insulin resistance in *db/db* mice (77). Conversely, adenovirus-mediated expression of antisense ORP150 in the liver of normal mice induced ER stress response and insulin resistance (77). Hotamislegil and his colleagues (82) proposed that ER stress mediates obesity-associated JNK/SAPK activation, which in turn causes insulin resistance. This hypothesis is plausible, particularly in the liver. However, a different explanation might be necessary for insulin resistance in skeletal muscle of obese, diabetic mice, where JNK/SAPK is activated, but enhanced ER stress response was not observed.

In cultured insulin-sensitive cells, including skeletal muscle cells, adipocytes, and hepatocytes, serine phosphorylation of IRS-1, particularly at serine 307 by JNK/SAPK (9) and IKK (37, 38), has been postulated to be a major molecular event underlying inflammation/stress signaling–mediated insulin resistance. Increased serine phosphorylation of IRS-1 has been shown in tissues of insulin-resistant animals (47, 104). However, it remains to be determined whether serine/threonine phosphorylation of IRS-1 or IRS-2 can largely account for the insulin-desensitizing effects of JNK/SAPK, IKK, or ER stress on insulin resistance *in vivo*. Rather, a role of serine/threonine phosphorylation of IRS-1 in insulin resistance could be more complex. Of interest, a very recent study showed that knockin of IRS-1 mutant, in which serine 307 was substituted by alanine and therefore can no longer be phosphorylated, unexpectedly resulted in insulin resistance on both normal chow and high-fat diet (21), as opposed to the anticipation that the lack of phosphorylation of serine 307 would lead to increased, but not decreased, insulin sensitivity, based on the presumption of a pathogenic role of serine phosphorylation of IRS-1 at serine 307. Further studies will be required to clarify the role of serine phosphorylation of IRS-1 *in vivo*.

These recent findings of the critical role of IKK-NF-κB, JNK/SAPK, and ER stress have substantially enhanced our understanding of the etiology of insulin resistance. Nevertheless, three major questions remain to be answered: (a) how the activation of cellular stress signaling and inflammatory pathways is upregulated in obesity and type 2 diabetes; (b) what are downstream effectors of these signaling pathways; and (c) how these inflammatory response and stress-signaling cascades are interrelated to each other in the pathogenesis of insulin resistance.

INDUCIBLE NITRIC OXIDE SYNTHASE AND INSULIN RESISTANCE

Inducible nitric oxide synthase (iNOS, also termed NOS2) is a mediator of inflammation. iNOS was originally identified in activated macrophages (115), but is actually expressed in various tissues, including skeletal muscle, liver, and adipose tissue, even under a normal, unstressed condition (34, 103). A close biologic link exists between iNOS and insulin resistance. Most, if not all, inducers of insulin resistance increase iNOS expression (Fig. 1). These inducers of insulin resistance include obesity (26), free fatty acids (98), hyperglycemia (97), tumor necrosis factor-α (22), oxidative stress (12), endotoxin (103), and burn injury (36). Circulating level of nitrite (NO_2) and nitrate (NO_3) (NOx) is increased in patients with type 2 diabetes and obese subjects compared with lean, healthy individuals (65, 109). Weight-reduction surgery in morbid obesity patients resulted in decreased serum nitrite and nitrate (NOx) concentration in parallel with amelioration of insulin resistance (65). The IKK-NF-κB pathway (119) and JNK/SAPK (85) are major upregulators of iNOS expression. Consistently, iNOS expression is elevated in skeletal muscle of patients

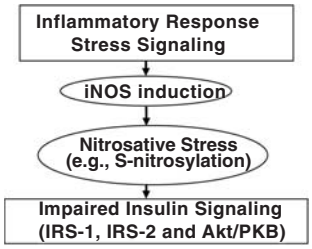

FIG. 1. Inducible nitric oxide synthase (iNOS) as a mediator of inflammatory response/stress signaling-induced insulin resistance. The expression of iNOS in insulin-sensitive tissues (*e.g.,* skeletal muscle, liver) is upregulated by obesity and most, if not all, inducers of insulin resistance, including proinflammatory cytokines, free fatty acid, hyperglycemia, endotoxin, and oxidative stress, all of which elicit stress/inflammatory responses. Increased nitrosative stress, particularly protein *S*-nitrosylation, has been proposed to be involved in the pathogenesis of iNOS-mediated insulin resistance by impairing intracellular insulin signaling.

with type 2 diabetes (109), and skeletal muscle, liver, and adipose tissue of *ob/ob* mice (18, 34, 102). Thiazolidinediones and metformin, insulin sensitizers, suppress iNOS expression in cultured cells, and also *in vivo* in diabetic rodents in association with improved insulin sensitivity (18, 88).

We and others have shown that gene disruption of iNOS or specific inhibitors for iNOS ameliorates insulin resistance induced by high-fat diet (79, 86), leptin deficiency (18, 34, 102) and lipopolysaccharide (LPS) (17, 103). Marette and his colleague (86) demonstrated that disruption of iNOS protected from high-fat diet–induced insulin resistance in mice. They found that a high-fat diet increased iNOS expression in skeletal muscle and adipose tissue, but not in liver, and that disruption of iNOS reversed impaired insulin signaling by a high-fat diet in skeletal muscle, but not in adipose tissue or liver (86). Kearney and his colleagues (79) found that iNOS deficiency prevented high-fat diet–induced defects in vasorelaxation response to insulin in the aorta, in association with reversal of whole-body insulin resistance. Similarly, we found that iNOS inhibition by gene disruption and pharmacologic inhibitors improved whole-body insulin sensitivity in *ob/ob* mice (34, 102) and LPS-administered rats (103). Consistent with the study in high-fat diet–fed mice (86), improved insulin signaling by iNOS inhibition was observed in skeletal muscle, but not in adipose tissue, of *ob/ob* mice. In regard to liver, however, the role of iNOS seems distinct between insulin resistance induced by high-fat diet (86) and leptin deficiency (34). iNOS expression was increased in the liver of *ob/ob* mice at 11 weeks of age (34). iNOS inhibitor, L-NIL, reversed fasting hyperglycemia and ameliorated fasting hyperinsulinemia, whole-body insulin resistance, and IRSs-mediated insulin signaling in the liver in *ob/ob* mice at 11 weeks of age (34). Although much scientific attention has not been paid, blood glucose level in *ob/ob* mice decreases as the mice age after they exhibit overt hyperglycemia at 10 to 12 weeks of age (34, 102). This finding is in agreement with a previous study (24) and an observation by the Jackson Laboratory (http://jaxmice.jax.org/library/notes/451b.html). At 24 weeks of age, the blood glucose level of *ob/ob* mice was decreased to a level indistinguishable from that observed in wild-type mice (102). Although mechanisms underlying age-related alterations in blood glucose in *ob/ob* mice are unknown, blood glucose level correlates with impaired insulin signaling in the liver of *ob/ob* mice. This is in accord with the primary role of hepatic insulin resistance in hyperglycemia (52, 105). IRS-1– and IRS-2–mediated insulin signaling and expression of IRS-1 and IRS-2 were reduced in the liver of *ob/ob* mice at 11 weeks of age when the animals exhibited overt hyperglycemia (34), but hepatic expression of IRS-1 and IRS-2 returned to the normal level at 24 weeks of age (H. Sugita and M. Kaneki, unpublished observation). Thus, consistent with the prominent role of hepatic insulin resistance in hyperglycemia, hyperglycemia and its remission correlates with reduced IRS-1 and IRS-2 expression and its restoration in the liver. Because plasma insulin concentration increases as *ob/ob* mice age (34, 102), age-related remission of hyperglycemia may be explained by pancreatic β-cell compensation. However, age-related reversal of reduced hepatic IRS-1 and IRS-2 expression cannot be accounted for by pancreatic β-cell compensation, because hyperinsulinemia promotes reduction in IRS-1 and IRS-2 expression. Thus, it remains unknown how hepatic expression of IRS-1 and IRS-2 is restored in *ob/ob* mice at 24 weeks of age. Of interest, however, our preliminary results revealed that hepatic iNOS expression decreased as *ob/ob* mice age, and that hepatic iNOS expression did not differ between *ob/ob* and wild-type mice at 24 weeks of age (unpublished observation, M. Fujimoto and M. Kaneki), in contrast to increased hepatic iNOS expression at 11 weeks of age (34). Therefore, age-related alterations in iNOS expression also correlate with those in blood glucose and attenuated hepatic insulin signaling.

By contrast, iNOS expression remained elevated in the skeletal muscle of *ob/ob* mice at both 11 and 24 weeks of age (34, 102). Unlike that in the liver, IRS-1 expression and IRS-1–mediated insulin signaling were attenuated in the skeletal muscle of *ob/ob* mice at both 11 and 24 weeks of age. iNOS inhibition ameliorated impaired insulin signaling in the skeletal muscle at both 11 and 24 weeks of age in *ob/ob* mice, whereas in the liver, iNOS inhibition improved insulin signaling at 11, but not 24, weeks of age. Moreover, LPS induced increased iNOS expression in the liver and overt hyperglycemia, and iNOS inhibitor reversed LPS-induced hyperglycemia and elevated hepatic glucose output (103). Combining these findings, increased iNOS expression, overt hyperglycemia, and reduced IRS-1 and IRS-2 expression in the liver correlated quite well with each other in insulin resistance. It is tempting to speculate, therefore, that increased iNOS expression in the liver may be required for overt hyperglycemia and hepatic insulin resistance. Additional data will be needed to confirm this hypothesis.

MOLECULAR MECHANISMS UNDERLYING iNOS-MEDIATED INSULIN RESISTANCE

As described earlier, previous studies have consistently demonstrated that iNOS plays an important role in obesity-

induced insulin resistance in rodents. However, molecular mechanisms by which iNOS mediates insulin resistance remain largely unknown, although iNOS has been shown to impair insulin signaling at multiple levels (Fig. 2). Marette and his colleague (86) showed that iNOS disruption reversed high-fat diet–induced impaired insulin-stimulated tyrosine phosphorylation of IR and IRS-1, IRS-1–associated PI3K activity, and phosphorylation of Akt/PKB in skeletal muscle. In their study, IRS-1 expression was not altered by high-fat diet or iNOS deficiency. Saad and his colleagues (18) found that antisense oligonucleotide for iNOS reversed decreased insulin-stimulated tyrosine phosphorylation of IR and IRS-1 and phosphorylation of Akt/PKB in skeletal muscle of *ob/ob* mice. In addition, they found that iNOS knockdown restored decreased IRS-1 expression in skeletal muscle of *ob/ob* mice (18). Consistently, we demonstrated that iNOS disruption and iNOS inhibitor restored decreased IRS-1 expression in skeletal muscle of *ob/ob* mice to the normal level and ameliorated depressed IRS-1–mediated insulin signaling (34, 102). However, unlike that in the study by Saad and his colleagues (18), iNOS inhibition did not improve insulin-stimulated tyrosine phosphorylation of IR in skeletal muscle of *ob/ob* mice in our study (102). Moreover, we found the protective effects of iNOS inhibition on attenuated insulin signaling in liver as well as in skeletal muscle. iNOS inhibition increased expression of IRS-1 and IRS-2, and improved IRS-1– and IRS-2–mediated insulin signaling in the liver of *ob/ob* mice, in parallel with reversal of fasting hyperglycemia and improved whole-body insulin sensitivity (34), as discussed earlier.

In cultured skeletal muscle cells, iNOS and NO donor reduce protein expression of IRS-1 by promoting ubiquitination-mediated, proteasome-dependent degradation of IRS-1, but did not affect IRS-2 expression (102). The inhibitory effects of NO donor are cGMP independent and facilitated by concomitant oxidative stress. Although the precise molecular mechanisms are unknown, NO donor and insulin are considered to promote IRS-1 protein degradation through distinct pathways, because inhibitors for PI3K, JNK/SAPK, and the mammalian target of rapamycin blocked reduction of IRS-1 expression by insulin, but not by an NO donor (102). In cultured hepatocytes, iNOS and an NO donor reduced both IRS-1 and IRS-2 protein expression (34). Thus, the effects of iNOS and an NO donor on IRS-2 expression are cell-type specific; it was reduced by iNOS and NO donor in hepatocytes, but not in skeletal muscle cells. In regard to mRNA levels, iNOS and NO donor did not affect mRNA of IRS-1 in both skeletal muscle and liver, whereas IRS-2 mRNA level was decreased by NO donor or iNOS in liver. The inhibitory effects of NO donor on IRS-1 and IRS-2 in hepatocytes are also cGMP independent and enhanced by concomitant oxidative stress. NO donor increased sterol regulatory element–binding protein (SREBP)-1c mRNA expression in cultured hepatocytes, as well. Consistently, iNOS inhibitor reduced SREBP-1c mRNA level in the liver of *ob/ob* mice (34). A previous study showed that SREBPs negatively regulate transcription of IRS-2 in liver (51), whereas transcription of genes involved in the lipid synthesis are upregulated by SREBPs. One can reasonably speculate, therefore, that iNOS may decrease IRS-2 expression, at least in part, by upregulating SREBP-1c expression, which reduces the transcription of IRS-2 in the liver. However, clarification of precise mechanisms by which iNOS reduces hepatic IRS-2 expression awaits further investigations.

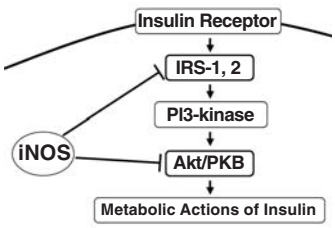

FIG. 2. iNOS impairs insulin signaling at multiple levels. iNOS has been shown to attenuate insulin-stimulated signal transduction at the levels of multiple components of insulin signaling, including IRS-1, IRS-2, and Akt/PKB in cultured cells and *in vivo* in obese, diabetic mice.

iNOS VERSUS cNOSs

Aforementioned results clearly revealed the insulin-desensitizing action of iNOS. However, this notion may not be generalized to the relation between insulin sensitivity and NO produced by NOS isoenzymes other than iNOS. Actually, the effects of NO on insulin sensitivity do not seem to be that simple, and rather differ dependent on the source of NO, although the role of iNOS in insulin resistance is obvious. NO is synthesized by three distinct genes, neuronal nitric oxide synthase (nNOS, also termed NOS1), endothelial nitric oxide synthase (eNOS, also termed NOS3), and iNOS. Although iNOS expression is induced by various inflammatory/stress signaling, nNOS and eNOS are constitutively expressed and thus categorized as constitutive nitric oxide synthases (cNOSs).

Of interest, and contrary to iNOS, gene disruption of eNOS or nNOS leads to insulin resistance (25, 96), although the effects of eNOS or nNOS deficiency alone on insulin sensitivity were relatively mild. With a high-fat diet, haplodeficiency of eNOS enhanced insulin resistance and increased plasma insulin concentration with an unaltered blood glucose level (19). However, hepatic glucose output was not elevated, and the authors concluded that whole-body insulin resistance in eNOS heterozygous knockout mice was mostly accounted for by decreased glucose uptake in peripheral tissue (19). Likewise, nonselective NOS inhibitors that block NO generation by both cNOSs and iNOS caused insulin resistance in normal rodents (8, 75, 95, 111), although controversial results were also reported about the effects of nonselective NOS inhibitors (5, 13, 14, 30, 75, 90, 92, 93, 106). The insulin-desensitizing effects of nonselective NOS inhibitors are quite a contrast to the improved insulin sensitivity and reversal of hyperglycemia by iNOS inhibitor in *ob/ob* mice. Thus, the effects of iNOS and cNOSs are obviously opposite, although all of the NOSs generate the same molecule, NO. It is noteworthy, however, that prolonged treatment with nonselective NOS inhibitor was reported to increase iNOS expression (68), and that pulmonary iNOS expression, but not nNOS expression,

was increased in eNOS knockout mice in association with increased exhaled NO concentration (20). However, the iNOS expression level in insulin-sensitive tissues of eNOS or nNOS knockout mice was not determined in the previous studies (19, 25, 96). Conversely, eNOS and nNOS expression were not altered by iNOS disruption in skeletal muscle, liver, and adipose tissue (34, 86, 102). One cannot rule out the possibility, therefore, that iNOS expression, which might be increased by eNOS or nNOS deficiency, could also contribute to insulin resistance in eNOS or nNOS knockout mice. Furthermore, NO itself induces glucose uptake independent of insulin or insulin signaling in cultured cells (6, 46, 56) and *in vivo* in rodents (6) and humans (45). NO has been proposed to mediate concentration (exercise)-induced glucose uptake in skeletal muscle (6), a major noninsulin stimulus for glucose transport, although it is controversial (46). Therefore, it is possible that NO produced by eNOS or nNOS might contribute to maintaining normal glycemic control and circulating insulin concentration by increasing insulin-independent glucose uptake. However, an impact of insulin-independent glucose uptake on *in vivo* insulin sensitivity, which is assessed by euglycemic hyperinsulinemic clamp or insulin tolerance test, is elusive.

To date, this dichotomy between iNOS and cNOSs has not been extensively discussed, and no hypothesis that can account for the differential roles of iNOS and cNOSs has been thus far proposed. However, it is conceivable that the difference may be related to the Janus-faced nature of actions of NO. Insulin resistance is not the only exception of such a functional disparity between iNOS and cNOSs. Indeed, iNOS and cNOSs function in biologically opposite directions in a variety of disease pathophysiology. In hypertension, atherosclerosis, heart failure, and stroke, iNOS has been implicated as pathogenic, whereas eNOS functions as protective.

PROTEIN *S*-NITROSYLATION

NO is a gaseous signaling molecule that is involved in a broad array of physiologic and pathologic processes in every organ in mammals. Effects of NO can be both protective and toxic to cells, depending on the nature of the NO and NO-derived species, the surrounding intracellular milieu, and the cellular context. A wide variety of actions of NO can be classified into two categories: (a) cGMP-dependent actions that are mediated by authentic NO and well exemplified by NO-mediated vasodilation, and (b) cGMP-independent effects that are in many cases mediated by nitrosative posttranslational modifications such as protein *S*-nitrosylation and tyrosine nitration. Reactive nitrogen species (RNS), such as peroxynitrite ($OONO^-$), nitrosonium ion (NO^+) equivalent, nitroxyl anion (NO^-), and dinitrogen trioxide (N_2O_3), are usually involved in nitrosative posttranslational modifications. Protein *S*-nitrosylation is a covalent attachment of nitrosonium ion (NO^+) to reactive thiol sulfhydryls and a prototype of redox-dependent posttranslational modifications. *S*-nitrosylation has recently emerged as a major mediator of NO biology (114). More than 100 proteins have been shown to be *S*-nitrosylated *in vitro* and in intact cells (101). In many cases, *S*-nitrosylation is associated with functional alterations of proteins, including those in enzymatic activities (63, 116), subcellular localization (44), and protein–protein interaction (44, 72). Many proteins contain multiple cysteine residues, but one or a few cysteines among them are sensitive to *S*-nitrosylation (101). For example, in human p21Ras that contains four cysteines, only cysteine 118 can be *S*-nitrosylated, leading to activation of p21Ras (63). Unlike phosphorylation, no particular consensus primary amino acid sequence has been established as a motif for *S*-nitrosylation (2, 42). Instead, three-dimensional structural features of the proteins have been proposed to be an important determinant of sensitivity of cysteine residues to *S*-nitrosylation (42).

S-nitrosylation can be regulated by proteins, such as ceruloplasmin (64), and electron acceptors, such as metal ions (40) and iron nitrosyl complex (113) in cells. However, no enzymatic activity is required for *S*-nitrosylation. In contrast, an enzyme negatively regulates *S*-nitrosylation in cells. Stamler and his colleagues (54, 66) revealed that glutathione-dependent formaldehyde dehydrogenase (also known as alcohol dehydrogenase class III) functions as *S*-nitrosoglutathione reductase (GSNOR), which decomposes *S*-nitrosylation of *S*-nitrosoglutathione (GSNO), exhibiting much greater catalytic activity toward GSNO than other substrates, including formaldehyde (48, 54, 66). Because GSNO and protein *S*-nitrosylation are in equilibrium, decomposition of GSNO results in decrease in *S*-nitrosylated proteins in cells (43, 67). Hence, *S*-nitrosylation/denitrosylation is coming to be recognized as a specific component of signal transduction, comparable to phosphorylation/dephosphorylation. Under normal, unstressed condition, *S*-nitrosylated proteins exist in cells and extracellular space, to which NO generated by eNOS and nNOS mainly contributes. However, once iNOS is induced by various stimuli, NO and NO-related species produced by iNOS further increase *S*-nitrosylation. NO generation by eNOS and nNOS requires calcium ion (Ca^{2+}) and is also regulated by phosphorylation, in addition to L-arginine, a substrate, and tetrahydrobiopterin, a cofactor for NO generation. In contrast, iNOS does not require Ca^{2+} or posttranslational modification (*e.g.*, phosphorylation) to produce NO, once induced. Therefore, iNOS induction results in a prolonged, exaggerated production of NO, up to >1,000-fold compared with eNOS and nNOS (78). On exposure to excess amounts of NO, the cGMP-dependent signaling pathway is readily downregulated (33). Increased iNOS expression is often, but not always, associated with attenuated cGMP-mediated signaling despite elevated NO production. However, such a negative feedback mechanism is unknown for *S*-nitrosylation. Thus, increased iNOS expression and subsequent overproduction of NO cause enhanced *S*-nitrosylation, whereas cGMP-mediated signaling is often impaired. It seems the case with type 2 diabetes, in which iNOS expression is increased. *S*-nitrosylated hemoglobin, reactive nitrogen species (RNS), and tyrosine nitration are elevated in patients with type 2 diabetes (39, 109), whereas cGMP-mediated signaling is impaired (23, 117). Our preliminary data showed that *S*-nitrosylated proteins were increased in the liver of *db/db* mice compared with wild-type mice (unpublished observation, N. Shimizu and M. Kaneki). Based on these observations, we hypothesized that increased *S*-nitrosylation plays a role in obesity-related, iNOS-involved insulin resistance.

A number of enzymes that are involved in glucose metabolism are *S*-nitrosylated *in vitro*, in cultured cells, and *in vivo*. These enzymes include glyceraldehyde-3-phosphate dehydrogenase (44), glucokinase (89), and aldose reductase (99). We have shown that Akt/PKB is reversibly inactivated by *S*-nitrosylation *in vitro* and in intact cells (116). Among seven cysteine residues in human Akt1/PKBα, cysteine 224 is a major *S*-nitrosylation acceptor site. Importantly, *S*-nitrosylated Akt/PKB was increased in skeletal muscle of *db/db* mice compared with wild-type mice, and *in vitro* incubation with a reducing agent, dithiothreitol (20 m*M*), partially restored attenuated insulin-stimulated Akt/PKB activity in *db/db* mice, but not in wild-type mice. Furthermore, Saad and his colleagues demonstrated that IR, IRS-1 and Akt/PKB were *S*-nitrosylated in skeletal muscle of *ob/ob* mice (18) and LPS-administered mice (17), which was reverted by antisense oligonucleotide for iNOS. A thiazolidinedione, rosiglitazone, also reversed increased *S*-nitrosylation of IR, IRS-1 and Akt/PKB in *ob/ob* mice, in association with suppression of iNOS expression (18). Collectively, these findings indicate that the role of *S*-nitrosylation in insulin resistance deserves further investigation.

In contrast, it is possible that cGMP-mediated signaling may be essential for the protective effects of eNOS and nNOS on insulin sensitivity, as is the case with the salutary effects of eNOS in vasculature. It is noteworthy that the insulin-sensitizing effects of eNOS and nNOS have been thus far found systemically only *in vivo*, but not in cultured cells. Whereas several independent groups demonstrated that NO donor and iNOS induced insulin resistance in cultured skeletal muscle cells, adipocytes and hepatocytes (3, 10, 18, 34, 56, 102, 116), insulin-sensitizing effects of NO or cGMP have not been reported *in vitro* or in cultured cells. Moreover, neither nonselective NOS inhibitor nor eNOS heterozygous knockout impaired insulin-stimulated glucose uptake *ex vivo* in skeletal muscle preparations of normal rats (4) and mice fed normal chow and high-fat diet (19). These findings of the systemic effects suggest the possibility, therefore, that the cardiovascular and/or nervous system may be required for the beneficial effects of eNOS and nNOS. In line with this, Baron and his colleagues (95) showed that a much smaller dose of nonselective NOS inhibitor was sufficient to induce whole-body insulin resistance when injected into intracerebroventicular space, as compared with systemic administration. Microcirculation (61), sympathetic activity (94), and vagus nerve–mediated neurotransmission (112) have been shown to modulate insulin sensitivity, although limited knowledge is currently available about their roles in obesity-induced insulin resistance and type 2 diabetes. The NO-cGMP signaling plays an essential role in microcirculation, neurotransmission, and inhibition of sympathetic nerve activity. In eNOS knockout mice, insulin resistance was associated with impaired muscle blood flow (25). In addition, eNOS heterozygous knockout mice exhibited sympathetic overactivity compared with wild-type mice (19), suggesting that reduced NO-cGMP signaling by eNOS haplodeficiency may contribute to insulin resistance, in part, by increasing sympathetic activity. These observations seem consistent with the hypothesis that cGMP may mediate the protective effects of eNOS and nNOS on insulin sensitivity. Nonetheless, a possible role of cGMP-mediated signaling in whole-body insulin sensitivity remains an open issue.

OXIDATIVE STRESS AND NITROSATIVE STRESS

Both reactive oxygen species (ROS) and RNS are important functional molecules that transduce intracellular signals in various biologic processes, including insulin-stimulated glucose uptake, under physiologic conditions. Conversely, excessive production of ROS and RNS, or failure of cellular defense mechanisms against deleterious effects of ROS and RNS, or the combination of both leads to deleterious or disadvantageous biologic consequences and is referred to as oxidative and nitrosative stress. Oxidative and nitrosative insults share common molecular events and pathophysiologic consequences, although, depending on the cellular context, oxidative and nitrosative stress can be distinct in biologic outcomes as well as in chemical reactions. Moreover, most defense mechanisms against ROS and RNS are common; these include superoxide dismutases, catalase, glutathione peroxidase, glutathione/glutathione reductase, and thioredoxin/thioredoxin reductase. Hence, oxidative and nitrosative stress can extensively cross-talk with each other in many ways at various levels. For instance, ROS induces iNOS expression, and superoxide (O_2^-) readily reacts with NO, leading to generation of peroxynitrite (27), a highly reactive RNS. Pathophysiologic levels of concomitant oxidative stress facilitate *S*-nitrosylation (31). RNS increases ROS generation by mitochondria (41). RNS can cause oxidative protein modifications, including formation of disulfide bond and sulfonic acid (70). Glutathione reductase is reversibly inactivated by *S*-nitrosylation (15). Depletion of glutathione also results in impaired decomposition of GSNO by GSNOR (22, 43). Because of such a wide variety of in-depth interactions, oxidative and nitrosative stress can often be difficult to differentiate from each other. In reality, oxidative and nitrosative always coexist, and presumably work in concert and/or in a synergistic manner under most, if not all, pathophysiologic conditions. The results of an earlier study seems consistent with a potential synergistic action between NO and oxidative stress on insulin sensitivity. Infusion of an NO donor resulted in decreased insulin-stimulated glucose disposal in elderly healthy subjects, although an impaired insulin response was not observed in young healthy subjects (74). It is conceivable that increased oxidative stress in the elderly might contribute to insulin resistance by an NO donor, which was not found in the young individuals.

Reflecting the great extent of overlap, the definition of oxidative and nitrosative stress appears to be somewhat confusing. The original concepts of them were stressful conditions induced by ROS and RNS. However, precise detection of ROS and RNS is problematic (108) and in reality almost impossible *in vivo*. Thus, another part of the definition could be the stress mediated by oxidative and nitrosative modifications of macromolecules, including proteins, lipids, and DNA, in cells.

Oxidative stress has been considered to be involved in obesity-induced insulin resistance. Several lines of evidence

indicate a role of oxidative stress in insulin resistance. Increased oxidative stress is associated with obesity and type 2 diabetes (35). In cultured cells, ROS impairs insulin response (80), and antioxidants rescue insulin resistance (29, 57). Antioxidants, such as α-lipoic acid, exerted beneficial effects in rodent models of obesity-induced insulin resistance (7, 60) and in patients with type 2 diabetes (53, 83). An inhibitor of NADPH oxidase increased plasma adiponectin concentration, an insulin-sensitizing adipokine, and decreased blood glucose and insulin levels in obese KKAy mice (35). In a recent study (49), a cell-permeable superoxide dismutase mimetic, MnTBAP, was used to investigate a role for oxidative stress, because α-lipoic acid *per se* induces insulin-independent glucose uptake via a mechanism unrelated to the antioxidant property (57). Lander and his colleagues (49) demonstrated that MnTBAP significantly ameliorates insulin resistance and hyperglycemia in *ob/ob* mice, although serum insulin concentration was not decreased by MnTBAP (49). These findings clearly indicate that oxidative stress has an important role in the pathogenesis of obesity-related insulin resistance. However, the beneficial effects of MnTBAP appeared to be possibly milder compared with the insulin-sensitizing effects of iNOS inhibition, based on the observations that iNOS inhibitor also reduced plasma insulin concentration in addition to the reversal of hyperglycemia in *ob/ob* mice (34), and that iNOS deficiency completely blocked high-fat diet–induced hyperglycemia and hyperinsulinemia (79, 86). Parenthetically, insulin also stimulates ROS generation, which is physiologically required for insulin signaling (69). In line with this, overexpression of glutathione peroxidase, which reduces hydrogen peroxide (H_2O_2) and organic hydroperoxides, resulted in insulin resistance, in association with reduced oxidative stress (73). Taken together, these findings suggest that not only increased ROS but also overquenching ROS may reduce insulin sensitivity.

Considering in-depth cross-talks and a broad range of overlap between oxidative and nitrosative stress, it is reasonably conceivable that oxidative stress and iNOS may exert insulin-desensitizing effects via both shared and distinct mechanisms. Oxidative stress contributes to the development of insulin resistance, possibly in part by enhancing *S*-nitrosylation. Conversely, increased iNOS expression and *S*-nitrosylation lead to accentuated oxidative stress. Thus, interactions and biologic outcome of oxidative and nitrosative stress are indeed complex, and biologic consequences of them are often indistinguishable. Proteomic analyses of nitrosative and oxidative protein modifications and their implications in the pathogenesis of insulin resistance are expected to help us understand more precisely the molecular mechanisms underlying obesity-induced insulin resistance and type 2 diabetes.

Oxidative stress has been proposed to cause and/or exacerbate insulin resistance by activating inflammatory/stress signaling, particularly JNK/SAPK (11, 49). It is possible, therefore, that iNOS may function as a downstream effector of oxidative stress–induced inflammatory/stress response. Conversely, given the similarity between oxidative and nitrosative stress in various biologic conditions, it is reasonable to speculate that, like oxidative stress, iNOS and resultant nitrosative stress may also contribute to the development of insulin resistance by enhancing sustained activation of inflammatory/stress-signaling pathways (Fig. 3). *S*-nitrosylation plays an essential role in nitrosative stress (59), as well as in physiologic processes. In agreement with this, iNOS and NO donors induce and/or amplify ER stress (81), JNK/SAPK (87), and the IKK-NF-κB (120) in various cells types, although controversial results were also reported in regard to JNK/SAPK (84) and the IKK-NF-κB activation (71). A recent study showed that *S*-nitrosylation of protein disulfide isomerase, which is an important enzyme for ER function, causes ER stress in neuronal cells and promotes neurodegeneration (110), indicating a direct role of *S*-nitrosylation in ER stress. Regardless, the effects of iNOS and NO donor on these inflammatory/stress-signaling pathways have not yet been investigated in skeletal muscle cells, hepatocytes, or adipocytes. Such a possibility remains an open issue.

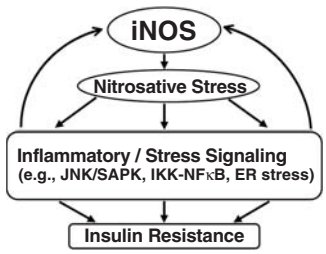

FIG. 3. iNOS as a potential enhancer of sustained activation of inflammatory/stress signaling pathways, establishing a vicious cycle. Activation of the IKK–NFκB pathway, JNK/SAPK, and ER stress response has been shown to play a critical role in obesity-induced insulin resistance. However, it remains to be clarified how activation of these signaling pathways is initiated and sustained. In addition to the functional role of iNOS as a downstream effector of inflammatory response and stress signaling, as shown in Fig. 1, iNOS may also contribute to the sustained activation of multiple stress-signaling pathways as an upstream enhancer.

CONCLUSIONS

iNOS has emerged as an important player in the pathogenesis of obesity-related insulin resistance. Although the molecular mechanisms responsible for iNOS-mediated insulin resistance remain to be elucidated, the role of protein *S*-nitrosylation has been proposed as a mediator of obesity-induced insulin resistance and type 2 diabetes. Several lines of circumstantial evidence suggest that iNOS may function as both a downstream effector and an upstream amplifier of sustained activation of inflammatory/stress-signaling pathways, forming a vicious cycle, which causes and/or exacerbates insulin resistance (see Figs. 1 and 3). These possibilities await further investigation.

ABBREVIATIONS

cGMP, cyclic guanosine monophosphate; CRP, c-reactive protein; eIF2α, eukaryotic initiation factor-2α; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum;

Grp78, glucose-regulated protein; IKK, inhibitory κB kinase; iNOS, inducible nitric oxide synthase; IR, insulin receptor; IRS, insulin receptor substrate; JNK/SAPK, c-Jun amino-terminal kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PERK, PKR-like kinase; PI3K, phosphatidylinositol-3 kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SREBP, sterol regulatory element–binding protein; XBP1, X-box–binding protein 1.

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