Inducible Nitric-oxide Synthase and NO Donor Induce Insulin Receptor Substrate-1 Degradation in Skeletal Muscle Cells*

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Chronic inflammation plays an important role in insulin resistance. Inducible nitric-oxide synthase (iNOS), a mediator of inflammation, has been implicated in many human diseases including insulin resistance. However, the molecular mechanisms by which iNOS mediates insulin resistance remain largely unknown. Here we demonstrate that exposure to NO donor or iNOS transfection reduced insulin receptor substrate (IRS)-1 protein expression without altering the mRNA level in cultured skeletal muscle cells. NO donor increased IRS-1 ubiquitination, and proteasome inhibitors blocked NO donor-induced reduction in IRS-1 expression in cultured skeletal muscle cells. The effect of NO donor on IRS-1 expression was cGMP-independent and accentuated by concomitant oxidative stress. Inhibitors for phosphatidylinositol-3 kinase, mammalian target of rapamycin, and c-Jun amino-terminal kinase failed to block NO donor-induced IRS-1 reduction, whereas these inhibitors prevented insulin-stimulated IRS-1 decrease. Moreover, iNOS expression was increased in skeletal muscle of diabetic (ob/ob) mice compared with lean wild-type mice. iNOS gene disruption or treatment with iNOS inhibitor ameliorated depressed IRS-1 expression in skeletal muscle of diabetic (ob/ob) mice. These findings indicate that iNOS reduces IRS-1 expression in skeletal muscle via proteasome-mediated degradation and thereby may contribute to obesity-related insulin resistance.

Insulin resistance, reduced cellular responsiveness to insulin, is a major causative factor for type 2 diabetes, which accounts for over 90% of patients with hyperglycemia. Chronic, low grade inflammation has been implicated in type 2 diabetes and other human diseases including atherosclerosis, neurodegenerative disorders, and cancer. Although the molecular bases underlying the pathogenesis of chronic inflammation-mediated diseases remains to be determined, inducible nitric-oxide synthase (iNOS), a mediator of inflammation, has been shown to be involved in atherosclerosis (1), neurodegenerative disorders (2), and cancer (3). Recently accumulating evidence indicates a close link between iNOS and insulin resistance. Most, if not all, inducers of insulin resistance increase iNOS expression. These inducers of insulin resistance include obesity (4), free fatty acids (5), hyperglycemia (6, 7), tumor necrosis factor-α, oxidative stress, endotoxin, and burn injury. iNOS mediates the impaired insulin-stimulated glucose uptake by treatment with tumor necrosis factor-α and lipopolysaccharide in cultured muscle cells (8). iNOS expression is elevated in skeletal muscle of patients with type 2 diabetes (9, 10) and high fat diet-induced diabetic mice (11). Moreover, we demonstrated that iNOS inhibitor prevents lipopolysaccharide-induced insulin resistance in rats (12). Perreault and Mareette (11) showed that the disruption of iNOS gene protects against high fat diet-induced insulin resistance in mice. However, how iNOS causes or exacerbates insulin resistance remains largely unknown.

Insulin receptor substrate (IRS)-1 is a key molecule in insulin signaling that transduces a signal from insulin receptor (IR) to phosphatidylinositol-3-kinase (PI3K) (13, 14). This pathway plays a central role in metabolic actions of insulin, including stimulation of glucose uptake, synthesis of glycogen and protein, and inhibition of gluconeogenesis (15). Gene knock-out of IRS-1 causes insulin resistance in mice (16, 17), and tissuespecific gene knock-out of IRS-1 and IRS-2 revealed that IRS-1, but not IRS-2, plays a prominent role in metabolic actions of insulin in skeletal muscle (18–20), which is the major site of glucose utilization. In humans, polymorphism of IRS-1 is assumed to have a pathogenic role in the development of type 2 diabetes (18).

Several lines of evidence indicate an important role for reduced IRS-1 expression in insulin resistance and type 2 diabetest. IRS-1 expression is decreased in rodent models of obesity-related insulin resistance and type 2 diabetes, including ob/ob mice (21, 22), gold-thioglucose-induced obese mice (23), high fat diet-fed rats (24), Zucker fatty rats (25), and obese spontaneously hypertensive rats (26). Importantly down-regulated pro-

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S The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. 1–3.

S The abbreviations used are: iNOS, inducible nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSNO, S-nitrosoglutathione; IR, insulin receptor; IRS, insulin receptor substrate; JNK/ASK, c-Jun amino-terminal kinase (also termed stress-activated protein kinase); mTOR, mammalian target of rapamycin; nNOS, neuronal nitric-oxide synthase; NOS, nitric-oxide synthase; P38K, phosphatidylinositol 3-kinase; SNAP, S-nitroso-N-acetylpenicillamine; t-NIL, 1-N^2-homoarginine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-c]quinazolin-1-one; PKB, protein kinase B; VASP, vasodilator-stimulated phosphoprotein; BW, body weight; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propane sulfonic acid; PKG, cGMP-dependent protein kinase.

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tein expression of IRS-1 was also observed in skeletal muscle and adipose tissue of patients with diabetes (27–30) and was proposed to be a marker to identify individuals at risk for overt diabetes (27, 31). Although recent studies revealed that insulin decreases IRS-1 expression via ubiquitin-mediated degradation in cultured adipocytes and hepatoma cells (32–37), the molecular mechanisms underlying reduced IRS-1 expression in insulin resistance and type 2 diabetes in vivo still remain to be clarified.

NO is a signaling molecule that is involved in a variety of physiological and pathological cellular processes in various tissues including vasculature, central nervous system, and skeletal muscle (38). NO is produced by three distinct genes: neuronal and endothelial nitric oxide synthases (nNOS and eNOS, also termed NOS1 and NOS3, respectively) and iNOS (also termed NOS2). In contrast to the activities of nNOS and eNOS that are tightly regulated by calcium-dependent calmodulin binding, iNOS does not require calcium ion or posttranslational modification for its activity. Therefore, iNOS expression is associated with prolonged, exaggerated NO generation up to over 1,000-fold compared with nNOS and eNOS (39, 40). Although iNOS expression is increased by various stimuli including acute inflammation, recent studies revealed that iNOS is expressed even in normal conditions in many tissues including skeletal muscle (11, 41–44). Diverse actions of NO can be classified into two categories: cGMP-dependent actions that are well exemplified by NO-mediated vasodilatation and cGMP-independent effects. In most cases, NO and NO-related compounds exert cGMP-independent effects through reactive nitrogen species-mediated nitrosative modifications of proteins, lipids, or DNA (45, 46). Concomitant oxidative stress facilitates cGMP-independent nitrosative modifications (40) but inhibits cGMP-dependent actions of NO (47). Of clinical importance, reactive nitrogen species and nitrosative protein modifications such as S-nitrosylation and tyrosine nitration are up-regulated in patients with diabetes (9, 10, 48–50), while cGMP-mediated signaling is impaired (51, 52).

Here we demonstrate that exposure to NO donor or transfection of iNOS caused a reduction in IRS-1 protein expression in a proteasome-dependent manner without altering the mRNA level of IRS-1. Furthermore, we found that protein expression of iNOS was increased in skeletal muscle of genetically obese (ob/ob) mice compared with lean wild-type mice and that iNOS gene disruption improved the reduced IRS-1 expression in skeletal muscle of ob/ob mice. These data provide new insights into the molecular bases underlying chronic inflammation–involved pathogenesis of insulin resistance.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lactacystin, MG132, cycloheximide, rapamycin, wortmannin, and SP600125 (Calbiochem); 8-bromo-cGMP, S-nitrosoglutathione (GSNO), S-nitroso-N-acetylpenicillamine (SNAP), ODQ, and 1-NIL (Cayman Chemical, Ann Arbor, MI); insulin, glucose oxidase, serum at 37 °C under a humidified atmosphere of 5% CO2. To induce differentiation into myotubes, after C2C12 myoblasts became confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum for 7 days.

**Cell Culture**—Mouse C2C12 myoblasts were obtained from American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C under a humidified atmosphere of 5% CO2. To induce differentiation into myotubes, after C2C12 myoblasts became confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum for 7 days.

C2C12 myotubes were treated with the indicated concentrations of GSNO (10–1000 μM) or insulin (100 μM) in the presence or absence of wortmannin (100 nM), rapamycin (50 nM), SP600125 (25 μM), glucose oxidase (1 or 3.3 milliunits/ml), or ODQ (1 μM) for 24 h unless otherwise indicated. SNAP (500 μM), whose half-life is shorter than that of GSNO, was added twice to the culture media at 12-h intervals during the incubation for 24 h.

**Cell Transfection**—C2C12 myoblasts were transfected with pcDNA3/iNOS or pcDNA3 using Lipofectamine 2000 (Invitrogen). pcDNA3/iNOS (53) was kindly provided by Dr. B. C. Kone. At 24 h after transfection, the cells were harvested.

Animals—iNOS knock-out (−/−) mice, genetically obese (ob/ob) mice, and wild-type C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). iNOS−/− mice and ob/ob mice were backcrossed onto wild-type C57BL/6J mice at least 10 and 30 generations, respectively. The Institutional Animal Care Committee approved the IL, respectively. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice were housed in mesh cages in a room maintained at 25 °C and illuminated in 12:12-h light-dark cycles; the mice were provided with standard rodent chow and water ad libitum.

We mated heterozygous female +/ob C57BL/6J mice with male iNOS−/− C57BL/6J mice to obtain double heterozygotes with the genotype iNOS+/−/+ob. Mature interlitters of iNOS+/−/+ob, and iNOS+/+ob C57BL/6J mice, which were generated by intercrossing double heterozygous iNOS+/−/+ob mice, were used for the study. Blood samples were collected under fed condition to measure glucose and insulin levels. The concentrations of blood glucose and plasma insulin were measured by Glucometer Elite (Bayer, Elkhart, IN) and enzyme-linked immunosorbent assay (Crystal Chem, Chicago, IL), respectively. After an overnight fast, under anesthesia with pentobarbital sodium (70 mg/kg BW, intraperitoneal), wild-type, iNOS+/+ob, and iNOS−/− ob/ob mice at 24 weeks of age received the insulin injection (humulin R, Eli Lilly, 5 units/kg BW) via the portal vein at 5 min after insulin injection, skeletal muscle (gastrocnemius) was taken for biochemical analyses.

**Genotype Determination**—Genotype was determined by PCR analysis. Genomic DNA was extracted from the tip of the tail. The primers and PCR conditions were as follows. For genotyping of iNOS gene, the sense primer was 5′-ACA TGCGAATGAGTACC GG-3′, and the antisense primer was 5′-TCAACATCTCTGGTGGAAC-3′ for wild-type allele. The allele primer was 5′-ATTATGGGAA GTTGACCTCG-3′ for iNOS knock-out allele. The PCR analysis began with denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C each for 1 min with a final extension at 72 °C for 10 min. The wild-type allele gave 108 base pairs, and the recombinant allele gave 270 base pairs. For genotyping of leptin gene, sense primer 5′-TGACCTGGGAATCTCTCC-3′ and antisense primer 5′-CATCCAGGCTCTTGCGG-3′ for wild type were used. Sense primer 5′-TGACCTGGGAATCTCTCC-3′ and antisense primer 5′-CATCCAGGCTCTTGCGG-3′ for wild type were used. The PCR analysis began with denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C each for 30 s with a final extension at 72 °C for 2 min. Both wild-type and mutant alleles of leptin gene gave 100 base pairs.

**Insulin Tolerance Test**—Insulin (15 units/kg BW) was intraperitoneally administered to fed ob/ob mice at the age of 23 weeks. Blood samples were collected at 0, 15, 30, 60, 90, and 120 min after insulin injection.

**1-NIL Treatment**—Obese (ob/ob) mice at 18 weeks of age received the intraperitoneal injection of iNOS inhibitor 1-NIL (80 mg/kg BW) or phosphate-buffered saline twice (every 12 h) daily for 10 days. After the treatment, following an overnight fast, the animals were anesthetized with pentobarbital sodium (70 mg/kg BW, intraperitoneal), and then skeletal muscle (gastrocnemius) was taken for biochemical analyses.

**Tissue and Cell Homogenates**—Tissue samples were homogenized as described previously (12). Briefly tissues were powdered under liquid nitrogen and homogenized in ice-cold buffer (50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Following the incubation on ice for 30 min, the homogenized samples were centrifuged at 13,000 × g for 30 min.

Cell lysates were obtained as described previously (54). Briefly cells were lysed with cell lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μM β-glycerophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM dithio-
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Fig. 1. The effects of NO donor or ectopic expression of iNOS on IRS-1 expression. A, C2C12 myotubes were treated with the indicated concentrations of NO donors GSNO or SNAP (500 μM) for 24 h. GSNO reduced IRS-1 expression in a dose-dependent manner. SNAP also decreased IRS-1 expression. However, the expressions of p85 PI3K, Akt/PKB, α-sarcomeric actin, and myosin heavy chain, however, were not affected. B, GSNO (1 mM) reduced IRS-1 expression in a time-dependent manner. C, while NO donor GSNO reduced IRS-1 expression, treatment with GSH or GSSG for 24 h did not decrease IRS-1 expression in C2C12 myotubes. D, after treatment with or without GSNO (1 mM) for 24 h, C2C12 myotubes were serum-starved for 2 h and then exposed to insulin (100 nm) for 1 min. Immunoprecipitation (IP) with anti-IRS-1 antibody followed by immunoblotting (IB) with anti-phosphotyrosine (PY) and IRS-1 antibodies revealed that reduced IRS-1 expression by NO donor was accompanied by attenuated insulin-stimulated tyrosine phosphorylation of IRS-1. E, PI3K activity was evaluated by in vitro phosphorylation of phosphatidylinositol-3-phosphate (PIP). Insulin-stimulated IRS-1-associated PI3K activity was reduced in C2C12 myotubes treated with GSNO (1 mM) for 24 h compared with untreated cells. F, C2C12 myoblasts were transfected with mammalian expression vector containing cDNA for iNOS or vector alone. At 24 h after transfection, IRS-1 expression was significantly decreased in the cells transfected with iNOS compared with vector alone, while p85 PI3K expression was unaltered.

RESULTS

Proteasome-dependent Degradation of IRS-1 by NO Donor or iNOS Transfection in Cultured Skeletal Muscle Cells—Differentiated C2C12 myotubes were treated with NO donor. NO donor GSNO decreased IRS-1 expression in a dose- and time-dependent manner in C2C12 myotubes (Fig. 1, A and B). GSNO at concentrations of 100 μM and 1 mM effectively reduced IRS-1 protein expression (Fig. 1A). GSNO (1 mM) decreased protein expression of IRS-1 at 3 h and beyond (Fig. 1B). In contrast, reduced and oxidized glutathione (1 mM) failed to decrease IRS-1 expression (Fig. 1C), indicating the specificity of the effects of GSNO.

Northern Blotting—Total RNA was prepared using TRIzol (Invitrogen) according to the manufacturer’s instructions. Northern hybridization was performed as described previously (55) using 32P-labeled cDNA probes for IRS-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). cDNA probes for IRS-1 (56) and GAPDH (55) were kindly provided by Drs. K. Ueki and K. Yokote, respectively. The probes were labeled with [32P]dCTP (PerkinElmer Life Sciences) using a random primer DNA labeling kit version 2 (Panvera-TAKARA, Madison, WI).

Phosphatidylinositol 3-Kinase Assay—PI3K activity in the immunoprecipitates with anti-IRS-1 antibody was measured by in vitro phosphorylation assay using phosphatidylinositol (Sigma) as a substrate as described previously (57) with minor modifications. Briefly 5 μl of 100 μM MgCl₂ and 10 μl of phosphatidylinositol (2 mg/ml) sonicated in kinase buffer (10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1 mM sodium vanadate) were added to the immunoprecipitates. The PI3K reaction was initiated by the addition of 1.5 μl of 1.5 mM ATP containing 10 μCi of [γ-32P]ATP. After incubation for 10 min at 37 °C, the reaction was stopped by the addition of 10 μl of 6 n HCl and 80 μl of CHCl₃/methanol (1:1). The samples were centrifuged, and the lower organic phase was applied to a silica gel TLC plate (Sigma), which had been prebaked for 1 h. The plate was developed in CHCl₃/CH₃OH/H₂O/NH₄OH (100:78:19:3.3), dried, and visualized by autoradiography.

Statistical Analysis—The data were compared using one-way analysis of variance followed by Fisher’s protected least significant difference test. A value of p < 0.05 was considered statistically significant. All values are expressed as mean ± S.E.
Decreased IRS-1 expression by GSNO was associated with attenuated tyrosine phosphorylation in response to insulin in C2C12 myotubes (Fig. 1D). Insulin-stimulated P13K activity was also decreased by GSNO treatment (Fig. 1E). NO donors, however, did not affect the expression of p85 P13K, Akt/PKB, α-sarcomeric actin, and myosin heavy chain (Fig. 1A) or the morphology of C2C12 myotubes (data not shown). Treatment with GSNO (1 mM) or SNAP (500 μM) for 24 h did not affect viability of the cells as judged by trypan blue exclusion test.

These findings indicate that NO donor caused a reduction in IRS-1 protein expression without altering differentiation status or viability of cultured muscle cells. In addition, ectopic expression of iNOS also reduced IRS-1 expression in C2C12 myoblasts without altering the expression of p85 P13K (Fig. 1F).

Treatment with GSNO for 24 h did not alter IRS-2 expression, whereas insulin decreased IRS-2 as well as IRS-1 expression in C2C12 myotubes (Fig. 2). Similarly SNAP did not decrease IRS-2 expression in C2C12 myotubes either (data not shown). Expression of IRS was not modulated by GSNO or insulin in C2C12 myotubes (Fig. 2, A and B). Of interest, the combined administration of GSNO (1 mM) with insulin caused a further reduction in IRS-1 expression compared with that induced by the various concentrations (up to 1 μM) of insulin alone (Fig. 2, A and C, and Supplemental Fig. 1). On the other hand, the combination of GSNO and insulin was not associated with a further decrease in IRS-2 expression compared with that induced by insulin alone (Fig. 2, A and D). The combination of GSNO and insulin did not affect the expression of Akt/PKB, α-sarcomeric actin, and myosin heavy chain (Fig. 2A). These observations suggest that NO donor and insulin may exert their effects on expression of IRSs via distinct mechanisms.

In contrast to the changes in protein expression of IRS-1, if any, there was little difference in IRS-1 mRNA level between C2C12 myotubes treated with or without GSNO (Fig. 3A). Insulin did not alter the IRS-1 mRNA level in C2C12 myotubes either. These observations suggest an involvement of up-regulated protein degradation of IRS-1. This assumption was supported by the evidence that GSNO augmented ubiquitination of IRS-1 (Fig. 3B) and that proteasome inhibitors lactacystin and MG132 blocked GSNO-induced decrease in IRS-1 expression in C2C12 myotubes (Fig. 3, C and D). When C2C12 myotubes were treated with NO donor SNAP (500 μM) for 5 h in the presence of MG132, NO donor-induced ubiquitination of IRS-1 was enhanced compared with the cells treated with NO donor in the absence of proteasome inhibitor (Supplemental Fig. 2).

When protein synthesis was blocked by cycloheximide (30 μg/ml), GSNO (1 mM) increased the turnover rate by more than 50% relative to C2C12 myotubes without GSNO treatment (Fig. 4A).

Previous studies showed that insulin-stimulated IRS-1 degradation is blocked by inhibitors for P13K and mammalian target of rapamycin (mTOR) (36, 58). In agreement with previous studies, wortmannin and rapamycin, inhibitors for P13K and mTOR, respectively, significantly attenuated insulin-induced reduction of IRS-1 (Fig. 4E). However, wortmannin and rapamycin failed to block IRS-1 reduction by NO donor GSNO.
Reduced stability of IRS-1 by NO donor and the effects of kinase inhibitors on NO donor-induced IRS-1 reduction. A, in the presence of cycloheximide (CHX, 30 μg/ml), an inhibitor for protein synthesis, the protein stability of IRS-1 was examined in C2C12 myotubes. Treatment with GSNO (1 mM) enhanced the time-dependent decrease in IRS-1 abundance. *, p < 0.05; **, p < 0.01 versus control. B, C, D, and E, wortmannin (100 nM), a PI3K inhibitor (B), rapamycin (50 nM), an mTOR inhibitor (C), and SP600125 (25 μM), a JNK/SAPK inhibitor (D), were added to the culture media 30 min prior to treatment with or without GSNO (1 mM) or insulin (100 nM). These inhibitors failed to block GSNO-induced reduction in IRS-1 expression, although these inhibitors attenuated insulin-induced decrease in IRS-1 expression. **, p < 0.01 versus the cells without GSNO. F, GSNO (1 mM) did not increase phosphorylation of IRS-1 at serine 307, whereas insulin (100 nM) caused serine phosphorylation of IRS-1. IB, immunoblot analysis; p-IRS-1, phosphorylated IRS-1.

FIG. 4.

To further investigate the mechanisms by which NO donor reduces IRS-1 expression, we examined the effects of a specific inhibitor for soluble guanylate cyclase, ODQ, ODQ did not inhibit GSNO-induced IRS-1 decrease (Fig. 4A), indicating that the effect of NO donor is cGMP-dependent. In agreement with this, the cell-permeable cGMP analog 8-bromo-cGMP did not affect IRS-1 expression, although cGMP analog induced a marked phosphorylation of VASP, an endogenous substrate of cGMP-dependent protein kinase. Of interest, phosphorylation of VASP was not detected in the cells treated with GSNO for 24 h, while increased phosphorylation of VASP was observed at 10 min after the addition of GSNO (1 mM) to the culture (data not shown). These findings appear to be in accordance with the previous observations that prolonged exposure to NO donor or the induction of iNOS results in down-regulated cGMP-PKG signaling (59, 60), whereas short exposure activates this signaling pathway.

Since obesity and diabetes are associated with increased oxidative stress and concomitant oxidative stress facilitates cGMP-independent, nitrosative modification-mediated effects of NO and NO-related compounds, we examined the effects of co-administration of glucose oxidase, which generates hydrogen peroxide. Glucose oxidase enhanced the effects of GSNO on IRS-1 expression but did not alter IRS-1 expression in the absence of GSNO (Fig. 5B). By contrast, glucose oxidase did not affect the effects of insulin on IRS-1 expression (Fig. 5C). These findings indicate that concomitant oxidative stress accentuates the effect of NO donor on IRS-1 but not that of insulin, thereby suggesting that nitrosative stress is likely to be involved in iNOS-mediated but not insulin-stimulated IRS-1 degradation.

Amelioration of Depressed IRS-1 Expression by iNOS Gene Disruption or iNOS Inhibitor in Skeletal Muscle of Genetically Obese (ob/ob) Mice—To investigate the biological relevance of NO donor- or iNOS transfection-induced reduction in IRS-1 expression, we examined the effects of iNOS gene disruption or iNOS inhibitor in vivo in genetically obese (ob/ob) mice. In skeletal muscle of ob/ob mice, the protein expression of iNOS was elevated compared with that in lean wild-type mice (Fig. 6A). The expression of cMOC and nMOC, however, did not differ between ob/ob and lean wild-type mice (Supplemental Fig. 3). No difference was found between iNOS+/+ ob/ob and iNOS−/− ob/ob mice in body weight (at 6 weeks of age, 33.2 ± 1.5 and 34.3 ± 1.0 g in iNOS+/+ ob/ob and iNOS−/− ob/ob mice, respectively; at 20 weeks of age, 62.9 ± 1.1 and 61.3 ± 1.0 g in iNOS+/+ ob/ob and iNOS−/− ob/ob mice, respectively), food intake (7.8 ± 0.6 and 7.5 ± 0.5 g/day in iNOS+/+ ob/ob
and iNOS−/−/ob mice, respectively), or epididymal fat weight (2.42 ± 0.17 and 2.55 ± 0.23 g in iNOS+/+ ob/ob and iNOS−/− ob/ob mice, respectively). At 20 weeks of age, there was no significant difference in blood glucose level under fed condition between iNOS+/+ ob/ob and iNOS−/− ob/ob mice (180.0 ± 15.1 and 169.1 ± 10.1 mg/dl in iNOS+/+ ob/ob and iNOS−/− ob/ob, respectively). Although the plasma insulin level appeared to be lower in iNOS−/− ob/ob mice than in iNOS+/+ ob/ob mice (147.9 ± 27.8 and 109.6 ± 16.4 ng/ml in iNOS+/+ ob/ob and iNOS−/− ob/ob mice, respectively), there was no statistically significant difference (p < 0.10). iNOS−/− ob/ob responded to insulin injection with more profound decreases in blood glucose compared with iNOS+/+ ob/ob and iNOS−/− ob/ob mice, indicating the improved insulin sensitivity by iNOS disruption (Fig. 6B).

The amelioration of insulin resistance was accompanied by restoration of depressed IRS-1 expression in skeletal muscle of ob/ob mice. The protein expression of IRS-1, but not that of IR or IRS-2, was markedly decreased in skeletal muscle of ob/ob mice as compared with lean wild-type mice (Fig. 7). iNOS disruption reverted depressed IRS-1 expression in skeletal muscle of ob/ob mice, whereas the expression of IR and IRS-2 was unaltered by iNOS disruption (Fig. 7). iNOS disruption, however, did not affect the mRNA level of IRS-1 (Fig. 8A). Moreover treatment with iNOS inhibitor L-NIL also improved IRS-1 expression in skeletal muscle of ob/ob mice without altering the expression of IR, IRS-2, and p85 PI3K (Fig. 8B and data not shown).

The restoration of IRS-1 expression by iNOS disruption was associated with the improvement in insulin-stimulated tyrosine phosphorylation of IRS-1 (Fig. 7B) and IRS-1-associated PI3K activity (Fig. 8C). Insulin-stimulated tyrosine phosphorylation of IR, however, was not significantly altered by iNOS disruption (Fig. 7A). The ratio of insulin-stimulated tyrosine phosphorylation of IRS-1/total IRS-1 expression level was not significantly altered by iNOS disruption either (data not shown). These findings indicate that improved tyrosine phosphorylation of IRS-1 by iNOS disruption may be attributable in large part to the reversal of depressed IRS-1 expression rather than increased IR tyrosine kinase activity.

**DISCUSSION**

Despite the intense investigation for a number of years, the molecular mechanisms responsible for obesity-induced insulin resistance still remain to be elucidated. We found that NO donor and ectopic expression of iNOS reduced IRS-1 expression via proteasome-dependent degradation in cultured skeletal muscle cells (Figs. 1–3). Moreover gene disruption or pharmacological inhibition of iNOS significantly improved depressed IRS-1 protein expression without altering mRNA expression of IRS-1 in skeletal muscle of ob/ob mice (Figs. 7 and 8). In accordance with previous studies, iNOS expression was increased in skeletal muscle of ob/ob mice compared with lean wild-type mice. These findings indicate that iNOS contributes to depressed expression of IRS-1 in ob/ob mice.

Our observation of insulin-induced IRS-1 degradation in C2C12 myotubes (Figs. 2, 3, and 5) is in agreement with previous studies in adipocytes and hepatoma cells (32–37). Both NO donor and insulin promote proteasome-mediated degradation of IRS-1, but NO donor and insulin seem to exert the effects on IRS-1 via different pathways. Although the molecular mechanisms responsible for insulin-stimulated IRS-1 degradation still remain to be determined, previous studies demonstrated that the activation of PI3K and mTOR is required for IRS-1 degradation by insulin. Consistent with this, our study confirmed that inhibitors for PI3K and mTOR inhibited insulin-induced reduction in IRS-1 in cultured muscle cells. In contrast, these inhibitors failed to block the effects of NO donor on IRS-1 (Fig. 4). These results indicate that, unlike insulin, NO donor does not require PI3K and mTOR activity to reduce IRS-1 expression. In addition, recent studies have demonstrated an involvement of JNK/SAPK and phosphorylation of IRS-1 at serine 307 in IRS-1 degradation (58, 61). However, our results argue against an involvement of JNK/SAPK and phosphorylation of IRS-1 at serine 307 in NO donor-induced IRS-1 reduction.

Insulin reduces both IRS-1 and IRS-2 expression. By contrast, the effect of NO donor was restricted to IRS-1, and IRS-2 expression remained unaltered upon exposure to NO donor in the presence and absence of insulin in cultured skeletal muscle cells. Moreover the combined administration of GSNO and insulin resulted in a greater reduction in IRS-1 expression than that induced by the maximal dose of insulin (1 μM) alone (Supplemental Fig. 1). Taken together, our findings indicate that the molecular mechanisms involved in NO donor-induced IRS-1 degradation may be distinct from those of insulin.

The effect of NO donor on IRS-1 was cGMP-independent and was accelerated by concomitant oxidative stress (Fig. 5). Since oxidative stress facilitates cGMP-independent, nitrosative modification-mediated actions of NO (62–64), these findings suggest that iNOS and NO donor induce IRS-1 degradation probably via nitrosative modification-involved mechanisms. Oxidative stress is postulated to be involved in the pathogen-
Inhibitor L-NIL on obesity-related decrease in IRS-1 expression was examined in wild-type mice. iNOS disruption increased significantly IRS-1-associated PI3K activity in phosphatidylinositol into phosphatidylinositol-3 phosphate (PI3P) but not that of IR in ob/ob mice. The protein expression of IRS-1 was restored in ob/ob mice. On the other hand, the protein expression of IRS-1, and IRS-2 were assessed by immunoblotting (IB). Insulin-stimulated tyrosine phosphorylation of IR (A) and IRS-1 (B) was attenuated in skeletal muscle of iNOS+/+ ob/ob mice compared with lean wild-type (WT) mice. iNOS disruption ameliorated significantly the attenuated insulin-stimulated tyrosine phosphorylation of IRS-1 but not that of IR in ob/ob mice. The protein expression of IRS-1 was decreased in skeletal muscle of iNOS+/+ ob/ob mice compared with lean wild-type mice. iNOS disruption restored IRS-1 expression in ob/ob mice to the level comparable to that in lean wild-type mice. On the other hand, the protein expression of IR (A) and IRS-2 (C) in skeletal muscle did not differ between the groups. * p < 0.05; **, p < 0.01; *** p < 0.001 versus wild-type mice; #, p < 0.05 versus iNOS+/+ ob/ob mice.

The effects of iNOS disruption or iNOS inhibitor on IRS-1 expression and IRS-1-associated PI3K activity. A, mRNA expression of IRS-1 and GAPDH in skeletal muscle was assessed by Northern blotting. The abundance of IRS-1 mRNA was normalized to GAPDH mRNA level. There was no difference in IRS-1 mRNA level between wild-type (WT), iNOS+/+ ob/ob, and iNOS−/− ob/ob mice. B, the effect of iNOS inhibitor L-NIL on obesity-related decrease in IRS-1 expression was examined in ob/ob mice. Immunoblot analysis (IB) revealed that L-NIL treatment for 10 days significantly increased IRS-1 expression in skeletal muscle of ob/ob mice. * p < 0.05; **, p < 0.0001 versus wild-type mice; #, p < 0.05 versus ob/ob mice without L-NIL. C, IRS-1-associated PI3K activity in skeletal muscle was assessed by in vitro phosphorylation of phosphatidylinositol into phosphatidylinositol-3 phosphate (PIP). IRS-1-associated PI3K activity was lower in iNOS+/+ ob/ob mice than in wild-type mice. iNOS disruption increased significantly IRS-1-associated PI3K activity in ob/ob mice. *, p < 0.05; **, p < 0.0001 versus wild-type mice; #, p < 0.05 versus iNOS+/+ ob/ob mice.

The in vivo biological relevance of the pharmacological doses of NO donor has also been an issue of discussion (69–71). In addition to oxidative stress, low oxygen tension (10–20 mm Hg), which is physiologically observed in normal tissues including skeletal muscle, accelerates nitrosative protein modifications such as S-nitrosylation (69). Moreover concentrations of NO and reactive nitrogen species are higher in membrane compartments in cells than in cytosolic fraction due to their higher solubility in lipid bilayers (70, 72). Therefore, proteins associated with membrane, which include IRS-1, are presumably exposed to higher local concentrations of NO-related species. The concentration of nitrite (NO2−) plus nitrate (NO3−), stable derivatives of NO, in the supernatants of the cells treated with SNAP (1 mM) for 1 h was ~150–200 μM2 and is comparable to those in the sera of healthy adults (20 ± 3 μM) and patients with sepsis (144 ± 39 μM) (73), healthy subjects (35 ± 9 μM) (74), elderly healthy subjects (39–121 μM; average, 73 μM) (75), and patients with coronary artery disease (20–96 μM; average, 51 μM) (76) respectively.

Fig. 7. The effects of iNOS disruption on IRS-1 expression in skeletal muscle of obese (ob/ob) mice. Insulin-stimulated tyrosine phosphorylation (PY) of IR and IRS-1 and expression of IR, IRS-1, and IRS-2 were assessed by immunoprecipitation (IP) and immunoblotting (IB). Insulin-stimulated tyrosine phosphorylation of IR (A) and IRS-1 (B) was attenuated in skeletal muscle of iNOS+/+ ob/ob mice compared with lean wild-type (WT) mice. iNOS disruption ameliorated significantly the attenuated insulin-stimulated tyrosine phosphorylation of IRS-1 but not that of IR in ob/ob mice. The protein expression of IRS-1 was decreased in skeletal muscle of iNOS+/+ ob/ob mice compared with lean wild-type mice. iNOS disruption restored IRS-1 expression in ob/ob mice to the level comparable to that in lean wild-type mice. On the other hand, the protein expression of IR (A) and IRS-2 (C) in skeletal muscle did not differ between the groups. * p < 0.05; **, p < 0.01; *** p < 0.001 versus wild-type mice; #, p < 0.05 versus iNOS+/+ ob/ob mice.

Fig. 8. The effects of iNOS disruption or iNOS inhibitor on IRS-1 expression and IRS-1-associated PI3K activity. A, mRNA expression of IRS-1 and GAPDH in skeletal muscle was assessed by Northern blotting. The abundance of IRS-1 mRNA was normalized to GAPDH mRNA level. There was no difference in IRS-1 mRNA level between wild-type (WT), iNOS+/+ ob/ob, and iNOS−/− ob/ob mice. B, the effect of iNOS inhibitor L-NIL on obesity-related decrease in IRS-1 expression was examined in ob/ob mice. Immunoblot analysis (IB) revealed that L-NIL treatment for 10 days significantly increased IRS-1 expression in skeletal muscle of ob/ob mice. * p < 0.05; **, p < 0.0001 versus wild-type mice; #, p < 0.05 versus ob/ob mice without L-NIL. C, IRS-1-associated PI3K activity in skeletal muscle was assessed by in vitro phosphorylation of phosphatidylinositol into phosphatidylinositol-3 phosphate (PIP). IRS-1-associated PI3K activity was lower in iNOS+/+ ob/ob mice than in wild-type mice. iNOS disruption increased significantly IRS-1-associated PI3K activity in ob/ob mice. *, p < 0.05; **, p < 0.0001 versus wild-type mice; #, p < 0.05 versus iNOS+/+ ob/ob mice.

Oxidative stress, in skeletal muscle of iNOS−/− mice, accelerates nitrosative protein modifications (66–68). Therefore, the doses of GSNO (100 μM and 1 mM) required to reduce IRS-1 expression in C2C12 myotubes (Fig. 1) seem to be consistent with the concentrations needed for nitrosative protein modification-involved effects of NO donor.

The in vivo biological relevance of the pharmacological doses of NO donor has also been an issue of discussion (69–71). In addition to oxidative stress, low oxygen tension (~10–20 mm Hg), which is physiologically observed in normal tissues including skeletal muscle, accelerates nitrosative protein modifications such as S-nitrosylation (69). Moreover concentrations of NO and reactive nitrogen species are higher in membrane compartments in cells than in cytosolic fraction due to their higher solubility in lipid bilayers (70, 72). Therefore, proteins associated with membrane, which include IRS-1, are presumably exposed to higher local concentrations of NO-related species. The concentration of nitrite (NO2−) plus nitrate (NO3−), stable derivatives of NO, in the supernatants of the cells treated with SNAP (1 mM) for 1 h was ~150–200 μM2 and is comparable to those in the sera of healthy adults (20 ± 3 μM) and patients with sepsis (144 ± 39 μM) (73), healthy subjects (35 ± 9 μM) (74), elderly healthy subjects (39–121 μM; average, 73 μM) (75), and patients with coronary artery disease (20–96 μM; average, 51 μM) (76).

M. Kaneki, unpublished observation.
average, 50 μM (76). Of note, our preliminary results demonstrated that the abundance of nitrosotriols, which was measured by photolysis-chemiluminescence assay as previously described (77), in C2C12 myoblasts treated with GSNO (1 μM) for 24 h was comparable to that observed in skeletal muscle of ob/ob mice (control C2C12 myoblasts, 5.3; GSNO (1 μM)-treated C2C12 myoblasts, 12.6; skeletal muscle of ob/ob mice, 8.1 ± 0.5 pmol/mg of protein). From a point of view of the level of intracellular nitrosative protein modifications, our preliminary observations, therefore, seem to support the pathophysiological relevance of the pharmacological doses of NO donors used in the experiments with cultured muscle cells.

Based on the inhibitory effects of insulin in cultured cells, hyperinsulinemia has been postulated to be involved in decreased expression of IRS-1 and IRS-2 in animal models of insulin resistance and patients with type 2 diabetes. Therefore, increased expression of IRS-1 and IRS-2 in animal models of hyperinsulinemia has been postulated to be involved in decreased IRS-1 expression associated with insulin resistance and patients with type 2 diabetes. Therefore, increased expression of IRS-1 and IRS-2 in animal models of hyperinsulinemia has been postulated to be involved in decreased IRS-1 expression associated with insulin resistance and patients with type 2 diabetes.

For example, the effects of iNOS transfection and NO donors in cultured muscle cells, the present data favor the assumption that the direct effect of iNOS in muscle, rather than the amelioration of hyperinsulinemia, played a major role in the reversal of depressed IRS-1 expression associated with iNOS deficiency in ob/ob mice.

The present data in ob/ob mice are in accordance with the previous study by Perreault and Marette (11) showing that iNOS expression was increased in skeletal muscle and adipose tissue, but not in liver, of mice fed a high fat diet, that iNOS knock-out mice were resistant to high fat diet-induced insulin resistance, and that iNOS disruption reverted impaired insulin signaling by high fat diet in skeletal muscle but not in adipose tissue or liver. In contrast to our data, however, in the study by Perreault and Marette (11), iNOS deficiency was not associated with alteration in IRS-1 expression. This difference might be attributable to the differences in experimental models to induce insulin resistance (high fat diet versus leptin mutation in ob/ob mice) and animal strains used. We used iNOS−/− mice that were backcrossed onto wild-type C57BL/6J background at least 10 generations; Perreault and Marette (11) used iNOS−/− mice in C57BL-6 × 129SvEv background in most parts of their study, including the experiment showing unaltered IRS-1 mRNA level by iNOS knock-out mice via as yet determined mechanisms. How-

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42. Gath, I., Closs, E. I., Godtel-Armbrust, U., Schmitt, S., Nakane, M., Wessler, I., and Forstermann, U. (1996) *FASEB J.* 10, 1614–1620.

43. Gath, I., Ebert, J., Godtel-Armbrust, U., Ross, R., Reske-Kunz, A. B., and Forstermann, U. (1999) *Biochem. J.* 340, 723–728.

44. Park, C. S., Park, R., and Krishna, G. (1996) *Life Sci.* 59, 219–225.

45. Stamler, J. S., Lamas, S., and Fang, F. C. (2001) *Cell* 106, 675–683.

46. Drew, B., and Leeuwenburgh, C. (2002) *Ann. N. Y. Acad. Sci.* 959, 66–81.

47. Hamilton, C. A., Brosnan, M. J., McIntyre, M., Graham, D., and Dominiczak, A. F. (2001) *Hypertension* 37, 529–534.

48. Lyall, F., Gibson, J. L., Greer, I. A., Brockman, D. E., Eis, A. L., and Myatt, L. (1998) *Diabetes Care* 21, 1753–1758.

49. Padron, J., Peiro, C., Cercas, E., Llergo, J. L., and Sanchez-Ferrer, C. F. (2000) *Biochem. Biophys. Res. Commun.* 271, 217–221.

50. Honing, M. L., Morrison, P. J., Banga, J. D., Stroes, E. S., and Rabelink, T. J. (1998) *Diabetes Metab. Rev.* 14, 241–249.

51. Kuncewicz, T., Balakrishnan, P., Snuggs, M. B., and Kone, B. C. (2001) *Am. J. Physiol.* 281, F326–F336.

52. Kaneki, M., Kharbanda, S., Pandey, P., Yoshida, K., Takekawa, M., Liou, J. R., Stone, R., and Kufe, D. (1999) *Mol. Cell. Biol.* 19, 461–470.

53. Fujimoto, M., Maezawa, Y., Yokote, K., Joh, K., Kobayashi, K., Kawamura, H., Nishimura, M., Putti, S., and Finazzi Agro, A. (1997) *FEBS Lett.* 410, 470–476.

54. Lei, S. Z., Pan, Z. H., Aggarwal, S. K., Chen, H. S., Hartman, J., Sucher, N. J., and Lipton, S. A. (1992) *Neuron* 8, 1087–1099.

55. Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, G. M. (2003) *Diabetes* 52, 1–8.

56. Gopalakrishna, R., Chen, Z. H., and Gundimeda, U. (1993) *J. Biol. Chem.* 268, 21780–21785.

57. Straw, O. A., Leone, A., Giercksky, K. E., and Kirkeboen, K. A. (2000) *Crit. Care Med.* 28, E1235–E1241.

58. Greene, M. W., Sakaue, H., Wang, L., Alessi, D. R., and Roth, R. A. (2003) *J. Biol. Chem.* 278, 8199–8211.

59. Warnholtz, A., Mollnau, H., Heitzer, T., Kontush, A., Moller-Bertram, T., Lavall, D., Giard, A., Beazegel, U., Marklund, S. L., Walter, U., Menertz, T., and Munzel, T. (2002) *Am. J. Physiol. Cardiol.* 40, 1356–1363.

60. Takata, M., Filippov, G., Liu, H., Ichinose, F., Jansens, S., Bloch, D. B., and Bloch, K. D. (2001) *Am. J. Physiol.* 280, L272–L278.

61. Gao, Z., Zhang, X., Zuberi, A., Hwang, D., Quon, M. J., Lefevre, M., and Ye, J. (2004) *Mol. Endocrinol.* 18, 2024–2034.

62. Beltran, B., Orsi, A., Clemen, E., and Moncada, S. (2000) *Br. J. Pharmacol.* 129, 953–960.

63. Blatt, P., and Lamas, S. (2000) *Eur. J. Biochem.* 276, 4928–4944.

64. Squadrito, G. L., and Pryor, W. A. (1998) *Free. Radic. Biol. Med.* 25, 392–403.

65. Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, G. M. (2003) *Diabetes* 52, 1–8.

66. Foster, M. W., McMahon, T. J., and Stamler, J. S. (2003) *Trends Mol. Med.* 9, 160–168.

67. Liu, X., Miller, M. J., Joshi, M. S., Thomas, D. D., and Lancaster, J. R., Jr. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 2175–2179.

68. Strand, O. A., Leone, A., Giercksky, K. E., and Kirkeboen, K. A. (2000) *Crit. Care Med.* 28, 2779–2785.

69. Fujiwara, N., Osanai, T., Kamada, T., Kato, T., Takahashi, K., and Okumura, K. (2000) *Circulation* 101, 856–861.

70. Yoon, Y., Song, J., Hong, S. H., and Kim, J. Q. (2000) *Clin. Chem.* 46, 1626–1630.

71. Watanabe, T., Akishita, M., Toba, K., Kozaki, K., Eto, M., Sugimoto, N., Kiuchi, T., Hashimoto, M., Shirakawa, W., and Ouchi, Y. (2000) *Clin. Chim. Acta* 301, 169–179.

72. Mannick, J. B., Hausladen, A., Liu, L., Hess, D. T., Zeng, M., Miao, Q. X., Kane, L. S., Gow, A. J., and Stamler, J. S. (1999) *Science* 284, 651–654.