S-Nitrosation of the Insulin Receptor, Insulin Receptor Substrate 1, and Protein Kinase B/Akt

A Novel Mechanism of Insulin Resistance

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Evidence demonstrates that exogenous nitric oxide (NO) and the NO produced by inducible nitric oxide synthase (iNOS) can induce insulin resistance in muscle. Here, we investigated whether this insulin resistance could be mediated by S-nitrosation of proteins involved in early steps of the insulin signal transduction pathway. Exogenous NO donated by S-nitrosoglutathione (GSNO) induced in vitro and in vivo S-nitrosation of the insulin receptor β subunit (IRβ) and protein kinase B/Akt (Akt) and reduced their kinase activity in muscle. Insulin receptor substrate (IRS)-1 was also rapidly S-nitrosated, and its expression was reduced after chronic GSNO treatment. In two distinct models of insulin resistance associated with enhanced iNOS expression—diet-induced obesity and the *ob/ob* diabetic mice—we observed enhanced S-nitrosation of IRβ/IRS-1 and Akt in muscle. Reversal of S-nitrosation of these proteins by reducing iNOS expression yielded an improvement in insulin action in both animal models. Thus, S-nitrosation of proteins involved in insulin signal transduction is a novel molecular mechanism of iNOS-induced insulin resistance. *Diabetes* 54:959–967, 2005

Nitric oxide (NO) is a free radical gas and biological signaling molecule produced by the intracellular enzyme NO synthase (1). The reactivity of NO toward molecular oxygen, thiols, transition metal centers, and other biological targets enables NO to act as an ubiquitous cell-signaling molecule with diverse physiological and pathophysiological roles (1,2). In this regard, NO can react with cysteine residues in the presence of O_2 to form S-nitrosothiol adducts (3,4), altering the activity of proteins including H-ras (5), the olfactory cyclic nucleotide-gated channel (6), and glyceraldehyde-3-phosphate dehydrogenase (7). The reversible regulation of protein function by S-nitrosation (see AUTHORS' NOTE at the end of the article) has led to the proposal that S-nitrosothiols function as posttranslational modifications, analogous to those created by phosphorylation or acetylation (4).

Evidence demonstrates that exogenous NO and the NO produced by inducible nitric oxide synthase (iNOS) can modulate insulin action in muscle. NO donors induce dose-dependent inhibition of maximal insulin-stimulated glucose transport in isolated muscles and in cultured L6 muscle cells, without affecting insulin binding to its receptor (8). iNOS was not detected in resting muscle; however, its induction has been associated with impaired insulin-stimulated glucose uptake in isolated rat muscles (8).

iNOS induction may be involved in the pathogenesis of some situations of insulin resistance, such as obesity-linked type 2 diabetes (9–11) and LPS-induced endotoxemia (12). In obese human subjects (13,14) and in several animal models of obesity (15,16), insulin resistance is also associated with increased systemic and tissue concentrations of proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-6.

It is well established that proinflammatory cytokines (tumor necrosis factor-α and interleukin-6) and endotoxins synergistically increase NO production via increased expression of iNOS in rat skeletal muscle and cultured myocytes and adipocytes (8,17,18). Recently, Perreault and Marette (19) demonstrated that genetic disruption of iNOS protects against obesity-linked insulin resistance, preventing impairments in phosphatidylinositol 3-kinase and protein kinase B/Akt (Akt) activation by insulin in muscle.

In light of the evidence that NO and increased iNOS expression are associated with reduced insulin action and that S-nitrosation is a posttranslational modification that can modulate protein function, we investigated whether insulin resistance could be mediated by S-nitrosation of proteins involved in the early steps of the insulin signal transduction pathway.

RESEARCH DESIGN AND METHODS

Antiphosphotyrosine, anti–insulin receptor β subunit (IRβ), anti–insulin receptor substrate (IRS)-1, and anti-Akt antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p[ser^{473}]Akt antibody was from Cell Signaling Technology (Beverly, MA). The anti-nitrosocystein antibody was from Calbiochem (Darmstadt, Germany). Human recombinant insulin (Humulin R) was purchased from Eli Lilly (Indianapolis, IN). Rosiglitazone was from

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Akt, protein kinase B/Akt; ASO, antisense oligonucleotides; DIO, diet-induced obese; GSK, glycogen synthase kinase; GSNO, S-nitrosoglutathione; iNOS, inducible nitric oxide synthase; IRβ, insulin receptor β subunit; IRS, insulin receptor substrate; SNO, S-nitrosothiol.

GlaxoSmithKline (Harlow, U.K.). Routine reagents were purchased from Sigma unless otherwise specified. ^{125}I-protein A, D-[U-^{14}C]glucose, and 2-deoxy-D-[2,6-^{3}H]glucose were from Amersham (Amersham, U.K.). L-[1-^{14}C]glucose was obtained from NEN Life Sciences Products (Boston, MA).

Male Wistar rats and *ob*/*ob* diabetic mice were obtained from the UNICAMP Central Animal Breeding Center (Campinas, São Paulo, Brazil). Animals were allowed free access to standard rodent food and water ad libitum. Acute experiments were performed with 8-week-old Wistar rats and *ob*/*ob* diabetic mice. Diet-induced obese (DIO) animals were obtained by high-fat diet administration to one group of Wistar rats, which started at 8 weeks of age for 24 weeks, with controls of the same age. The high-fat diet consisted of 55% calories derived from fat, 29% from carbohydrate, and 16% from protein similar to that previously described (20). Food was withdrawn 6 h before the experiments. One group of DIO animals and one group of its controls were treated with 4 mg/kg rosiglitazone by oral gavage for 10 days before tissue extraction. The ethics committee at the University of Campinas approved all experiments involving animals.

iNOS antisense oligonucleotide treatment. Phosphothiolate-modified oligonucleotides for iNOS (sense, 5′-GCATACCTGAAGGTG-3′ and antisense 5′-GCATACCTGAAGGTG-3′) were obtained from Invitrogen (Gaithersburg, MD). The sequence was obtained from the NCBI Entrez Nucleotide Bank based on the *Mus musculus* iNOS mRNA complete code. Adult *ob*/*ob* diabetic and control mice were treated with sense or antisense iNOS oligonucleotides diluted in Tris/EDTA buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA) and injected once a day at 10:00 A.M. (10.0 nmol/dose, 24, 48, and 72 h after the onset of the experimental period).

S-nitrosoglutathione treatment. S-nitrosoglutathione (GSNO) was prepared by the reaction of glutathione with sodium nitrite in acidic solution, as previously reported (21). Acute treatment was performed by GSNO injection intraperitoneally 30 min before muscle extraction. Chronic treatment was performed by 0.1 mol/l GSNO intraperitoneally injected every 2 h, until completing four doses in 8 h.

Glucose uptake and glycogen synthesis measurements. Thirty minutes before the beginning of the experiment, GSNO (GSNO group) or PBS (control group) was injected in the peritoneal cavity of the animals. Soleus muscles were isolated and incubated as previously described (22). The muscles were incubated in Krebs-Ringer bicarbonate buffer containing 5.6 mmol/l glucose, 0.2 μCi/ml 2-deoxy-D-[2,6-^{3}H]glucose, and 0.3 μCi/ml D-[U-^{14}C]glucose, with 95% O_2/5% CO_2, at 37°C and 120 rpm. Incubation was performed for 1 h in the absence or presence of 10 mUI/ml insulin. GSNO was present in the incubation media of the GSNO group. 2-Deoxy-D-[2,6-^{3}H]glucose uptake and [^{14}C]glycogen synthesis were determined as previously described (23,24).

The 30-min insulin tolerance test. Rats or mice were fasted for 6 h and submitted to an insulin tolerance test. Briefly, 1.5 IU/kg insulin was infused intraperitoneally to anesthetized rats or mice, and glucose was measured at 0 (basal), 5, 10, 15, 20, 25, and 30 min thereafter. Glucose disappearance rate (K_{itt}) was calculated from the formula $0.693/t_{1/2}$. The glucose $t_{1/2}$ was calculated from the slope of the least square analysis of blood glucose concentration during the linear phase of decline (25).

IRβ, IRS-1, and S-nitrosothiol immunoprecipitation and Western blot analysis. Wistar rats or mice were injected with either saline or insulin (10^{-5} mol/l), and 90 s later, soleus muscle was removed and homogenized as already described (26).

In immunoprecipitation studies, muscle lysates were incubated with anti-IRβ (0.3 mg/ml), anti–IRS-1 (1:1,000), or anti–S-nitrosothiol (SNO) (1:200) antibodies for 2 h and then incubated with protein A Sepharose for a further 2 h. Beads were then washed with Tris containing 1% Triton X-100, boiled in Laemmli buffer for 5 min, and subjected to Western blotting analysis. Anti-nitrosocystein immunoprecipitates were always handled in the dark to decrease SNO auto-degradation.

Samples from whole-tissue extracts, immunoprecipitates, or biotinylated nitrosocysteines were subjected to SDS-PAGE electrophoresis, and immunoblotting was performed as described (26). Immunoreactive bands were detected by the enhanced chemiluminescence method (RPN 2108 ECL Western blotting analysis system; Amersham Biosciences).

Detection of S-nitrosated proteins by biotin switch method. The biotin switch assay was performed essentially as previously described (27,28). Extracts were adjusted to 0.5 mg/ml of protein, and equal amounts were blocked with four volumes of blocking buffer (225 mmol/l HEPES, pH 7.7, 0.9 mmol/l neocuproine, 2.5% SDS, and 20 mmol/l methylmethanethiosulfonate) at 50°C for 30 min with agitation. After blocking, extracts were precipitated with two volumes of cold acetone (−20°C), chilled at −20°C for 10 min, centrifuged at 2,000*g* at 4°C for 5 min, washed with acetone, dried out, and resuspended in 0.1 ml HENS buffer (250 mmol/l HEPES, pH 7.7, 1 mmol/l EDTA, 0.1 mmol/l neocuproine, and 1% SDS) per milligram of protein. Until this point, all operations were carried out in the dark. A 1/3 volume of 4 mmol/l biotin-HPDP and 2.5 mmol/l ascorbic acid was added and incubated for 1 h at room temperature. Proteins were acetone-precipitated again and resuspended in the same volume of HENS buffer.

For purification of biotinylated proteins, samples from the biotin switch assay were diluted with two volumes of neutralization buffer (20 mmol/l HEPES, pH 7.7, 100 mmol/l NaCl, 1 mmol/l EDTA, and 0.5% Triton X-100), and 15 μl neutravidin-agarose per milligram protein in the initial extract was added and incubated for 1 h at room temperature with agitation. Beads were washed five times with washing buffer (20 mmol/l HEPES, pH 7.7, 600 mmol/l NaCl, 1 mmol/l EDTA, and 0.5% Triton X-100) and incubated with elution buffer (20 mmol/l HEPES, pH 7.7, 100 mmol/l NaCl, 1 mmol/l EDTA, and 100 mmol/l 2-mercaptoethanol) for 20 min at 37°C with gentle stirring. Supernatants were collected, and proteins were separated by SDS-PAGE.

Detection of S-nitrosation by fluorimetry. Muscle lysates were submitted to immunoprecipitation using the anti-IRβ antibody. The immunoprecipitated pellets were washed five times with lysis buffer (29) and then twice with PBS. The pellets were incubated with 1.3 mmol/l $HgCl_2$ and 7.8 mmol/l 2,3-diaminonaphthalene for 30 min at 37°C and centrifuged, and then 0.75 mol/l NaOH was added. The quantity of fluorescent naphtotriazole generated from the reaction between 2,3-diaminonaphthalene and NO released from S-nitrosated IRβ was monitored by fluorimetry, using a Perkin-Elmer HTS 7000 spectrofluorimeter with excitation wavelength at 375 nm and emission measured at a wavelength of 450 nm (30,31).

Insulin receptor autophosphorylation and tyrosine kinase activity. IRβ tyrosine kinase activity was measured in vitro by autophosphorylation and by its ability to induce tyrosine phosphorylation of its natural substrate IRS-1. The IRβ was immunoprecipitated from rat muscle with or without previous 10^{-9} mol/l insulin infusion in the cava vein. This dose of insulin can induce conformational change of IRβ but not its autophosphorylation. After immunoprecipitation, half of the aliquots were treated with 10^{-2} mol/l GSNO diluted in PBS and the other half with PBS only for 30 min. After extensive washing of immunoprecipitates, a kinase assay was performed by adding 15 μmol/l ATP (32) and the same amount of immunopurified IRS-1 to each immunoprecipitate (33) to measure the ability of IRβ to phosphorylate IRS-1. The IRS-1 was immunopurified, as previously described, from the livers of control rats (33). Tyrosine phosphorylation was measured by immunoblotting with antiphosphotyrosine antibody.

Akt activity. Soleus muscles were removed from rats treated, or not treated, with 10^{-5} mol/l insulin. Akt activity was measured with the Akt Kinase Assay Kit (Cell Signaling catalog no. 9840). Briefly, Akt was immunoprecipitated from the muscle of rats, with or without previous 10^{-5} mol/l insulin infusion in the cava vein. The immunoprecipitates were incubated with 10^{-2} mol/l GSNO diluted in PBS or with PBS alone for 30 min. After extensive washing, the immunoprecipitates were then incubated with glycogen synthase kinase (GSK)-α/β, which is a substrate to Akt, and Akt activity was measured by immunoblotting to phospho–GSK-α/β.

Statistical analysis. The results of blots are presented as direct comparisons of bands or dots in autoradiographs and quantified by densitometry using the Scion Image software (ScionCorp, Frederick, MD). Data were analyzed by the two-tailed unpaired Student's *t* test or by repeat-measures ANOVA (one-way or two-way ANOVA) followed by post hoc analysis of significance (Bonferroni test) when appropriate, comparing experimental and control groups. The level of significance was set at $P < 0.05$.

RESULTS

GSNO induces insulin resistance in isolated and in vivo muscle by means of S-nitrosation. Isolated soleus muscle was treated with increasing GSNO doses for 30 min and then with insulin or saline for a further 1 h. Insulin-stimulated glucose uptake (Fig. 1*A*) and glycogen synthesis (Fig. 1*B*) were progressively reduced when GSNO doses were increased. We hypothesized that NO can modulate insulin action in muscle by inducing S-nitrosation of proteins involved in the early steps of insulin signaling. The ability of NO donors to induce S-nitrosation of IRβ was investigated by three different methods after the treatment of isolated soleus muscle with 10 mmol/l GSNO, a dose that induced maximal reduction of insulin-stimulated glucose uptake and glycogen synthesis.

GSNO induces S-nitrosation of IRβ as demonstrated by the biotin switch method (Fig. 1*C*), by immunoprecipitation with anti-SNO and immunoblotting with anti-IRβ (Fig.

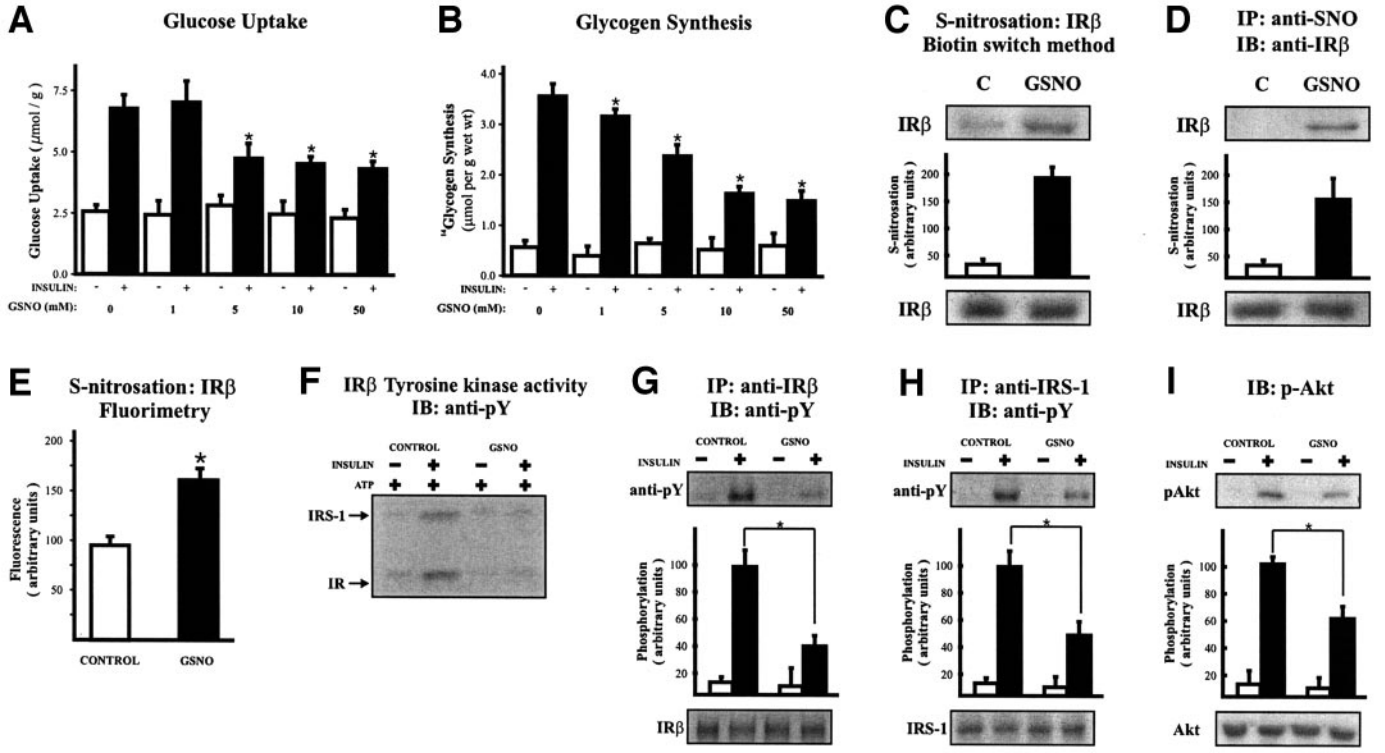

FIG. 1. Effect of GSNO on insulin sensitivity and IRβ S-nitrosation in isolated soleus muscle. Dose response of GSNO treatment on glucose uptake (*A*) and glycogen synthesis (*B*) in isolated rat soleus muscle is shown. GSNO induced S-nitrosation of IRβ, as demonstrated by the biotin switch method (*C*), immunoprecipitation (IP) with anti-SNO followed by immunoblotting (IB) with anti-IRβ (*D*), and fluorimetry after incubation with diaminonaphtalen (*E*) are indicated. *F*: Effect of GSNO treatment in vitro on in vivo insulin-induced IRβ tyrosine kinase activity measured by autophosphorylation and by its ability to phosphorylate immunopurified IRS-1. Insulin-induced tyrosine phosphorylation of IRβ (*G*) and IRS-1 (*H*) and serine phosphorylation of Akt (*I*) after insulin (10 mUI/ml) stimulation for 5 min are indicated. *$P < 0.05$, control vs. GSNO-treated soleus. Bars in *A*, *B*, *E*, *G*, *H*, and *I* represent means ± SE from six to eight isolated soleus.

1*D*), and by fluorimetry (Fig. 1*E*). The results were reproduced by the three methods; however, the biotin switch method was slightly more sensitive.

In parallel with S-nitrosation of IRβ, in vitro GSNO treatment reduced insulin-induced IRβ autophosphorylation and IRβ kinase activity, as demonstrated by the reduction in purified IRS-1 tyrosine phosphorylation, which was decreased by 70% (Fig. 1*F*).

The effect of GSNO on IRβ and IRS-1 tyrosine phosphorylation and Akt serine phosphorylation was investigated in isolated soleus muscle. A 50–60% decrease in insulin-induced IRβ and IRS-1 tyrosine phosphorylation in GSNO-treated muscle (Fig. 1*G* and *H*) was found. GSNO treatment also reduced insulin-induced Akt serine phosphorylation by 40%. (Fig. 1*I*).

Male Wistar rats were also treated with GSNO to investigate whether this NO donor could induce insulin resistance in vivo. Thirty minutes after GSNO administration, an insulin resistance condition was established, as indicated by a lower plasma glucose disappearance rate after the 30 min of the insulin tolerance test (K_{itt}) (Fig. 2*A*). Insulin resistance was associated with enhanced S-nitrosation of the IRβ (Fig. 2*B*). S-nitrosocysteine, another NO donor, when administered to rats, also induced insulin resistance and IRβ S-nitrosation (data not shown).

Treatment with GSNO for 30 min (Fig. 2*C* and *D*) also led to S-nitrosation of IRS-1, as demonstrated by the biotin switch method, but did not change IRS-1 protein levels and IRS-1 serine phosphorylation levels (data not shown). However, when GSNO was given during 8 h, the increased S-nitrosation of IRS-1 (Fig. 2*C*) was associated with a reduced concentration of this protein in the muscle (Fig. 2*D*).

We also observed enhanced S-nitrosation of Akt after acute treatment with GSNO (Fig. 2*E*). A reduction in insulin-induced Akt activity after in vitro GSNO treatment, as demonstrated by decreased GSK-α/β phosphorylation, was found (Fig. 2*F*). Because insulin was administrated in vivo and GSNO treatment was performed in vitro, we can conclude that S-nitrosation of Akt directly inhibits its kinase activity. We did not observe S-nitrosation of IRS-2 or p85 or p110 subunits of phosphatidylinositol 3-kinase (data not shown).

We demonstrated that GSNO administration to rats induced an ~50% reduction in insulin-induced IRβ and IRS-1 tyrosine phosphorylation (Fig. 2*G* and *H*) and a 60% reduction in insulin-induced Akt serine phosphorylation (Fig. 2*I*) in muscle. In preliminary experiments, the S-nitrosation levels of IRβ, IRS-1, and Akt were also investigated in other muscles with different fiber composition, such as gastrocnemius and adutor longus, and the findings were similar, independently of the muscle used.

Diet-induced obesity is associated with enhanced S-nitrosation. Table 1 shows comparative data regarding controls, DIO rats, DIO rats submitted to rosiglitazone treatment, and *ob*/*ob* mice and their respective controls.

Soleus muscles of male Wistar rats treated with a high-fat diet for 24 weeks were removed, and S-nitrosated proteins were determined and compared with those of controls that received standard rodent food for the same

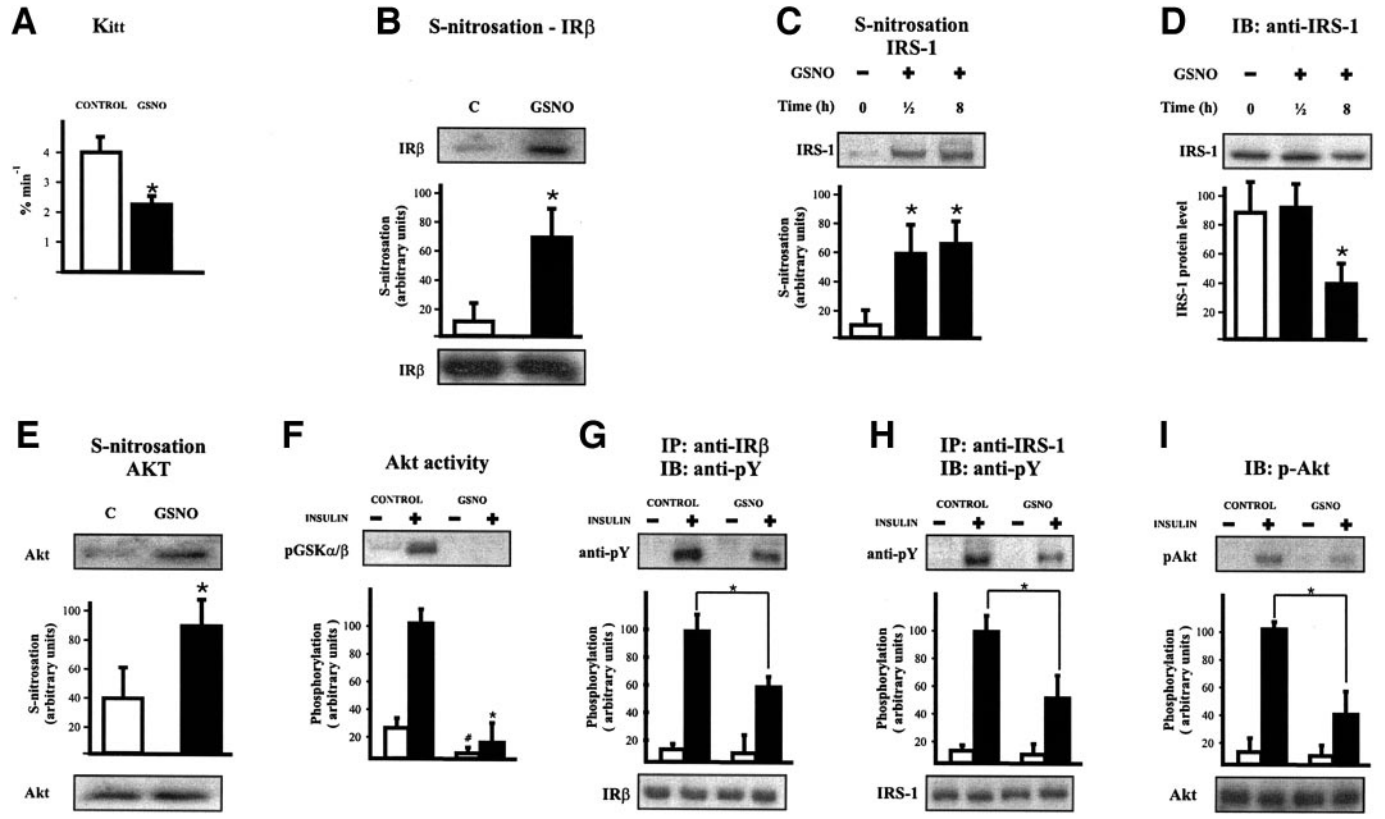

FIG. 2. Effect of GSNO on insulin sensitivity and S-nitrosation in muscle of intact rat. *A*: In vivo effect of acute GSNO (100 mmol/l) treatment on glucose disappearance rates, measured by the 30-min insulin tolerance test. S-nitrosation of IRβ (*B*) after acute GSNO treatment is shown. S-nitrosation of IRS-1 (*C*) and IRS-1 protein level (*D*) after acute and chronic GSNO treatment is indicated. *E*: Akt S-nitrosation after acute GSNO treatment. *F*: Immunoprecipitated Akt activity after insulin stimulation in vivo and GSNO treatment in vitro, as demonstrated by its capability to phosphorylate its purified substrate GSK-α/β. Insulin-induced tyrosine phosphorylation of IRβ (*G*) and IRS-1 (*H*) and serine phosphorylation of Akt (*I*) after acute GSNO treatment are shown. S-nitrosation was determined in all experiments by the biotin switch method. *$P < 0.05$, insulin control vs. insulin GSNO. #$P < 0.05$, basal control vs. basal GSNO. Bars in *A*, *G*, *H*, *I*, and *J* represent means ± SE from four to eight rats. IB, immunoblotting; IP, immunoprecipitated.

period. We observed an increase in iNOS protein expression in the muscle of obese animals (Fig. 3*A*). This increase in iNOS expression was associated with enhanced S-nitrosation of IRβ, IRS-1, and Akt (Fig. 3*B–D*).

Insulin resistance was demonstrated by a reduction in the K_{itt} of obese animals (Fig. 3*E*). Insulin-stimulated tyrosine phosphorylation of IRβ was reduced in the skeletal muscle of obese animals by 50% (Fig. 3*F*). Insulin-stimulated IRS-1 tyrosine phosphorylation was also reduced by 50% (Fig. 3*G*), and this was accompanied by a 40% reduction in IRS-1 protein content in the skeletal muscle (Fig. 3*H*). Insulin-induced Akt activation was reduced by 40% when compared with controls (Fig. 3*I*). The experiments were also repeated with rats treated on the high-fat diet for 4 weeks with very similar results of S-nitrosation and insulin-induced tyrosine phosphorylation but without reductions in IRS-1 protein expression (data not shown).

TABLE 1
Characteristics of rats and mice studied

	n	Body weight (g)	Plasma glucose (mg/dl)	Insulin (μU/ml)
Control	10	532 ± 38*	112 ± 4	11 ± 2*
DIO	12	953 ± 61	122 ± 5	23 ± 3
DIO/rosiglitazone	12	962 ± 58	118 ± 7	18 ± 6
ob/+	8	18.2 ± 0.6†	117 ± 17‡	12 ± 4‡
ob/*ob*	8	34.2 ± 0.4	335 ± 34	85 ± 21
ob/*ob* treated with iNOS ASO	8	33.5 ± 0.6	272 ± 28	27 ± 12

Data are means ± SE. *$P < 0.05$, control × DIO and control × rosiglitazone; †$P < 0.05$, *ob*/+ × *ob*/*ob* and *ob*/+ × *ob*/*ob* treated with iNOS ASO; ‡$P < 0.05$, *ob*/+ × *ob*/*ob*.

iNOS antisense oligonucleotide treatment reduces S-nitrosation and restores insulin signaling in the muscle of *ob*/*ob* diabetic mice. In the *ob*/*ob* mice, another model of insulin resistance, we found enhanced expression of iNOS in muscle (Fig. 4*A*). We treated *ob*/*ob* mice with iNOS antisense oligonucleotide (ASO) and studied S-nitrosation of these proteins and insulin signaling. In these experiments, *ob*/*ob* diabetic mice were also treated with sense oligonucleotide, yielding identical results to those of the untreated *ob*/*ob* diabetic mice (data not shown). iNOS ASO efficiently blocked the expression of the protein, as demonstrated by a reduction of almost 80% in iNOS protein levels in the muscle (Fig. 4*A*). In *ob*/*ob* mice treated with iNOS ASO, no changes were observed in endothelial NOS (eNOS) or neuronal NOS (nNOS) protein expression in muscle, suggesting that the ASO used was specific for iNOS (data not shown).

Treatment of *ob*/*ob* diabetic mice with iNOS ASO for 3 days lowered the plasma glucose level from 335 ± 34 to 272 ± 28 ($P < 0.05$). As shown in Fig. 4*B*, the glucose

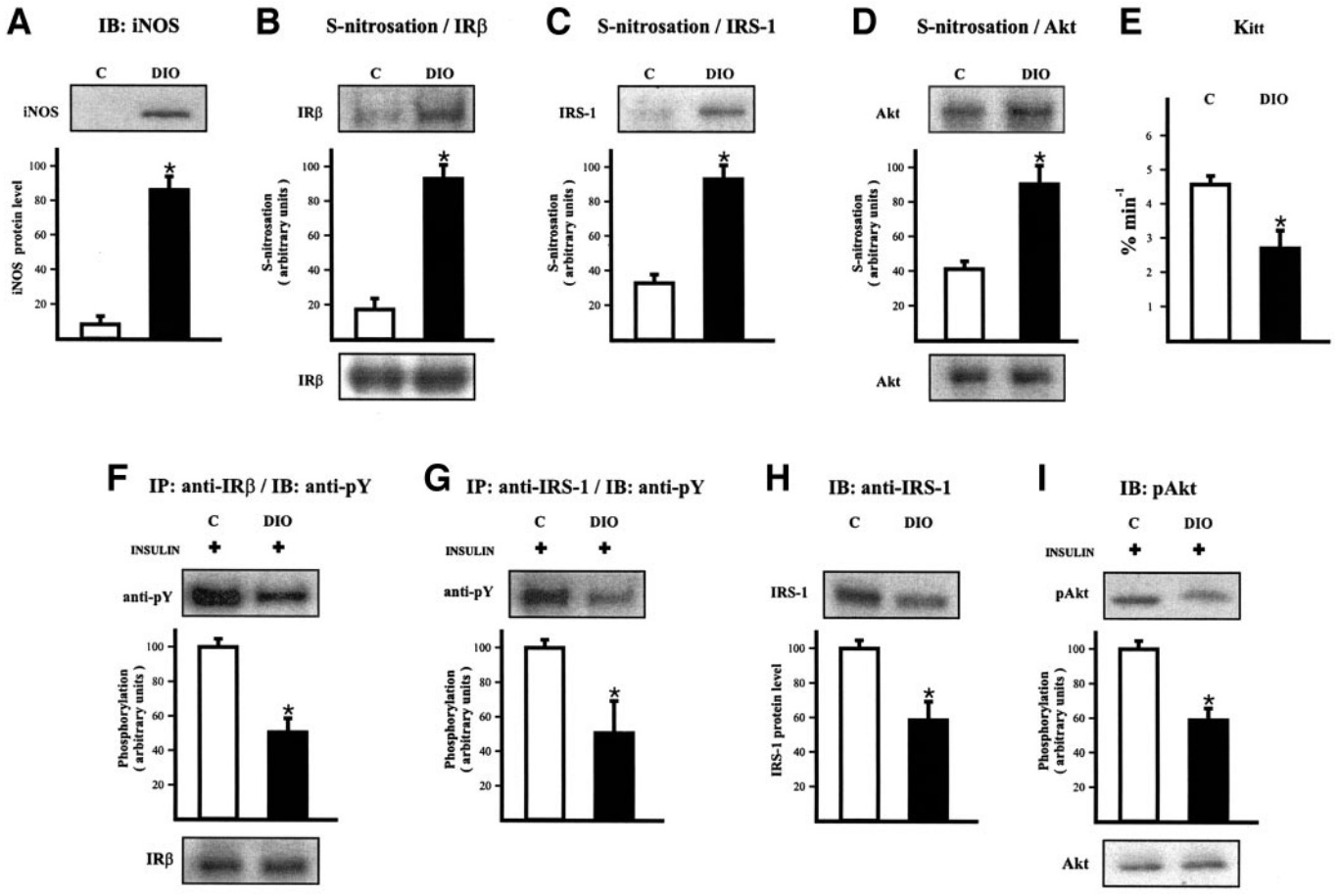

FIG. 3. Effect of DIO on insulin sensitivity and S-nitrosation of IRβ, IRS-1, and Akt in muscle. *A*: iNOS expression in muscle of DIO animals. S-nitrosation of IRβ (*B*), IRS-1 (*C*), and Akt (*D*) is shown, determined by the biotin switch method. *E*: Insulin sensitivity evaluated by glucose disappearance rates measured by the 30-min insulin tolerance test. Insulin-stimulated tyrosine phosphorylation of IRβ (*F*) and IRS-1 (*G*), IRS-1 protein level (*H*), and Akt serine phosphorylation (*I*) in muscle of DIO animals are shown. *$P < 0.05$, control vs. DIO. Bars represent means ± SE from six to eight rats. IB, immunoblotting; IP, immunoprecipitated.

disappearance rate at 30 min in the insulin tolerance test was significantly lower in *ob*/*ob* diabetic mice, and this reduction was reversed by iNOS ASO treatment, indicating an improvement in insulin sensitivity. We demonstrated an enhanced S-nitrosation of IRβ, IRS-1, and Akt in the muscle of *ob*/*ob* diabetic animals, which was reversed in iNOS ASO–treated *ob*/*ob* mice (Fig. 4*C*–*E*).

The insulin-stimulated IRβ and IRS-1 tyrosine phosphorylation were reduced in muscle of the *ob*/*ob* mice, and these reductions were reversed in the muscle of the iNOS ASO–treated *ob*/*ob* mice (Fig. 4*F* and *G*). iNOS antisense treatment also restored the IRS-1 protein content in muscle (Fig. 4*H*). The insulin-stimulated serine phosphorylation of Akt was reduced in the muscle of *ob*/*ob* mice but not in the muscle of iNOS ASO–treated *ob*/*ob* mice (Fig. 4*I*).

Rosiglitazone reduces S-nitrosation of IRβ/IRS-1/Akt and improves insulin sensitivity in diet-induced obesity. Thiazolidinediones, ligands of peroxisome proliferator–activated receptor-γ, which have an insulin-sensitizing effect, were recently found to be inhibitors of the expression of iNOS (34).

Male Wistar rats received a high-fat diet for 24 weeks, and a subgroup of these animals received rosiglitazone at 4 mg/kg body wt for 10 days. Figure 5*A* shows that rosiglitazone improved insulin sensitivity in animals with diet-induced obesity. Rosiglitazone did not improve insulin sensitivity in control rats (data not shown). The results also demonstrated that rosiglitazone treatment decreased iNOS expression in the muscle of treated animals (Fig. 5*B*) and attenuated the S-nitrosation of IRβ, IRS-1, and Akt (Fig. 5*C*–*E*), in parallel with an improvement in insulin signaling (Fig. 5*F*–*I*).

DISCUSSION

S-nitrosation, the formation of SNO by covalent addition to cysteine residues of a NO moiety, shares many features in common with phosphorylation, the prototypic post-translational modification involved in signal transduction regulation, and has been shown to regulate the function of a broad spectrum of proteins in intact cells (35). Important recent findings include demonstrations of major roles for S-nitrosation in vesicle-mediated insulin release (36), in protein processing associated with neurodegeneration of Parkinson's disease (37), and in the essential mechanisms of vectorial membrane trafficking (38). In the present study, we demonstrated that some proteins involved in early steps of insulin action can be S-nitrosated and that this posttranslational modification alters their function, suggesting a mechanism for iNOS-induced insulin resistance.

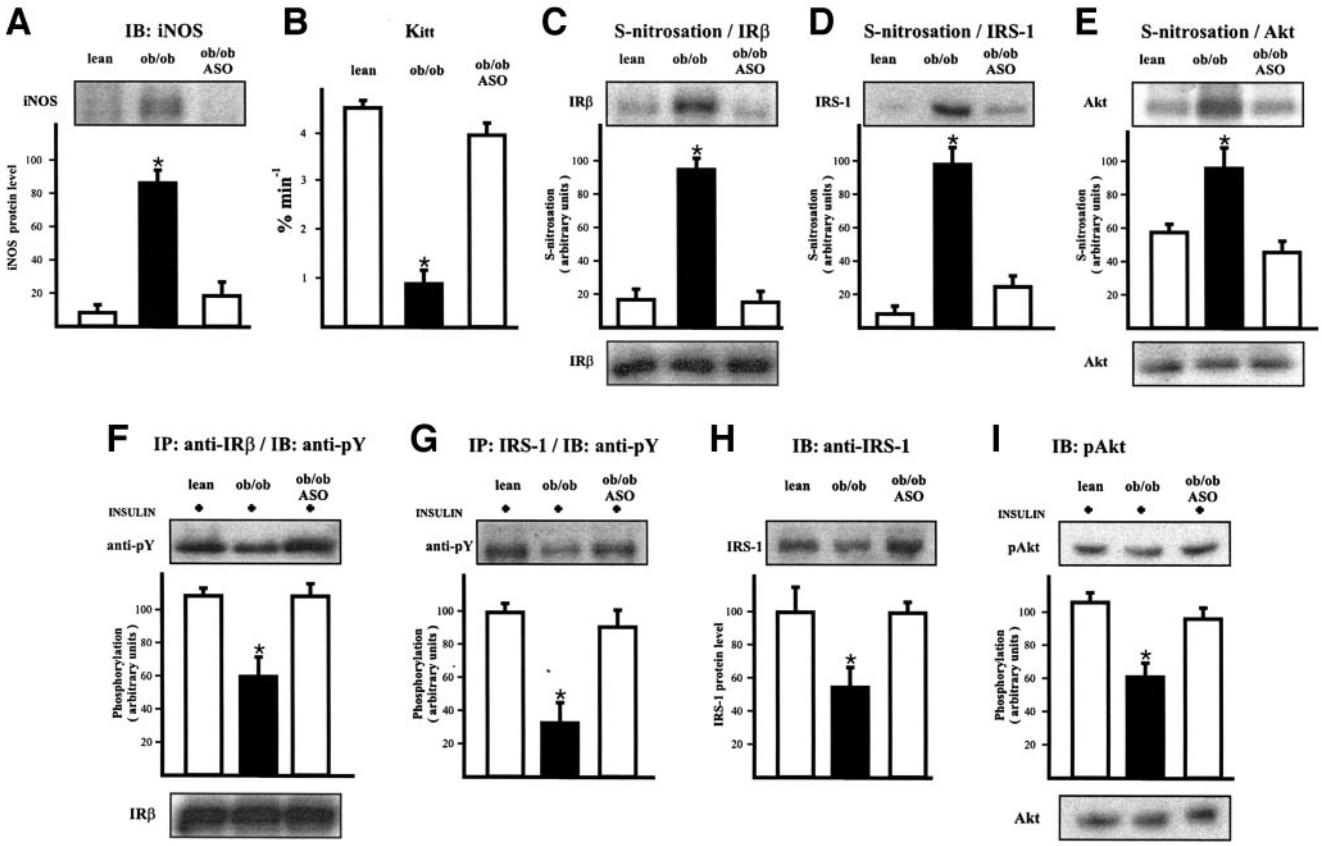

FIG. 4. Effect of iNOS antisense oligonucleotide (ASO) on iNOS expression, S-nitrosation of IRβ/IRS-1/Akt, and insulin signaling in muscle of *ob/ob* diabetic mice. *A*: iNOS protein level in *ob/ob* diabetic mice compared with iNOS ASO–treated *ob/ob* mice. *B*: Insulin sensitivity evaluated by glucose disappearance rates, measured by the 30-min insulin tolerance test. S-nitrosation of IRβ (*C*), IRS-1 (*D*), and Akt (*E*) is shown, determined by the biotin switch method. Insulin-stimulated tyrosine phosphorylation of IRβ (*F*) and IRS-1 (*G*), IRS-1 protein levels (*H*), and insulin-stimulated serine phosphorylation of Akt (*I*) is indicated. *$P < 0.05$, *ob/ob* vs. lean and *ob/ob* ASO. Bars represent means ± SE from six to eight mice. IB, immunoblotting; IP, immunoprecipitated.

Initially, we demonstrated that the NO donor, GSNO, reduces insulin-stimulated glucose metabolism in incubated rat soleus muscle. These effects were associated with increased S-nitrosation of IRβ in both isolated muscle and in the intact rat. This posttranslational modification was accompanied by a reduction in insulin-induced insulin receptor autophosphorylation and tyrosine kinase activity. The administration of GSNO also leads to S-nitrosation of IRS-1. Interestingly, the administration of this NO donor for 8 h, which allows a more prolonged S-nitrosation of IRS-1, is also accompanied by a reduction in IRS-1 protein content. A recent report demonstrated that the administration of NO donors to C2C12 myocytes induced IRS-1 degradation and, consequently, reduced protein content in a time- and dose-dependent manner (39). Taking these data together, it may be suggested that an increase in IRS-1 S-nitrosation may favor its degradation and explain the reduced protein content observed in the muscle of the intact rats that received the NO donor. This may be important, since in some situations of insulin resistance, there is a reduction in IRS-1 expression in muscle, contributing to reduced insulin sensitivity (40,41). In addition, GSNO administration also led to Akt S-nitrosation, which was accompanied by a reduction of its basal and insulin-stimulated kinase activity. The S-nitrosation of IRβ and Akt, and their reduced kinase activity, is associated with an important reduction of insulin signaling, which may play an important role in the reduced glucose transport and glycogen storage in isolated muscle treated with the NO donor and also in the insulin resistance of the animals that received this drug.

Two previously described molecular mechanisms related to insulin resistance are increased phosphotyrosine phosphatase (PTPase) activity, mainly protein tyrosine phosphatase 1B (42), and increased serine phosphorylation of IRSs (43), possibly mediated by many kinases, including protein kinase C (44), mitogen-activated protein kinase (45), and c-Jun NH_2-terminal kinase (46). We suggest that S-nitrosation is a novel mechanism of iNOS-induced insulin resistance, since the administration of an NO donor, which increased S-nitrosation of IRS-1, did not change the serine phosphorylation level of this protein, and recent data show that the administration of NO donors to different cell types reduces PTP1B activity (47). The insulin resistance induced by S-nitrosation seems to occur in at least three different steps of insulin action, i.e., by reducing IRβ autophosphorylation and tyrosine kinase activity, by reducing IRS-1 protein content, and by reducing Akt activity.

Recently, Gow et al. (48) demonstrated that protein S-nitrosation is coupled with the activity of all major forms of NO synthase. Furthermore, previous data have shown that iNOS expression is induced in several situations associated with insulin resistance, including DIO, genetic

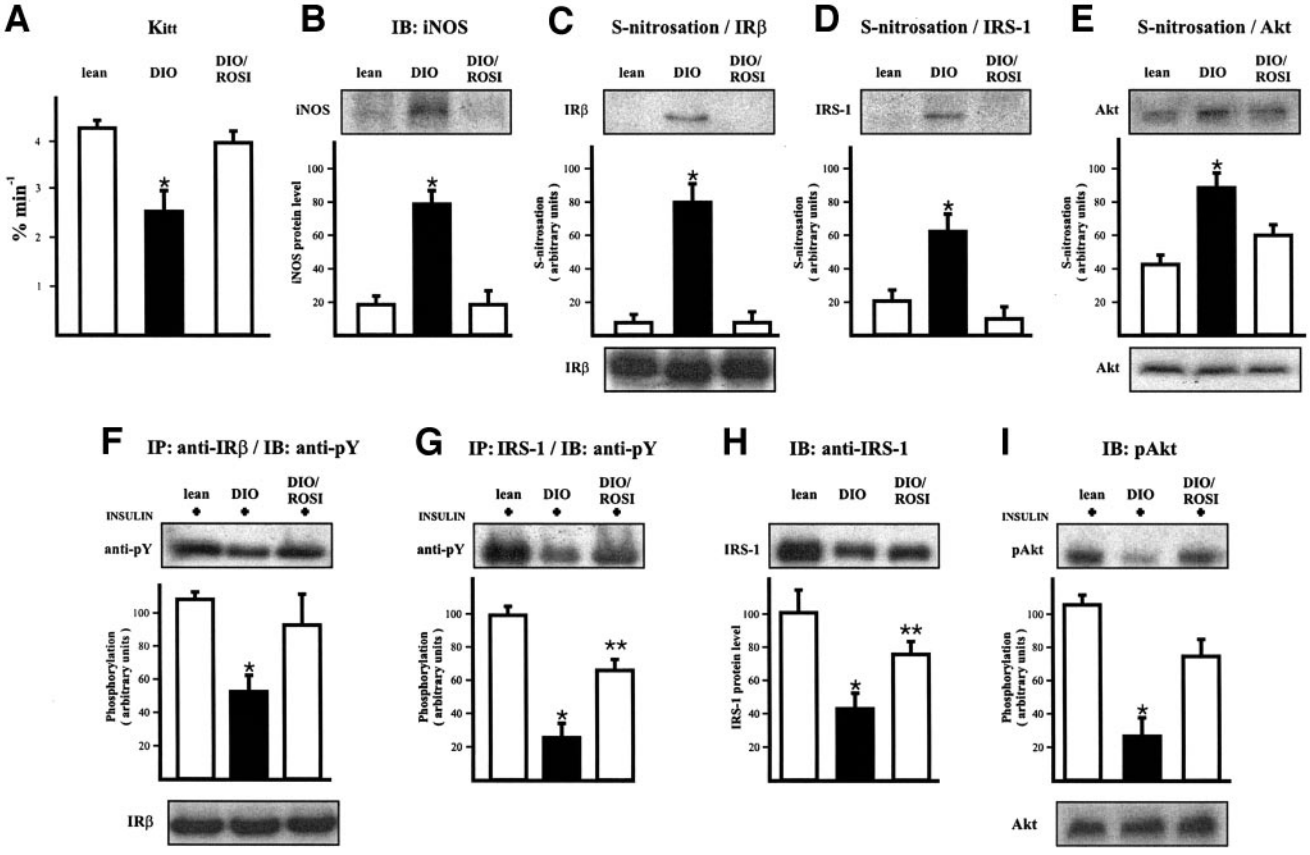

FIG. 5. Effect of rosiglitazone (ROSI) on insulin sensitivity, iNOS protein level, S-nitrosation of IRβ/IRS-1/Akt, and insulin signaling in muscle of DIO animals. *A*: Insulin sensitivity, as evaluated by glucose disappearance rates and measured by the 30-min insulin tolerance test. *B*: iNOS protein level in the muscle of DIO animals compared with rosiglitazone-treated DIO animals. S-nitrosation of IRβ (*C*), IRS-1 (*D*), and Akt (*E*) is shown, as determined by the biotin switch method. Insulin-stimulated tyrosine phosphorylation of IRβ (*F*) and IRS-1 (*G*), IRS-1 protein levels (*H*), and insulin-stimulated serine phosphorylation of Akt (*I*) are indicated. **P* < 0.05, DIO vs. lean. ***P* < 0.05, DIO vs. DIO/ROSI. Bars represent means ± SE from six to eight rats. IB, immunoblotting; IP, immunoprecipitated.

obesity, and endotoxemia (12,17,19). Here we investigated whether the phenomenon of S-nitrosation could also be observed in the muscle of animal models of insulin resistance. In DIO and *ob*/*ob* diabetic mice, there was an increase in iNOS expression in muscle, with a parallel increase in the S-nitrosation of IRβ, IRS-1, and Akt. As previously described, we also observed a reduced insulin signaling in the muscle of these animals (26). In *ob*/*ob* diabetic mice, the reduction of iNOS expression by ASO is accompanied by a reduction of S-nitrosation and improvement of insulin signaling through IRβ, IRS-1, and Akt and a reversal of insulin resistance. These data demonstrate that the insulin resistance observed in DIO and *ob*/*ob* diabetic mice is accompanied by an increase in iNOS expression and in S-nitrosation of proteins involved in early steps of insulin action. Moreover, in *ob*/*ob* mice, the reduction in iNOS expression is followed by the reduction in S-nitrosation of these proteins and reversal of insulin resistance. Targeted disruption of iNOS prevents whole-body and skeletal muscle insulin resistance in mice rendered obese by feeding a high-fat diet, which seems to be secondary to preservation of insulin-induced tyrosine phosphorylation of IRβ and IRS-1 and phosphatidylinositol 3-kinase activity in skeletal muscle of obese iNOS knockout mice (19). Although our data suggest that S-nitrosation of these proteins is a potential mechanism of iNOS-induced insulin resistance, we cannot rule out that tyrosine nitration may also contribute to NO-mediated insulin resistance in these models.

Other molecular mechanisms of insulin resistance have been demonstrated in the muscle of DIO rats and *ob*/*ob* mice, including an increase in IRS serine phosphorylation and also an increase in protein tyrosine phosphatase 1B activity (42,43). It is possible that multiple mechanisms can contribute to insulin resistance, and reversal of one of them can improve insulin action, as we demonstrate here and as previously demonstrated for other mechanisms (42,46,49).

Finally, our data raise the possibility that agents that reduce iNOS expression or activity and, consequently, reduce S-nitrosation might have beneficial effects on obesity- and septicemia-linked insulin resistance and associated complications. Recently, thiazolidinediones, a class of synthetic ligands of the peroxisome proliferator–activated receptor-γ with insulin-sensitizing effects, were found to be potent inhibitors of iNOS induction in several cell types (34,50,51). When administered to diet-induced obese animals, rosiglitazone was able to reduce iNOS expression, improve insulin signaling, reduce insulin resistance, and decrease the S-nitrosation observed in these obese animals. These effects may explain part of the improving effects of thiazolidinedione on insulin sensitivity in obese diabetic subjects.

The results presented herein may represent a potential

mechanism involved in the iNOS-induced insulin resistance. The increase in iNOS expression provokes enhanced production of NO that can induce S-nitrosate IRβ, IRS-1, and Akt, inducing downregulation of insulin signaling.

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AUTHORS' NOTE

Despite the lack of a consensus about the use of nitrosation or nitrosylation to describe the modifications induced in proteins and other substrates through their reaction with NO, we are using "S-nitrosation" throughout this article to emphasize that the modification we are referring to is a nitrosative modification of the cysteine residues of the insulin receptor, IRS-1, and Akt. This modification is associated with the formation of an SNO group and rules out the coordination of NO to a metal center as a nitrosyl ligand. In the opinion of the authors, only this last class of reactions should be named "nitrosylation" reactions.

REFERENCES

1. Lane P, Gross SS: Cell signaling by nitric oxide. *Semin Nephrol* 19:215–229, 1999
2. Gross SS, Wolin MS: Nitric oxide: pathophysiological mechanisms. *Annu Rev Physiol* 57:737–769, 1995
3. Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, Loscalzo J: S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci U S A* 89:444–448, 1992
4. Stamler JS, Toone EJ, Lipton SA, Sucher NJ: (S)NO signals: translocation, regulation, and a consensus motif. *Neuron* 18:691–696, 1997
5. Lander HM, Ogiste JS, Pearce SF, Levi R, Novogrodsky A: Nitric oxide-stimulated guanine nucleotide exchange on p21ras. *J Biol Chem* 270:7017–7020, 1995
6. Broillet MC, Firestein S: Direct activation of the olfactory cyclic nucleotide-gated channel through modification of sulfhydryl groups by NO compounds. *Neuron* 16:377–385, 1996
7. Molina y Vedia L, McDonald B, Reep B, Brune B, Di Silvio M, Billiar TR, Lapetina EG: Nitric oxide-induced S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase inhibits enzymatic activity and increases endogenous ADP-ribosylation. *J Biol Chem* 267:24929–24932, 1992
8. Kapur S, Bedard S, Marcotte B, Cote CH, Marette A: Expression of nitric oxide synthase in skeletal muscle: a novel role for nitric oxide as a modulator of insulin action. *Diabetes* 46:1691–1700, 1997
9. Shimabukuro M, Ohneda M, Lee Y, Unger RH: Role of nitric oxide in obesity-induced beta cell disease. *J Clin Invest* 100:290–295, 1997
10. Shimabukuro M, Zhou YT, Levi M, Unger RH: Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 95:2498–2502, 1998
11. Zhou YT, Grayburn P, Karim A, Shimabukuro M, Higa M, Baetens D, Orci L, Unger RH: Lipotoxic heart disease in obese rats: implications for human obesity. *Proc Natl Acad Sci U S A* 97:1784–1789, 2000
12. Sugita H, Kaneki M, Tokunaga E, Sugita M, Koike C, Yasuhara S, Tompkins RG, Martyn JA: Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance. *Am J Physiol Endocrinol Metab* 282:E386–E394, 2002
13. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415, 1995
14. Yudkin JS, Stehouwer CD, Emeis JJ, Coppack SW: C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Biol* 19:972–978, 1999
15. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259:87–91, 1993
16. Hotamisligil GS, Spiegelman BM: Tumor necrosis factor-α: a key component of the obesity-diabetes link. *Diabetes* 43:1271–1278, 1994
17. Bedard S, Marcotte B, Marette A: Cytokines modulate glucose transport in skeletal muscle by inducing the expression of inducible nitric oxide synthase. *Biochem J* 325:487–493, 1997
18. Kapur S, Marcotte B, Marette A: Mechanism of adipose tissue iNOS induction in endotoxemia. *Am J Physiol* 276:E635–E641, 1999
19. Perreault M, Marette A: Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med* 7:1138–1143, 2001
20. Storlien LH, James DE, Burleigh KM, Chisholm DJ, Kraegen EW: Fat feeding causes widespread in vivo insulin resistance, decreased energy expenditure, and obesity in rats. *Am J Physiol* 251:E576–E583, 1986
21. Oliveira MG, Shishido SM, Seabra AB, Morgon NH: Thermal stability of primary S-nitrosothiols: roles of autocatalysis and structural effects on the rate of nitric oxid release. *J Phys Chem A* 106:8963–8970, 2002
22. Crettaz M, Prentki M, Zaninetti D, Jeanrenaud B: Insulin resistance in soleus muscle from obese Zucker rats: involvement of several defective sites. *Biochem J* 186:525–534, 1980
23. Ceddia RB, William WN Jr, Curi R: Comparing effects of leptin and insulin on glucose metabolism in skeletal muscle: evidence for an effect of leptin on glucose uptake and decarboxylation. *Int J Obes Relat Metab Disord* 23:75–82, 1999
24. Leighton B, Cooper GJ: Pancreatic amylin and calcitonin gene-related peptide cause resistance to insulin in skeletal muscle in vitro. *Nature* 335:632–635, 1988
25. Bonora E, Moghetti P, Zancanaro C, Cigolini M, Querena M, Cacciatori V, Corgnati A, Muggeo M: Estimates of in vivo insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies. *J Clin Endocrinol Metab* 68:374–378, 1989
26. Saad MJ, Araki E, Miralpeix M, Rothenberg PL, White MF, Kahn CR: Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. *J Clin Invest* 90:1839–1849, 1992
27. Jaffrey SR, Snyder SH: The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE* 2001:PL1, 2001
28. Martinez-Ruiz A, Lamas S: Detection and proteomic identification of S-nitrosylated proteins in endothelial cells. *Arch Biochem Biophys* 423: 192–199, 2004
29. Park HS, Park E, Kim MS, Ahn K, Kim IY, Choi EJ: Selenite inhibits the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) through a thiol redox mechanism. *J Biol Chem* 275:2527–2531, 2000
30. Miles AM, Wink DA, Cook JC, Grisham MB: Determination of nitric oxide using fluorescence spectroscopy. *Methods Enzymol* 268:105–120, 1996
31. Wink DA, Grisham MB, Mitchell JB, Ford PC: Direct and indirect effects of nitric oxide in chemical reactions relevant to biology. *Methods Enzymol* 268:12–31, 1996
32. Saad MJ, Carvalho CR, Thirone AC, Velloso LA: Insulin induces tyrosine phosphorylation of JAK2 in insulin-sensitive tissues of the intact rat. *J Biol Chem* 271:22100–22104, 1996
33. Peraldi P, Hotamisligil GS, Buurman WA, White MF, Spiegelman BM: Tumor necrosis factor (TNF)-alpha inhibits insulin signaling through stimulation of the p55 TNF receptor and activation of sphingomyelinase. *J Biol Chem* 271:13018–13022, 1996
34. Li M, Pascual G, Glass CK: Peroxisome proliferator-activated receptor gamma-dependent repression of the inducible nitric oxide synthase gene. *Mol Cell Biol* 20:4699–4707, 2000
35. Stamler JS, Lamas S, Fang FC: Nitrosylation: the prototypic redox-based signaling mechanism. *Cell* 106:675–683, 2001
36. Rizzo MA, Piston DW: Regulation of beta cell glucokinase by S-nitrosylation and association with nitric oxide synthase. *J Cell Biol* 161:243–248, 2003
37. Chung KK, Thomas B, Li X, Pletnikova O, Troncoso JC, Marsh L, Dawson VL, Dawson TM: S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science* 304:1328–1331, 2004
38. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O'Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ: Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell* 115:139–150, 2003
39. Sugita H, Sugita M, Kaneki M: iNOS gene disruption restores depressed IRS-1 expression in skeletal muscle and ameliorates insulin resistance in (ob/ob) mice (Abstract). *Diabetes* 52 (Suppl. 1):A299, 2003
40. Saad MJ, Folli F, Kahn JA, Kahn CR: Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *J Clin Invest* 92:2065–2072, 1993
41. Zecchin HG, Bezerra RM, Carvalheira JB, Carvalho-Filho MA, Metze K, Franchini KG, Saad MJ: Insulin signalling pathways in aorta and muscle

from two animal models of insulin resistance: the obese middle-aged and the spontaneously hypertensive rats. *Diabetologia* 46:479–491, 2003
42. Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP: Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* 283:1544–1548, 1999
43. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 271:665–668, 1996
44. Chin JE, Liu F, Roth RA: Activation of protein kinase C alpha inhibits insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1. *Mol Endocrinol* 8:51–58, 1994
45. De Fea K, Roth RA: Modulation of insulin receptor substrate-1 tyrosine phosphorylation and function by mitogen-activated protein kinase. *J Biol Chem* 272:31400–31406, 1997
46. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: A central role for JNK in obesity and insulin resistance. *Nature* 420:333–336, 2002
47. Li S, Whorton AR: Regulation of protein tyrosine phosphatase 1B in intact cells by S-nitrosothiols. *Arch Biochem Biophys* 410:269–279, 2003
48. Gow AJ, Chen Q, Hess DT, Day BJ, Ischiropoulos H, Stamler JS: Basal and stimulated protein S-nitrosylation in multiple cell types and tissues. *J Biol Chem* 277:9637–9640, 2002
49. Klaman LD, Boss O, Peroni OD, Kim JK, Martino JL, Zabolotny JM, Moghal N, Lubkin M, Kim YB, Sharpe AH, Stricker-Krongrad A, Shulman GI, Neel BG, Kahn BB: Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. *Mol Cell Biol* 20:5479–5489, 2000
50. Kwon G, Xu G, Marshall CA, McDaniel ML: Tumor necrosis factor alpha-induced pancreatic beta-cell insulin resistance is mediated by nitric oxide and prevented by 15-deoxy-delta12,14-prostaglandin J2 and aminoguanidine: a role for peroxisome proliferator-activated receptor gamma activation and inos expression. *J Biol Chem* 274:18702–18708, 1999
51. Maggi LB Jr, Sadeghi H, Weigand C, Scarim AL, Heitmeier MR, Corbett JA: Anti-inflammatory actions of 15-deoxy-delta 12,14-prostaglandin J2 and troglitazone: evidence for heat shock-dependent and -independent inhibition of cytokine-induced inducible nitric oxide synthase expression. *Diabetes* 49:346–355, 2000