Targeted disruption of iNOS prevents LPS-induced S-nitrosation of IRβ/IRS-1 and Akt and insulin resistance in muscle of mice

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Carvalho-Filho, Marco A., Mirian Ueno, José B. C. Carvalheira, Lício A. Velloso, and Mario J. A. Saad. Targeted disruption of iNOS prevents LPS-induced S-nitrosation of IRβ/IRS-1 and Akt and insulin resistance in muscle of mice. Am J Physiol Endocrinol Metab 291: E476–E482, 2006. First published April 25, 2006; doi:10.1152/ajpendo.00422.2005.—We have previously demonstrated that the insulin resistance associated with inducible nitric oxide synthase (iNOS) induction in two different models of obesity, diet-induced obesity and the ob/ob mouse, is mediated by S-nitration of proteins involved in insulin signal transduction: insulin receptor β-subunit (IRβ), insulin receptor substrate 1 (IRS-1), and Akt. S-nitrosation of IRβ and Akt impairs their kinase activities, and S-nitrosation of IRS-1 reduces its tissue expression. In this study, we observed that LPS-induced insulin resistance in the muscle of wild-type mice, as demonstrated by reduced insulin-induced tyrosine phosphorylation of IRβ and IRS-1, reduced IRS-1 expression and reduced insulin-induced serine phosphorylation of Akt. This resistance occurred in parallel with enhanced iNOS expression, which was accompanied by S-nitration of IRβ/IRS-1 and Akt. In the muscle of iNOS−/− mice, we did not observe enhanced iNOS expression or any S-nitration of IRβ/IRS-1 and Akt after LPS treatment. Moreover, insulin resistance was not present. The preservation of insulin-induced tyrosine phosphorylation of IRβ and IRS-1, of IRS-1 protein expression, and of insulin-induced serine phosphorylation of Akt observed in LPS-treated iNOS−/− mice strongly suggests that the insulin resistance induced by LPS is iNOS mediated, probably through S-nitrosation of proteins of early steps of insulin signaling.

inducible nitric oxide synthase; lipopolysaccharide; insulin receptor β-subunit; insulin receptor substrate-1

SEPTIC PATIENTS AND ANIMAL MODELS of sepsis or endotoxemia exhibit many metabolic alterations, including attenuated responsiveness to insulin (10, 20, 21). The occurrence of insulin resistance during sepsis is demonstrated by diminished glucose tolerance and hyperinsulinemia (23, 24).

Administration of gram-negative bacterial lipopolysaccharide (LPS) has been used as a model of severe infection in humans and animals (5, 12). LPS is a key mediator of many of the host responses resulting from gram-negative bacteremia and sepsis, inducing many genes involved in the immune, inflammatory, and acute phase responses. Among these genes, inducible nitric oxide synthase (iNOS) has been implicated in both protective and detrimental host responses to sepsis and endotoxiaemia (18, 22, 25). LPS decreases glucose uptake and induces iNOS expression in isolated soleus (8) and induces insulin resistance in rats (1).

Accumulating evidence implicates a potential link between iNOS and insulin resistance. In several situations of insulin resistance, such as diet-induced, genetic obesity and endotoxemia, there is an iNOS induction in tissues classically related to insulin signaling (3, 8). Recently, Perreault and Marette (14) demonstrated that genetic disruption of iNOS protects against obesity-linked insulin resistance, preventing impairments in phosphatidylinositol 3-kinase (PI 3-kinase) and Akt activation by insulin in muscle.

It was recently demonstrated in our laboratory (3) that the insulin resistance associated with iNOS induction in two different models of obesity, diet-induced obesity and the ob/ob mouse, is mediated by S-nitration of proteins involved in insulin signal transduction, i.e., insulin receptor β-subunit (IRβ), insulin receptor substrate-1 (IRS-1), and Akt. S-nitrosation of IRβ reduces its autophosphorylation and tyrosine-kinase activity (3). In addition, S-nitrosation of IRS-1 is associated with its reduced tissue expression, possibly mediated by increased proteasome-mediated degradation (3, 17). Finally, S-nitrosation of Akt is associated with decreased serine-kinase activity of this enzyme, in basal states and after insulin stimulation (3, 26).

These previous data suggest that S-nitrosation may also contribute to the molecular mechanism of reduced insulin sensitivity induced by LPS or sepsis. In this study, we investigated whether LPS treatment induces insulin resistance by means of S-nitrosation of proteins involved in insulin signal transduction and whether genetic disruption of iNOS can prevent this state of insulin resistance.

METHODS

Materials. Anti-phosphotyrosine, anti-IRβ, anti-IRS-1, anti-protein kinase B (Akt), anti-iNOS, anti-α-tubulin, and anti-IRS-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pAkt was obtained from Cell Signaling Technology (Beverly, MA). Anti-pSER107IRS-1 was obtained from Upstate Biotechnology (Charlottesville, VA). Human recombinant insulin (Humulin R) was purchased from Eli Lilly (Indianapolis, IN). Routine reagents were purchased from Sigma unless otherwise specified. 125I-protein A was obtained from Amersham Biosciences (Amersham, UK).

Animals. The iNOS knockout mice used were C57BL/6-backcrossed iNOS−/− mice purchased from Jackson Laboratory (C57BL/6-Nos2tm1Lau colony). Animals were allowed free access to standard rodent chow and water ad libitum. All experiments were performed with 8-week-old C57BL/6 or iNOS−/− mice. Food was withdrawn 6 h before the experiments. The ethics committee at the University of Campinas approved all experiments involving animals.

LPS treatment. Eight hours before experiments, C57BL/6 and Nos2−/− mice received intraperitoneal injections of saline alone or saline plus LPS (10 mg/kg).

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TNF-α and IL-1β determination. TNF-α and IL-1β were determined in serum of wild-type and iNOS−/− mice after LPS treatment by enzyme-linked immunosorbent assay (ELISA) with Endogen mouse TNF-α and IL-1β ELISA kits from Pierce (Rockford, IL).

Thirty-minute insulin tolerance test. Mice were fasted for 6 h and submitted to an insulin tolerance test (ITT). Briefly, 1.5 IU/kg insulin was infused intraperitoneally in mice, and glucose was measured at 0 (basal), 5, 10, 15, 20, 25, and 30 min thereafter. The glucose disappearance rate (Km) was calculated from the formula 0.693/t1/2. The glucose t1/2 was calculated from the slope of the least-squares analysis of blood glucose concentration during the linear phase of decline (2).

IRβ/IRS-1/iNOS immunoprecipitation. Mice were injected intra-peritoneally with either saline or insulin (3.8 U/kg) 90 s after soleus muscle was removed and homogenized as described (19). Muscle lysates were incubated with anti-IRβ (0.3 mg/ml), anti-IRS-1 (1: 1,000), or anti-iNOS (1:1,000) 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 and phosphatase inhibitors, boiled 5 min in Laemml buffer, and subjected to Western blot analysis.

Western blot analysis. Muscle extracts, immunoprecipitates, or biotinylated nitrosocysteines were subjected to SDS-PAGE, and immunoblotting was performed as described (15). Immuneoreactive bands were detected using 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 (7, 11). Muscle tissue was extracted and homogenized in extraction buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine). After centrifugation at 9,000 g for 20 min, insoluble material was removed, extracts were adjusted to 0.5 mg/ml protein, and equal amounts were blocked with four volumes of blocking buffer (225 mM HEPES, pH 7.7, 0.9 mM neocuproine, 2.5% SDS, and 20 mM 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 of HENS buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, and 1% SDS) per milligram of protein. Until this point, all operations were carried out in the dark. A one-third volume of 4 mM N-6-(biotinamido)hexyl-3-(2-pyridyldithio)propionamide and 2.5 mM ascorbic acid were 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 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100), and 15 μl of neutralvidin-agarose per milligram of protein in the initial extract were added and incubated for 1 h at room temperature with agitation. Beads were washed five times with washing buffer (20 mM HEPES, pH 7.7, 600 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) and incubated with elution buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 100 mM 2-mercaptoethanol) for 20 min at 37°C with gentle stirring. Supernatants were collected, Laemml buffer was added, and proteins were separated by SDS-PAGE. Immunoblotting was performed as described above.

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 mice muscle 90 s after insulin (1.5 × 10−2 U/kg) or saline infusion in the cava vein of wild-type and iNOS−/− mice. This dose of insulin can induce a conformational change in IRβ, but not its autophosphorylation. After extensive washing of immunoprecipitates, a kinase assay was performed by adding 15 μM ATP (16) and the same amount of immunopurified IRS-1 to each immunoprecipitate (13) to measure the ability of IRβ to phosphorylate IRS-1. The IRS-1 was immunopurified, as previously described, from the muscle of control mice (13). Tyrosine phosphorylation was measured by immunoblotting with antiphosphotyrosine antibody.

Akt activity. Soleus muscles were removed from untreated mice or those treated with 10−5 M insulin. Akt activity was measured with the Akt kinase assay kit (catalog no. 9840; Cell Signaling). Briefly, Akt was immunoprecipitated from the muscle of mice, with or without previous 10−5 M insulin infusion in the cava vein. After extensive washing, the immunoprecipitates were then incubated with glycoprotein 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 in autoradiographs and were quantified by densitometry using the Scion Image software (Frederick, MD). Data were analyzed using the two-tailed unpaired Student’s t-test or by repeated-measures analysis of variance (1-way or 2-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

Effect of LPS on iNOS expression and insulin sensitivity. Initially, we investigated whether LPS treatment could induce iNOS protein expression in the muscle of wild-type and iNOS−/− mice. In the wild-type mice, we observed basal iNOS expression, which was enhanced after 8 h of LPS (10 mg/kg) infusion. In the iNOS−/− mice, we did not observe either basal iNOS expression or iNOS induction after LPS treatment (Fig. 1A).

To investigate whether enhanced iNOS expression might be associated with insulin resistance, we performed the intraperitoneal ITT in the wild-type and iNOS−/− mice. Although not significant, there was an increase of ~30% in insulin sensitivity, expressed by lower plasma glucose disappearance rates, measured using the 30-min ITT (Km) in the iNOS−/− mice compared with wild-type mice before LPS administration. This suggests that iNOS is a constitutive repressor of insulin-mediated glucose disposal, even in basal conditions. Eight hours after administration of LPS, we noticed insulin resistance in iNOS−/− mice, but not its autophosphorylation.

![Image](http://ajpendo.physiology.org/10.22118/ajpendo.2006.11002)
in the wild-type mice but not in the iNOS−/− mice, suggesting that iNOS induction by LPS treatment contributes to insulin resistance in this model (Fig. 1B). We did not observe any difference in TNF-α (wild type: 121 ± 32 ng/ml vs. iNOS−/−: 95 ± 28 ng/ml) or IL-1β (wild type: 1.7 ± 0.5 ng/ml vs. iNOS−/−: 1.3 ± 0.4 ng/ml) serum levels after LPS treatment in wild-type mice compared with iNOS−/− mice.

**Effect of LPS on insulin-induced IRβ autophosphorylation, tyrosine kinase activity, S-nitrosation, and protein level.** LPS treatment reduced insulin-induced IRβ autophosphorylation in the muscle of wild-type mice by 73% (P < 0.05) compared with wild-type controls. LPS treatment did not affect insulin-induced IRβ autophosphorylation in the muscle of iNOS−/− mice (Fig. 2A). This reduction in IRβ autophosphorylation

![Fig. 2. Effects of LPS on insulin receptor β-subunit (IRβ) tyrosine kinase activity, tyrosine phosphorylation, S-nitrosation, and protein level in muscle. A: effect of LPS (10 mg/kg) in vivo treatment on in vitro IRβ tyrosine kinase activity in muscle of WT and iNOS−/− mice as measured by autophosphorylation and by its ability to phospho-phorylate immunopurified insulin receptor substrate-1 (IRS-1). Anti-pY, anti-phosphotyrosine. B: IRβ tyrosine phosphorylation after insulin stimulation. C: IRβ S-nitrosation as detected using the biotin switch method as described in METHODS. D: IRβ protein level. Blotting against α-tubulin was employed as a loading control. *P < 0.05, WT + LPS vs. iNOS−/− + LPS. Data represent means ± SE from 6–8 experiments.](http://ajpendo.physiology.org/Downloaded From)
observed in the muscle of the wild-type mice after LPS treatment was accompanied by decreased insulin-induced IRβ tyrosine kinase activity, as demonstrated by reduced purified IRS-1 tyrosine phosphorylation by immunoprecipitated IRβ from muscle of LPS-treated wild-type mice (Fig. 2A). The insulin-induced IRβ tyrosine kinase activity was preserved in the muscle of iNOS−/− mice even after LPS treatment (Fig. 2A).

We also observed a reduction in insulin-induced IRβ tyrosine phosphorylation after LPS treatment in the muscle of wild-type mice but not in the muscle of iNOS−/− mice (Fig. 2B). LPS treatment was associated with enhanced S-nitrosation of IRβ in the muscle of wild-type mice but not in the muscle of iNOS−/− mice (Fig. 2C). The IRβ protein level was not affected by LPS treatment in the muscle of either wild-type or iNOS−/− mice (Fig. 2D).

Effect of LPS on IRS-1 tyrosine phosphorylation, serine phosphorylation, S-nitrosation, and protein level. IRS-1 tyrosine phosphorylation was reduced by 62% ($P < 0.05$) in the muscle of wild-type mice after LPS treatment compared with control wild-type mice. This reduction was not observed in the muscle of iNOS−/− mice after LPS treatment (Fig. 3A). In the muscle of the LPS-treated wild-type mice, we observed enhanced S-nitrosation of IRS-1, which was accompanied by a reduction in the IRS-1 protein level of 47% compared with control wild-type mice ($P < 0.05$). These alterations in IRS-1 S-nitrosation and protein level were not observed in the muscle of iNOS−/− mice after LPS treatment (Fig. 3, B and C).

LPS treatment induced IRS-1 serine 307 phosphorylation in a similar fashion in the muscle of wild-type and iNOS−/− mice (Fig. 3D). The insulin-induced IRS-2 tyrosine phosphorylation level after LPS treatment was similar in the muscles of both

Fig. 3. Effects of LPS on IRS-1 tyrosine phosphorylation, S-nitrosation, protein level, and serine 307 phosphorylation in muscle. A: IRS-1 tyrosine phosphorylation after insulin stimulation. B: IRS-1 S-nitrosation as detected using the biotin switch method as described in METHODS. C: IRS-1 protein level. D: IRS-1 serine 307 phosphorylation (IRS1PSER307). Blotting against α-tubulin was employed as a loading control. *$P < 0.05$, WT + LPS vs. iNOS−/− + LPS. Data represent means ± SE from 6–8 experiments.
wild-type and iNOS<sup>−/−</sup> mice. LPS treatment did not change IRS-2 protein expression in the muscle of wild-type or iNOS<sup>−/−</sup> mice (data not shown).

**Effect of LPS on Akt serine-kinase activity, serine phosphorylation, S-nitrosation, and protein level.** After 8 h of LPS treatment, we observed a reduction in immunoprecipitated Akt serine kinase activity from the muscle of LPS-treated wild-type mice, as demonstrated by its reduced capacity to phosphorylate its substrate GSK-α/β compared with Akt immunoprecipitated from the muscle of control wild-type mice. Immunoprecipitated Akt from the muscle of LPS-treated iNOS<sup>−/−</sup> mice did not present this reduction in serine kinase activity (Fig. 4A). Insulin-induced Akt serine phosphorylation also was reduced in the muscle of LPS-treated wild-type mice compared with its control. This reduced Akt serine phosphorylation was not observed in the muscle of iNOS<sup>−/−</sup> mice after LPS treatment (Fig. 4B).

LPS treatment led to increased S-nitrosation of Akt in the muscle of wild-type mice compared with its control. We did not observe enhanced Akt S-nitrosation in the muscle of iNOS<sup>−/−</sup> mice after LPS treatment (Fig. 4C). LPS treatment had no effect on Akt protein level in the muscle of either wild-type or iNOS<sup>−/−</sup> mice (Fig. 4D).

We also performed experiments to investigate whether the phenomenon of S-nitrosation occurs in other insulin-sensitive tissues, such as liver or adipocytes. We investigated S-nitrosation...
tion of Akt in the liver and adipose tissue of animals treated with LPS. Results demonstrated that LPS infusion induced Akt S-nitrosation in the liver and adipose tissues of mice in parallel with a decrease in insulin-induced Akt phosphorylation in these tissues (data not shown).

**DISCUSSION**

Recent studies have demonstrated that exogenous nitric oxide (NO) and the NO produced by 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). Moreover, the induction of iNOS has been associated with impaired insulin-stimulated glucose uptake in isolated rat muscles (8).

Endotoxemia is a clinical and experimental situation related to insulin resistance and iNOS induction. LPS impairs insulin-stimulated glucose uptake in skeletal muscle, the major tissue for insulin-mediated glucose uptake (23). However, the molecular mechanisms involved in this reduction were not completely understood. Therefore, because LPS induces iNOS expression in muscle, we decided to investigate whether the enhanced NO production by this enzyme could induce S-nitrosation of proteins involved in insulin signal transduction and, consequently, insulin resistance.

Recently, we demonstrated that iNOS induction in the muscle of diet-induced obese rats and ob/ob mice is associated with enhanced NO production and enhanced S-nitrosation of IRβ, IRS-1, and Akt. S-nitrosation of IRβ and Akt is associated with reductions in their kinase activities, downregulating the IRβ/IRS-1/PI 3-kinase/Akt pathway (3). Because this pathway plays a central role in metabolic actions of insulin in muscle, including stimulation of glucose uptake and glycogen synthesis, a downregulation of this pathway in muscle by S-nitrosation may be an important mechanism of iNOS-induced insulin resistance.

Initially, we observed that LPS treatment induced insulin resistance in parallel with augmented iNOS expression in the muscle of wild-type mice. Insulin-stimulated IRβ tyrosine kinase activity and tyrosine phosphorylation were reduced in this tissue, and its S-nitrosation was enhanced. As we have previously demonstrated, S-nitrosation of IRβ is associated with its reduced tyrosine kinase activity.

Investigators in our laboratory also previously observed that iNOS induction in diet-induced obesity and in ob/ob mice is associated with enhanced S-nitrosation of IRS-1 and its reduced protein level in muscle. Pharmacological or genetic blockade of iNOS prevented the reduction in the IRS-1 protein level in these two models of insulin resistance (3). LPS treatment induced S-nitrosation of IRS-1, which was accompanied by reduced IRS-1 protein expression in accordance with these previous observations. Recently, Sugita et al. (17) demonstrated that NO donors or induction of iNOS in C2C12 myotubes decreased IRS-1 protein expression without altering its mRNA expression through augmented ubiquitination and proteasome degradation of this protein. Moreover, S-nitrosation was identified as a novel posttranslational modification that targets a protein for recognition by the ubiquitin-proteasome system (9), similar to phosphorylation and hydroxylation, as a molecular switch that precisely activates the degradation of a specific protein. In light of these data, we may hypothesize that S-nitrosation of IRS-1 may target this protein to degradation via the ubiquitin-proteasome system; however, this observation requires further investigation. Conversely, we did not observe any modulation of IRβ or Akt protein expression by S-nitrosation, which suggests the specificity of this mechanism of protein expression regulation.

In accordance with our data, Yasukawa et al. (26) demonstrated that S-nitrosation of Akt after NO donor treatment of cultured myoblasts and in the muscle of db/db diabetic mice is associated with reduced serine kinase activity of this enzyme, although this S-nitrosation did not modulate Akt protein expression. Substitution of cysteine 224 of human Akt1/PKBα by serine conferred resistance to NO donor-induced S-nitrosation and inactivation of Akt1/PKBα, indicating that a major S-nitrosation acceptor site in Akt1/PKBα is cysteine 224, which is conserved in Akt1/Akt2 and Akt3. LPS treatment also leads to S-nitrosation of Akt, a serine kinase involved in glucose uptake induced by insulin in muscle. This S-nitrosation in LPS-treated mice was accompanied by reduced Akt kinase activity.

Targeted disruption of iNOS prevents the 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, as well as PI 3-kinase activity in skeletal muscle of obese iNOS knockout mice (14). The data presented in this study show that targeted disruption of iNOS also prevented LPS-induced skeletal muscle insulin resistance in addition to preventing S-nitrosation of IRβ, IRS-1, and Akt. Therefore, the preservation of insulin-induced tyrosine phosphorylation of IRβ and IRS-1 and the maintenance of IRS-1 protein expression, and of insulin-induced serine phosphorylation of Akt, observed in LPS-treated iNOS−/− mice strongly suggest that the insulin resistance induced by LPS is iNOS mediated, probably through S-nitrosation of proteins of early steps of insulin signaling.

Insulin resistance is certainly a metabolic situation related to several molecular mechanisms that act in parallel to downregulate insulin signaling. Endotoxemia and LPS treatment did not differ from other situations of insulin resistance, and there are probably a number of activated metabolic pathways that lead to negative feedback in insulin signaling. Our model shows one of these pathways, S-nitrosation of IRβ/IRS-1 and Akt, which was recently described for obesity-induced insulin resistance and also is important for LPS-induced insulin resistance. In addition, we cannot exclude the hypothesis that other molecular mechanisms may be contributing to the LPS-induced insulin resistance, although we did not observe any difference in IRS-1 serine 307 phosphorylation or in serum levels of IL-1β/TNF-α between LPS-treated wild-type and iNOS−/− mice.

Several authors have demonstrated that, although insulin resistance is mediated by different mechanisms, the disruption of one of these may restore insulin signaling almost completely (4, 6). In this study, we have shown that S-nitrosation of IRβ/IRS-1 and Akt is one of these mechanisms and that in LPS-mediated insulin resistance, this mechanism may be the central one in muscle. Together, our data suggest that skeletal muscle insulin resistance, induced by LPS treatment, is related to iNOS induction, augmented NO production, and S-nitrosa-
tion of proteins involved in insulin signal transduction: IRβ, IRS-1, and Akt.

REFERENCES


