A Role for iNOS in Fasting Hyperglycemia and Impaired Insulin Signaling in the Liver of Obese Diabetic Mice

Masaki Fujimoto,[1] Nobuyuki Shimizu,[1] Kaiko Kunii,[1] J.A. Jeevendra Martyn,[1] Kohjiro Ueki,[2] and Masao Kaneki[1]

Chronic inflammation has been postulated to play an important role in the pathogenesis of insulin resistance. Inducible nitric oxide synthase (iNOS) has been implicated in many human diseases associated with inflammation. iNOS deficiency was shown to prevent high-fat diet–induced insulin resistance in skeletal muscle but not in the liver. A role for iNOS in fasting hyperglycemia and hepatic insulin resistance, however, remains to be investigated in obesity-related diabetes. To address this issue, we examined the effects of a specific inhibitor for iNOS, L-NIL, in obese diabetic (*ob/ob*) mice. iNOS expression was increased in the liver of *ob/ob* mice compared with wild-type mice. Treatment with iNOS inhibitor reversed fasting hyperglycemia with concomitant amelioration of hyperinsulinemia and improved insulin sensitivity in *ob/ob* mice. iNOS inhibitor also increased the protein expression of insulin receptor substrate (IRS)-1 and -2 1.5- and 2-fold, respectively, and enhanced IRS-1– and IRS-2–mediated insulin signaling in the liver of *ob/ob* mice. Exposure to NO donor and ectopically expressed iNOS decreased the protein expression of IRS-1 and -2 in cultured hepatocytes. These results suggest that iNOS plays a role in fasting hyperglycemia and contributes to hepatic insulin resistance in *ob/ob* mice. *Diabetes* 54:1340–1348, 2005

Chronic low-grade inflammation has been proposed to be involved in the pathogenesis in obesity-related insulin resistance and type 2 diabetes. The expression of proinflammatory cytokines, including tumor necrosis factor-α (1) and interleukin-6 (2), is upregulated in animal models of and patients with type 2 diabetes. However, limited knowledge is thus far available about the molecular mechanisms by which chronic inflammation mediates insulin resistance and type 2 diabetes.

The activation of inhibitor κB kinase β (IKKβ)–nuclear factor-κB (NF-κB), a crucial signaling cascade for inflammatory response, has been highlighted as a mediator of insulin resistance. The pharmacological inhibition or gene disruption of IKKβ reversed obesity-related insulin resistance and fasting hyperglycemia in rodents and humans (3–5). However, little is known about genes that function as downstream effectors of the IKKb–NF-κB pathway to mediate insulin resistance.

Inducible nitric oxide synthase (iNOS; also termed NOS2), whose expression is regulated by IKKβ–NF-κB (6), is assumed to be one of the candidates that mediate inflammation-involved insulin resistance. Accumulating evidence indicates a close link between iNOS and insulin resistance. Although iNOS was originally identified in macrophages, it is now known that it is widely expressed in many tissues, including insulin-sensitive organs such as skeletal muscle, adipose tissue, and liver, in normal rodents and humans. The expression of iNOS is upregulated by most, if not all, inducers of insulin resistance, including proinflammatory cytokines, obesity (7), free fatty acids (8), hyperglycemia (9,10), endotoxins (6,11), and oxidative stress. In fact, elevated expression of iNOS was observed in skeletal muscle of high-fat diet–fed mice (12), in heart of Zucker diabetic fatty rats (13), and in skeletal muscle (14) and platelets of patients with type 2 diabetes (15). Nitrosative protein modifications, such as tyrosine nitration often associated with iNOS expression, were elevated in plasma (16), skeletal muscle (14), vasculature (17,18), and retina (19) of patients with and rodent models of type 2 or obesity-related diabetes. Furthermore, iNOS induction resulted in attenuated insulin-stimulated glucose uptake in cultured skeletal muscle cells (20). Thiazolidinediones, a class of insulin sensitizer, suppress iNOS expression in cultured cells and in vivo in rodents (21,22). Thus, inhibition of iNOS expression has been recently proposed to be a new mechanism of actions of insulin sensitizers (23,24).

Recently, we and others have shown that iNOS plays an important role in the pathogenesis of insulin resistance in vivo. We demonstrated that iNOS inhibitor prevented fasting hyperglycemia and mitigated insulin resistance in lipopolysaccharide (LPS)-administered rats (11). Perreault and Marette (12) reported that iNOS deficiency protected against high-fat diet–induced insulin resistance. With respect to a role of iNOS in insulin resistance, our previous findings in LPS-administered rats appear to agree with the data of Perreault and Marette in high-fat diet–fed mice.

From the [1]Department of Anesthesia and Critical Care, Massachusetts General Hospital, Shriners Hospital for Children, Harvard Medical School, Boston, Massachusetts; and the [2]Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Address correspondence and reprint requests to Masao Kaneki, Department of Anesthesia and Critical Care, Massachusetts General Hospital, Harvard Medical School, 149 13th St., Rm. 6604, Charlestown, MA 02129. E-mail: mkaneki@partners.org.

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Br-cGMP, 8-bromoguanosine-3′,5′-cyclic monophosphorothioate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSNO, S-nitrosoglutathione; IKK, inhibitor κB kinase; iNOS, inducible NO synthase; IR, insulin receptor; IRS, IR substrate; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PI3, phosphatidylinositol-3; SREBP, sterol regulatory element–binding protein.

However, differences also exist in these previous studies (11,12), particularly in regard to the tissues in which iNOS inhibition or deficiency exerts its insulin-sensitizing effects. We found that iNOS expression was elevated in the liver by LPS and that iNOS inhibitor blocked LPS-induced increase in hepatic glucose output in Sprague-Dawley rats (11). By contrast, Perreault and Marette observed that iNOS expression was increased in skeletal muscle and adipose tissue but not in the liver of C57BL/6X129SvEv mice fed a high-fat diet and that iNOS disruption restored high-fat diet–induced defects in insulin signaling in skeletal muscle but not in adipose tissue or liver (12). Moreover, in their study, iNOS deficiency did not alter blood glucose levels in mice fed a high-fat diet, whereas whole-body insulin resistance was alleviated, as judged by an insulin tolerance test. A question, therefore, remains to be answered whether a role of iNOS in obesity-related insulin resistance is restricted to skeletal muscle or whether iNOS also plays a role in the liver, which is the primary organ to determine fasting blood glucose levels (25–27). To address this issue, we examined the effects of iNOS inhibitor on fasting hyperglycemia and hepatic insulin signaling in genetically obese diabetic (*ob/ob*) mice.

RESEARCH DESIGN AND METHODS

Male *ob/ob* mice and lean wild-type C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The *ob/ob* mice were backcrossed onto wild-type C57BL/6J mice for at least 30 generations. The institutional animal care committee approved the study protocol. The mice were housed in mesh cages in a room maintained at 25°C and illuminated in 12:12-h light-dark cycles; they were provided with standard rodent diet and water ad libitum.

Treatment with iNOS inhibitor. Male *ob/ob* mice at 10 weeks of age were treated with a specific inhibitor of iNOS, L-NIL (60 mg/kg body wt, i.p., b.i.d.; Cayman Chemical, Ann Arbor, MI), or PBS for 10 days. Following 10 days of treatment, the animals treated with L-NIL ($n = 17$) or PBS ($n = 18$) were fasted overnight and then received an injection of insulin (Humulin R; Eli Lilly, Indianapolis, IN; 15 units/kg body wt in PBS with 0.1% BSA) or vehicle (PBS with 0.1% BSA) via the portal vein under anesthesia with pentobarbital sodium (70 mg/kg body wt, i.p.). At 5 min after insulin injection, the liver, gastrocnemius muscle, and epididymal fat were taken for biochemical analyses. Male *ob/ob* mice at 10 weeks of age were also treated with L-NIL or PBS for 10 days under a pair-fed condition. After 10 days of treatment, the liver was taken from pair-fed mice under fed condition or after overnight fasting. Twenty-four mice were randomly assigned into four groups (L-NIL fasted, PBS fasted, L-NIL fed, and PBS fed) of six animals. The average food intake of both L-NIL and PBS-treated animals under pair-fed condition was 5.6 g/day.

Insulin tolerance test. After 10 days of treatment, the animals treated with L-NIL ($n = 10$) or PBS ($n = 10$) were fasted for 4 h and then received an injection of insulin (2.5 units/kg body wt in PBS with 0.1% BSA, i.p.). At 0, 15, 30, 60, 90, and 120 min after insulin injection, blood samples were collected to measure glucose level.

Immunoprecipitation and immunoblotting. Liver samples were homogenized as previously described (28). Immunoprecipitation and immunoblotting were performed (29) using anti-IRβ, IRS-1 and -2, p85 phosphatidylinositol 3(PI3)-kinase, iNOS, endothelial NOS, neuronal NOS (Upstate, Lake Placid, NY), Akt, phosphorylated Akt (Ser473) (Cell Signaling, Beverly, MA), and phosphotyrosine antibodies (Santa Cruz, Santa Cruz, CA) (online appendix available at http://diabetes.diabetesjournals.org).

Partial purification of iNOS. Partial purification of iNOS was performed using immobilized 1400W beads (Calbiochem, San Diego, CA), which selectively bind to iNOS, according to the manufacturer's instruction (online appendix).

PI3-kinase assay. PI3-kinase activities in the immunoprecipitates with anti–IRS-1 or -2 antibody were measured in vitro (30).

Detection of tyrosine nitration. Immunostaining with anti-nitrotyrosine antibody (Upstate) was performed according to the manufacturer's instruction (online appendix).

RNase protection assay. RNase protection assay was performed as previously described (11) using the RPA III kit (Ambion, Austin, TX). mRNA levels of IRS-1 and -2 and sterol regulatory element–binding protein (SREBP)-1c were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (online appendix).

Cell culture. Mouse Hepa1c1c7 and human HepG2 cells (American Type Culture Collection, Manassas, VA) were maintained in α-minimum essential medium and minimum essential medium supplemented with 10% fetal bovine serum, respectively. After 8 h serum starvation, the cells were treated with the indicated concentrations of S-nitrosoglutathione (GSNO) or 3-morpholinosydnonimine (Cayman Chemical, Ann Arbor, MI) in the presence or absence of ODQ (1 μmol/l; Sigma) or 8-bromoguanosine-3′,5′-cyclic monophosphorothioate (Br-cGMP; 100 μmol/l; Axxora, San Diego, CA) for 24 h, unless otherwise indicated. Hepa1c1c7 cells were transfected with pCDNA3/iNOS or pCDNA3 using Lipofectamin 2000 (Invitrogen). pCDNA3/iNOS was kindly provided by Dr. Kone. At 48 h after transfection, the cells were harvested.

Measurement of blood glucose, insulin, triglycerides, glycogen, adiponectin, and free fatty acids. Blood glucose level and plasma insulin concentration were determined by the glucose oxidase method (Ascensia Elite Glucometer; Bayer, Elkhart, IN) and enzyme-linked immunosorbent assay (Crystal Chem, Chicago, IL), respectively. Glycogen content was measured using a glucose hexokinase assay kit (Sigma) (31). A Glycerol phosphate oxidase trinder reagent (Sigma) was used to measure triglyceride (32). The concentrations of adiponectin and free fatty acids in serum from fasted animals were determined using an enzyme-linked immunosorbent assay kit (Linco Research, St. Charles, MO) and an enzymatic colorimetric assay kit (Wako Chemicals, Richmond, VA), respectively.

Statistical analysis. Statistical analyses were performed using Student's t test or one-way ANOVA followed by Fisher's protected least significant differences test. Values of $P < 0.05$ were considered statistically significant. Data are expressed as means ± SE.

RESULTS

Increased expression of iNOS in the liver of diabetic mice. We found a 2.5-fold increase in iNOS protein expression in the liver of *ob/ob* mice compared with wild-type mice (Fig. 1*A*). No difference was found in the protein expression of endothelial or neuronal NOS between *ob/ob* and wild-type mice (online appendix Fig. 1). iNOS expression was also increased in skeletal muscle and adipose tissue of *ob/ob* mice compared with wild-type mice to the extent comparable to that in the liver (online appendix Fig. 2).

Reduced nitrotyrosine immunoreactivity by iNOS inhibitor. The immunoreactivity for nitrotyrosine, a marker of nitrosative stress, was also elevated in the liver of *ob/ob* mice compared with wild-type mice (Fig. 1*B*). Treatment with iNOS inhibitor (60 mg/kg body wt, i.p., b.i.d., for 10 days) reversed the elevated nitrotyrosine immunoreactivity in the liver of *ob/ob* mice compared with PBS. L-NIL treatment, however, did not alter the expression of iNOS and endothelial and neuronal NOS (data not shown). These results suggest that L-NIL, a competitive inhibitor for iNOS, effectively reduced the activity of iNOS in the liver of *ob/ob* mice without altering iNOS expression level, as expected.

Reversal of fasting hyperglycemia and amelioration of hyperinsulinemia by iNOS inhibitor in diabetic mice. At the inception of the treatment, there was no difference in fed blood glucose level (L-NIL: 292.8 ± 21.8; PBS: 290.3 ± 18.7 mg/dl), plasma insulin concentration (L-NIL: 57.0 ± 0.5; PBS: 57.0 ± 0.5 ng/ml), or body weight (L-NIL: 57.0 ± 0.5; PBS: 56.8 ± 0.6 g) between L-NIL –and PBS-administered animals. Treatment with L-NIL did not affect body weight at the time of its termination (L-NIL: 57.2 ± 0.5; PBS: 57.0 ± 0.6 g). There was no statistical difference in food intake between L-NIL –and PBS-treated animals (L-NIL: 5.8 ± 0.2; PBS: 6.4 ± 0.2 g/day), although food intake in L-NIL–treated animals appeared to be lower than in PBS-treated animals. However, L-NIL treatment

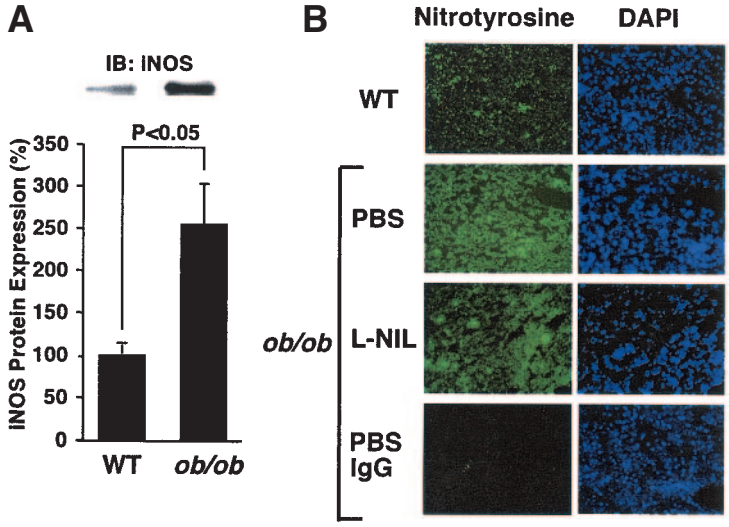

FIG. 1. Expression of iNOS and tyrosine nitration in *ob/ob* mice. *A*: Immunoblot analyses (IB) revealed that iNOS expression was increased in the liver of *ob/ob* mice compared with wild-type (WT) mice. *B*: Nitrotyrosine immunoreactivity was elevated in the liver of *ob/ob* mice treated with PBS compared with wild-type (WT) mice. L-NIL reduced nitrotyrosine immunoreactivity in *ob/ob* mice. Magnification: ×400.

reversed fasting hyperglycemia and significantly ameliorated fasting hyperinsulinemia in *ob/ob* mice (Fig. 2*A* and *B*). The insulin sensitivity index (fasting blood glucose × plasma insulin concentration) was also significantly improved by L-NIL (L-NIL: 16.9 ± 4.0; PBS: 51.0 ± 12.6 mg^2/l^2, $P < 0.05$). Improved insulin sensitivity by L-NIL treatment was confirmed by insulin tolerance test. *ob/ob* mice treated with L-NIL responded to insulin with a more profound decrease in blood glucose level than in PBS-administered *ob/ob* mice (Fig. 2*C*). Area under the curve analysis in response to the insulin tolerance test also revealed the beneficial effects of L-NIL (Fig. 2*D*).

To exclude a possible influence of food intake, the effects of L-NIL treatment were also examined in pair-fed *ob/ob* mice. The beneficial effects of L-NIL were corroborated in pair-fed mice. In pair-fed *ob/ob* mice, L-NIL treatment also led to the reversal of fasting hyperglycemia (L-NIL: 95.3 ± 13.4; PBS: 210.8 ± 18.1 mg/dl, $P < 0.05$) and significant amelioration of fasting hyperinsulinemia (L-NIL: 11.4 ± 3.2; PBS: 19.4 ± 1.9 ng/ml, $P < 0.05$) and insulin sensitivity index (L-NIL: 10.3 ± 2.8; PBS: 40.8 ± 5.9 mg^2/l^2, $P < 0.05$). Under fed condition, L-NIL treatment appeared to mitigate hyperglycemia in *ob/ob* mice (L-NIL: 195.7 ± 29.1; PBS: 272.2 ± 24.0, $P < 0.10$), although no statistical significance was found. Plasma insulin concentration under fed condition tended to be higher in L-NIL–treated animals than PBS-treated animals (L-NIL: 86.4 ± 7.0; PBS: 57.5 ± 12.4 ng/ml, $P < 0.10$), although there was no statistical difference.

iNOS inhibitor improved IRS-1– and IRS-2–mediated insulin signaling in the liver of diabetic mice. iNOS inhibitor, L-NIL, did not alter the protein expression of insulin receptor (IR). However, the protein expression of IRS-1 and -2 under a fasted condition was increased by 1.5- and 2-fold, respectively, compared with PBS-treated animals (Fig. 3*A*). Increased protein expression of IRS-1 and -2 by L-NIL was further confirmed in pair-fed mice. In pair-fed *ob/ob* mice, the protein expression of IRS-1 and -2 in L-NIL–treated animals was greater than in PBS-treated animals under both fasted and fed conditions (Fig. 3*B* and online appendix Fig. 3). L-NIL treatment resulted in upregulated mRNA level of IRS-2 under both fasted and fed

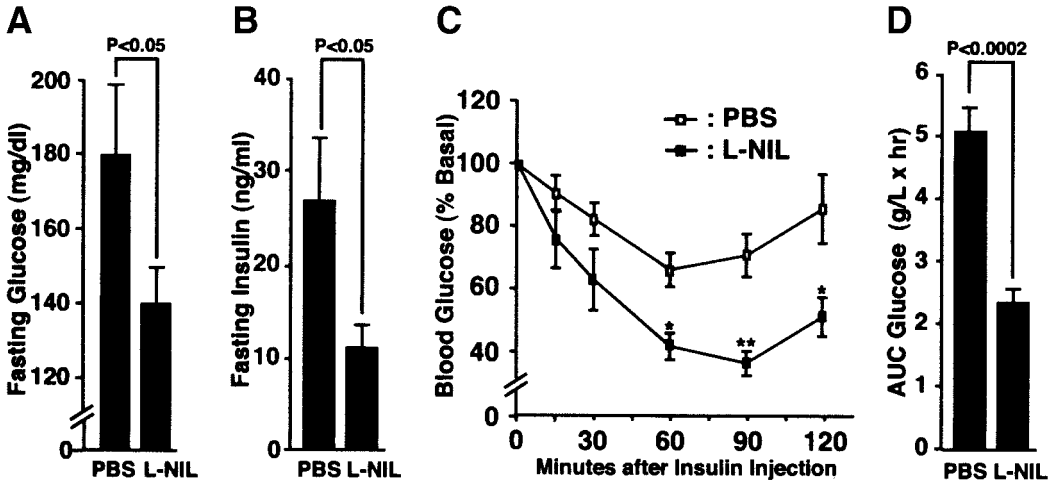

FIG. 2. Effects of iNOS inhibitor on glycemic control and insulin sensitivity in *ob/ob* mice. *A* and *B*: L-NIL treatment decreased fasting blood glucose level (*A*) and fasting plasma insulin concentration (*B*) in *ob/ob* mice compared with PBS. *C*: Percent decreases in blood glucose relative to the basal value just before insulin injection were greater in L-NIL–treated *ob/ob* mice (■) than in PBS-treated *ob/ob* mice (□) at 60, 90, and 120 min after insulin injection. *$P < 0.05$; **$P < 0.01$ vs. mice with PBS. *D*: L-NIL significantly reduced the area under the blood glucose curve (AUC Glucose) of the 120-min time course in response to an insulin tolerance test, compared with PBS.

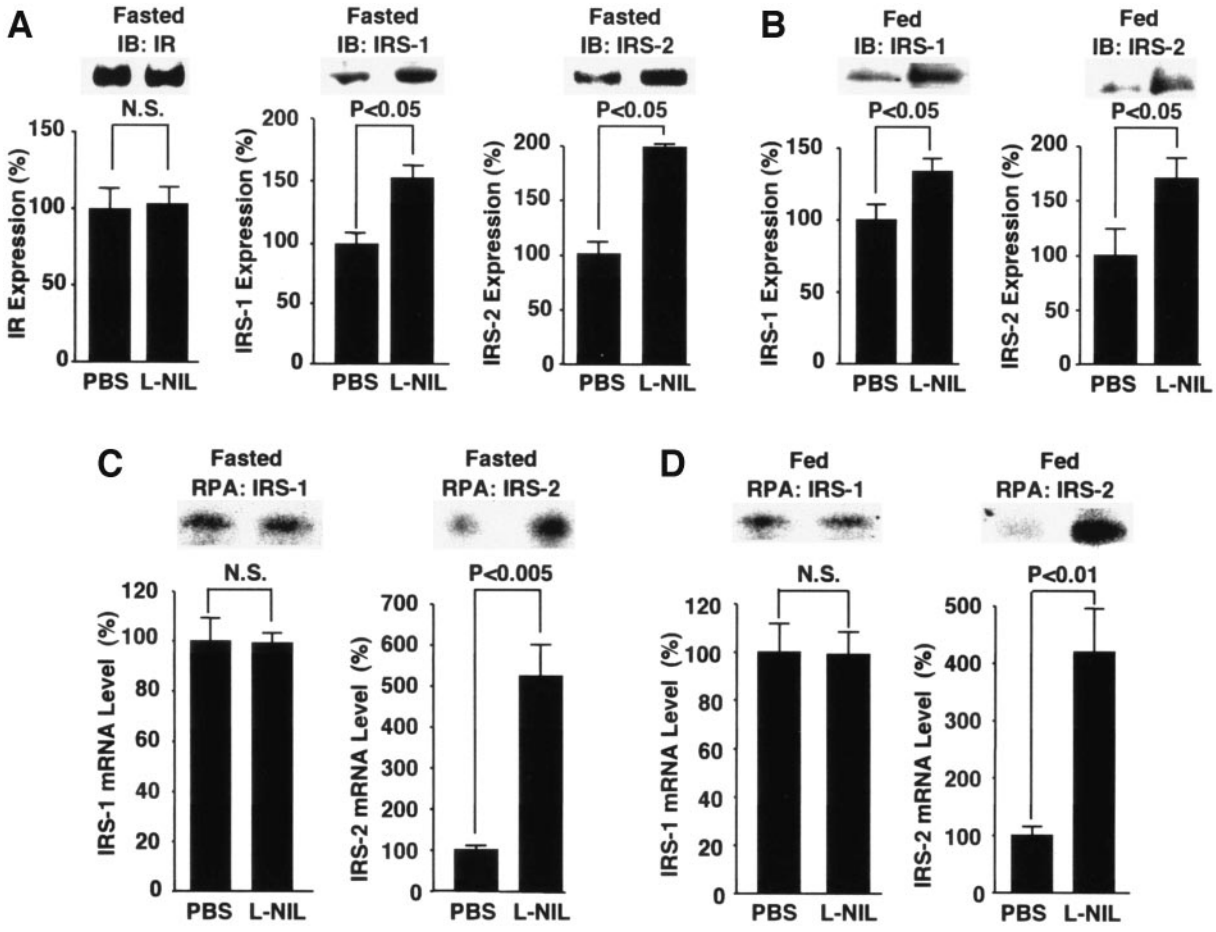

FIG. 3. Effects of iNOS inhibitor on expression of IR and IRS-1 and -2 in *ob/ob* mice. *A* and *B*: Immunoblot analyses (IB) revealed that the IRS-1 and -2 expression was greater in the liver of *ob/ob* mice treated with L-NIL compared with PBS under both fasted (*A*) and fed (*B*) conditions. *C* and *D*: RNase protection assay (RPA) demonstrated that L-NIL increased IRS-2 mRNA, but not IRS-1 mRNA, in the liver of *ob/ob* mice under both fasted (*C*) and fed (*D*) conditions.

conditions (Fig. 3*C* and *D*). However, the mRNA level of IRS-1 was not altered by L-NIL treatment. L-NIL did not affect GAPDH mRNA level (data not shown). No difference was found in the protein expression of p85, p55α, and p50α PI3-kinase in the liver between *ob/ob* mice treated with L-NIL and PBS (online appendix Fig. 4).

Insulin-stimulated tyrosine phosphorylation of IRS-1 and -2 in the liver was upregulated ~1.5- and 3-fold by iNOS inhibitor, respectively, compared with PBS (Fig. 4). However, iNOS inhibitor did not alter insulin-stimulated tyrosine phosphorylation of IR. L-NIL treatment did not significantly alter basal (insulin-unstimulated) tyrosine phosphorylation of IR and IRS-1 and -2. The ratio of insulin-stimulated tyrosine phosphorylation to protein abundance for IR and IRS-1 and -2 was not significantly altered by L-NIL treatment (online appendix Fig. 5). These results suggest that the improvement by iNOS inhibitor in insulin-stimulated tyrosine phosphorylation of IRS-1 and -2 may be mainly attributable to the increased expression of IRS-1 and -2. Insulin-stimulated IRS-1– and IRS-2–associated PI3-kinase activity was also enhanced by L-NIL up to 1.5- and 2.5-fold, respectively (Fig. 4*D* and *E*). L-NIL treatment also increased insulin-stimulated phosphorylation of Akt/protein kinase B, a downstream signaling molecule, while the expression of Akt/protein kinase B was not altered (Fig. 4*F* and *G*).

Glycogen content in the liver of fasted animals was significantly increased by L-NIL treatment (L-NIL: 14.9 ± 1.8; PBS: 7.3 ± 0.6 mg/g liver wt, $P < 0.01$). Triglyceride content in the liver of fasted animals appeared to be lower in L-NIL–treated animals than in PBS-treated animals (L-NIL: 52.9 ± 5.6; PBS: 65.7 ± 4.0 μg/g protein, $P < 0.10$), although there was no statistical difference. In skeletal muscle of *ob/ob* mice, L-NIL treatment increased IRS-1 protein expression, while IRS-2 abundance was unaltered. In adipose tissue, neither IRS-1 nor -2 expression was affected by L-NIL treatment (online appendix Fig. 6). We did not find a significant difference in circulating adiponectin (L-NIL: 6.32 ± 0.6; PBS: 6.00 ± 0.45 ng/ml) and free fatty acid (L-NIL: 1.83 ± 0.1; PBS: 1.71 ± 0.12 mEq/l) concentration between L-NIL– and PBS-administered *ob/ob* mice.

NO donor and iNOS reduced the expression of IRS-1 and -2 in cultured hepatocytes. Next, we asked whether iNOS can downregulate IRS-1 and -2 expression in cultured hepatocytes. Exposure to NO donor, GSNO (1 mmol/l), for 24 h decreased the protein expression of IRS-1 and -2 in Hepalc1c7 and HepG2 cells (Fig. 5 and online appendix Fig. 7), whereas the expression of IR and p85 PI3-kinase was not affected by NO donor. Treatment with another NO donor, 3-morpholino-sydnonimine-1 (0.5 mmol/l), for 24 h also decreased the expression of IRS-1 and -2 (online appendix Fig. 7). Ectopically expressed

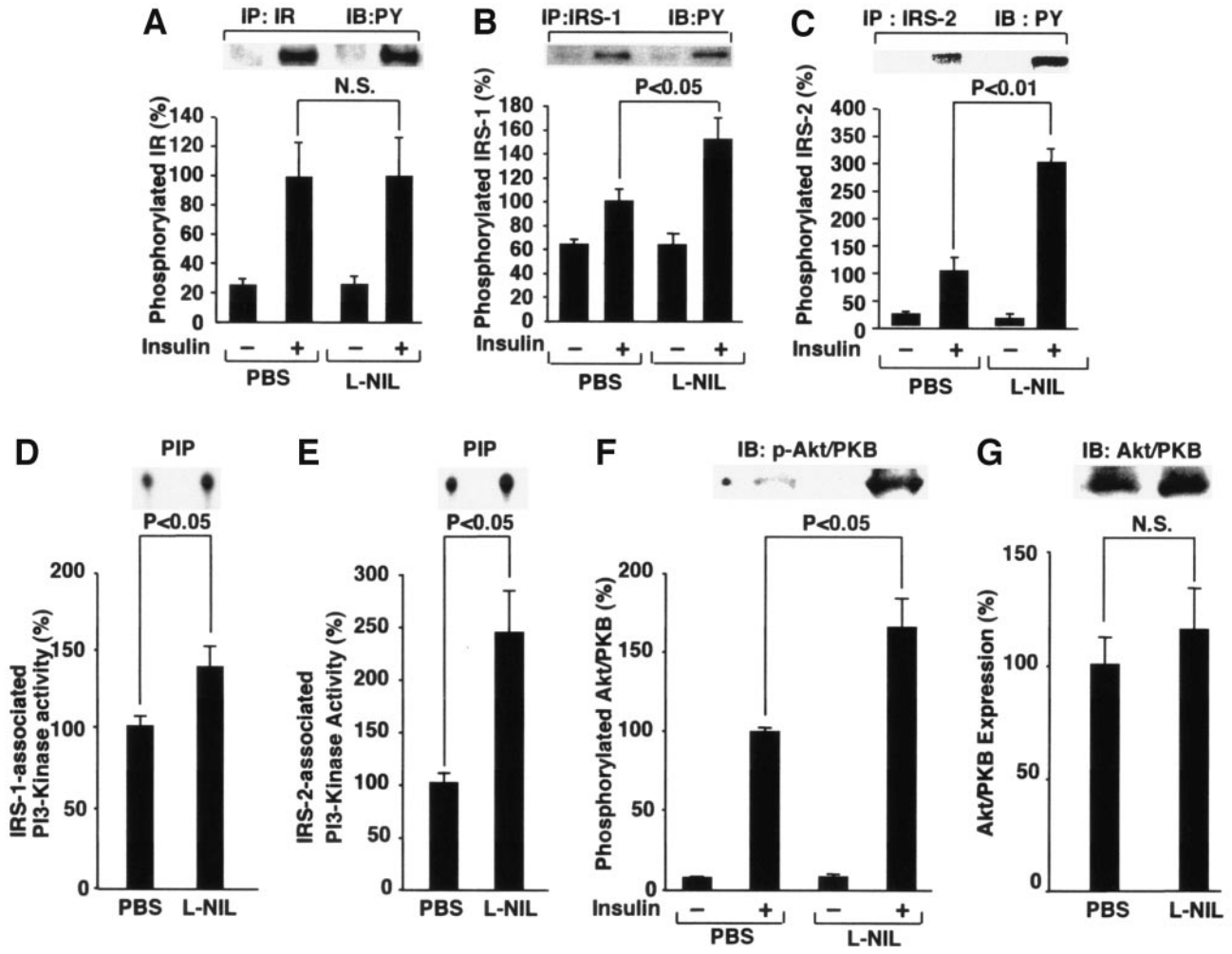

FIG. 4. Effects of iNOS inhibitor on insulin signaling in *ob/ob* mice. *A–C*: Phosphorylation status was evaluated by immunoprecipitation (IP) followed by immunoblotting (IB) with anti-phosphotyrosine (PY) antibody. L-NIL enhanced insulin-stimulated tyrosine phosphorylation of IRS-1 (*B*) and IRS-2 (*C*) compared with PBS. *D* and *E*: Insulin-stimulated IRS-1– and IRS-2–associated PI3-kinase activity was significantly greater in the liver of *ob/ob* mice treated with L-NIL compared with PBS, as judged by in vitro phosphorylation of phosphatidylinositol to phosphatidylinositol-3 phosphate (PIP). *F* and *G*: Insulin-stimulated phosphorylation of Akt/protein kinase B was increased in the liver of *ob/ob* mice treated with L-NIL compared with PBS.

iNOS reduced the expression of IRS-1 and -2 without altering p85 expression. The inhibitory effects of GSNO on IRS-1 and -2 expression were dose and time dependent (Fig. 6). In accordance with the greater increase in IRS-2 expression than in IRS-1 by L-NIL treatment in the liver of *ob/ob* mice (Fig. 3), the inhibitory effects of NO donor or iNOS transfection appeared to be more prominent on IRS-2 than -1 (Figs. 5 and 6). Treatment with 1 mmol/l GSNO for 24 h did not affect viability of the cells, as judged by the trypan blue exclusion test. Consistent with the effects of iNOS inhibitor on IRS-1 and -2 mRNA in the liver of *ob/ob* mice, 1 mmol/l GSNO decreased IRS-2 mRNA level but did not affect IRS-1 mRNA level in cultured Hepa1c1c7 cells (Fig. 7).

To further characterize the effects of NO donor on IRS-1 and -2, we examined the effects of ODQ, a soluble guanylate cyclase inhibitor, and Br-cGMP, a cell-permeable cGMP analog in Hepa1c1c7 cells. ODQ (1 μmol/l) failed to inhibit GSNO-induced reduction in IRS-1 and -2 expression. cGMP analog, Br-cGMP (100 μmol/l), did not affect the expression of IRS-1 and -2, either. These observations suggest that the inhibitory effects of GSNO are cGMP independent.

Since a recent study (33) revealed that SREBPs suppress the transcription of IRS-2 in the liver, we examined the effects of L-NIL and GSNO on SREBP-1c expression. SREBP-1c mRNA level was increased by GSNO (1 mmol/l) in cultured Hepa1c1c7 cells (Fig. 7*C*) and was reduced by L-NIL treatment in the liver of *ob/ob* mice under both fasted and fed conditions (Fig. 7*D* and *E*).

DISCUSSION

We found that iNOS expression was significantly elevated in the liver as well as skeletal muscle and adipose tissue in *ob/ob* mice compared with wild-type mice (Fig. 1) and that iNOS inhibitor reversed fasting hyperglycemia and ameliorated whole-body insulin resistance in *ob/ob* mice (Fig. 2). The improved glycemic control was accompanied by increased protein expression of IRS-1 and -2 and enhanced IRS-1– and IRS-2–mediated insulin signaling in the liver of *ob/ob* mice (Figs. 3 and 4). Moreover, we found that exposure to an NO donor and ectopically expressed iNOS

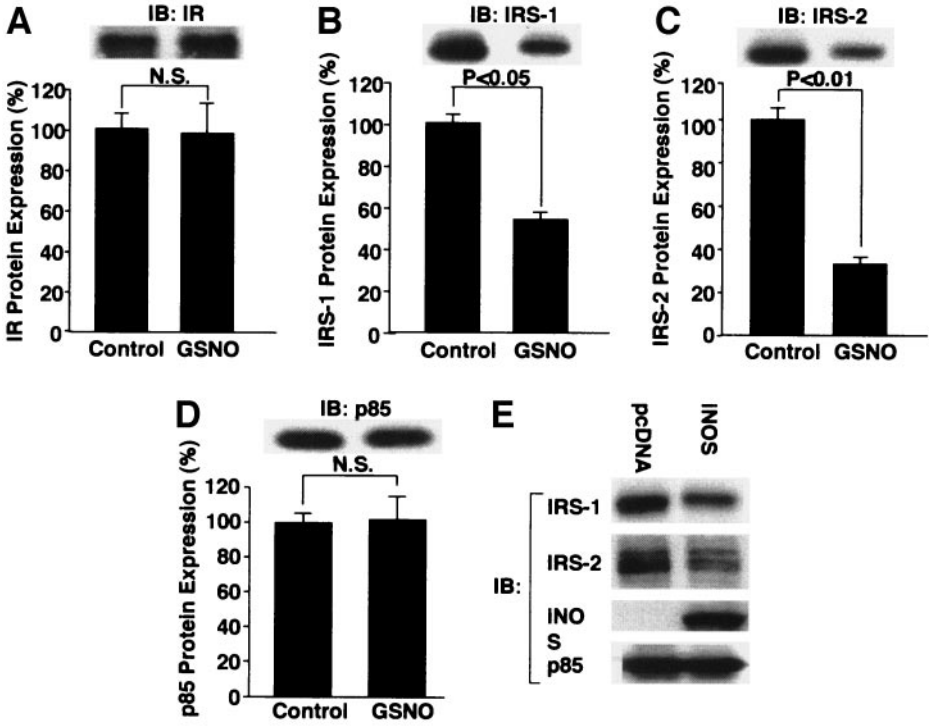

FIG. 5. Effects of NO donor and iNOS on IRS-1 and -2 expression in cultured hepatocytes. Immunoblot analysis (IB) revealed that GSNO (1 mmol/l) and ectopically expressed iNOS reduced IRS-1 and -2 expression in Hepa1c1c7 cells.

reduced the protein expression of IRS-1 and -2 in cultured hepatocytes (Figs. 5 and 6). These results indicate that increased expression of iNOS may play an important role in obesity-induced fasting hyperglycemia and suggest that iNOS may contribute to the defects in hepatic insulin signaling, in particular, depressed expression of IRS-1 and -2 in *ob/ob* mice.

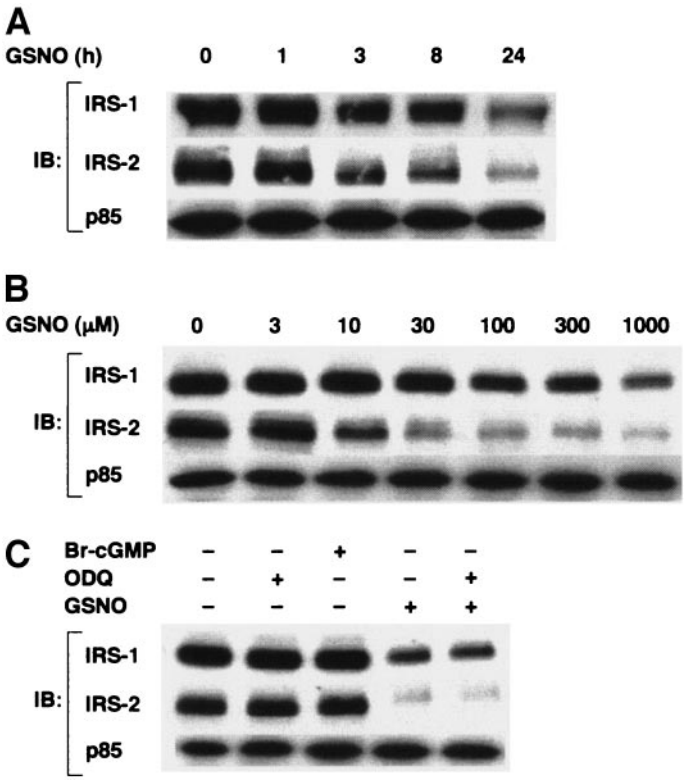

FIG. 6. NO donor-induced decreased expression of IRS-1 and -2 in cultured hepatocytes. *A* and *B*: Immunoblot analyses (IB) revealed that GSNO reduced the expression of IRS-1 and -2 in a time- and dose-dependent manner in Hepa1c1c7 cells. *C*: Hepa1c1c7 cells were treated with or without GSNO (1 mmol/l) in the presence or absence of cell-permeable cGMP analog, Br-cGMP (100 μmol/l), or soluble guanylate cyclase inhibitor ODQ (1 μmol/l).

Fasting blood glucose levels are determined primarily by glucose output from the liver, and hepatic glucose output is suppressed by insulin in insulin-sensitive animals (21,22,25). Therefore, fasting hyperglycemia is accounted for by the failure of insulin to inhibit the production and release of glucose by the liver. A predominant role for defects in hepatic insulin signaling in fasting hyperglycemia is also supported by previous studies in mice with tissue-specific gene targeting of IR. Tissue-specific gene disruption of IR in skeletal muscle (34) or adipose tissue (35) was not associated with hyperglycemia, whereas liver-specific knockout of IR did exhibit hyperglycemia (36,37). Taken together, our findings suggest that increased iNOS expression may contribute to fasting hyperglycemia, in part, by exacerbating deranged insulin signaling in the liver of *ob/ob* mice.

In the liver, IRS-2 rather than IRS-1 plays a prominent role in metabolic actions of insulin, including the inhibition of hepatic glucose output, whereas IRS-1 has a major role in skeletal muscle (38–41). Previous studies (42–44) showed a marked reduction in IRS-2 expression in the liver of *ob/ob* mice, whereas IRS-1 expression was unaltered or modestly decreased. These findings suggest that defective IRS-2–mediated insulin signaling is a major component of obesity-related hepatic insulin resistance.

iNOS inhibitor improved the protein expression of both IRS-1 and -2 in the liver of *ob/ob* mice. However, the beneficial effect of iNOS inhibitor on IRS-2 seemed more prominent compared with that on IRS-1 in the liver. Similarly, the reduction in protein expression induced by NO donor or iNOS transfection was more profound in IRS-2 compared with IRS-1 in Hepa1c1c7 and HepG2 cells.

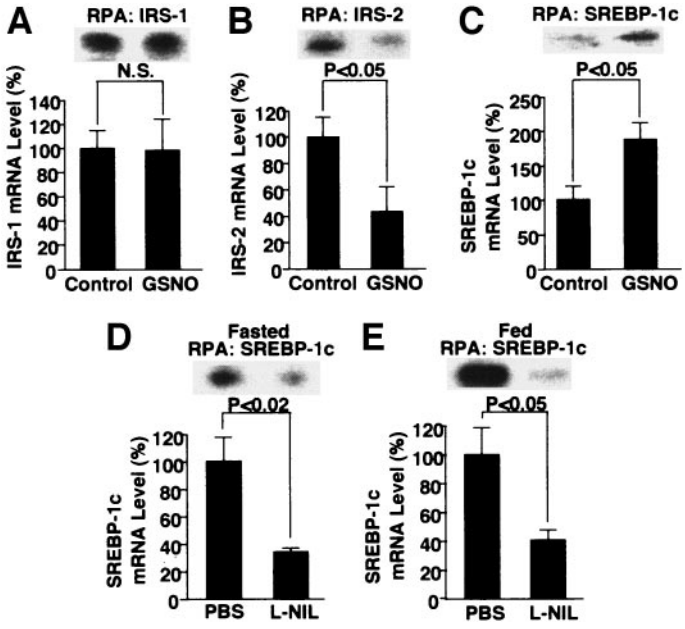

FIG. 7. Effects of NO donor and L-NIL on IRS-1 and -2 and SREBP-1c mRNA levels. RNase protection assay (RPA) revealed that GSNO (1 mmol/l) modulated IRS-1 (*A*), IRS-2 (*B*), and SREBP-1c (*C*) mRNA levels in Hepa1c1c7 cells and that L-NIL treatment decreased SREBP-1c mRNA level in the liver of *ob/ob* mice under both fasted (*D*) and fed (*E*) conditions.

These findings suggest that iNOS may downregulate preferentially the expression of IRS-2 and, to a lesser extent, that of IRS-1 in the liver.

To date, limited knowledge is available about the regulatory mechanisms of IRS-2 expression in normal and disease states such as insulin resistance and diabetes. Previous studies (25,45,46) showed that insulin downregulates IRS-2 expression. Therefore, one can hypothesize that the improved expression of IRS-2 might be due to amelioration of hyperinsulinemia by L-NIL via as yet determined mechanisms rather than direct effects of iNOS inhibitor in the liver. However, our data argue against this hypothesis. NO donor and iNOS transfection reduced IRS-2 expression in cultured hepatocytes, suggesting that increased iNOS expression without hyperinsulinemia can reduce IRS-2 expression in the liver. Moreover, L-NIL increased IRS-2 expression at both protein and mRNA levels under fed conditions as well as in fasted animals, although the effects of L-NIL on plasma insulin level appeared to differ between fasted and fed *ob/ob* mice. Fasting plasma insulin concentration was significantly lower in L-NIL–treated *ob/ob* mice than in PBS-treated animals, while plasma insulin concentration under fed condition tended to be higher in L-NIL–treated animals, albeit with no statistical significance.

Recently, SREBPs were shown to negatively regulate the transcription of IRS-2 in the liver (33). We found that SREBP-1c expression was reduced by L-NIL in the liver in vivo and was elevated by NO donor in cultured hepatocytes (Fig. 7). In aggregate, therefore, it is possible that increased expression of IRS-2 may be associated with reduced SREBP-1 expression by iNOS inhibitor in the liver of *ob/ob* mice.

Protein expression of IRS-1 was increased by iNOS inhibitor in the liver of *ob/ob* mice, while NO donor and ectopically expressed iNOS decreased IRS-1 in cultured hepatocytes. However, mRNA of IRS-1 was not altered by iNOS inhibitor or NO donor. This is consistent with our preliminary observation that iNOS and NO donor decreased IRS-1 expression via proteasome-dependent proteolysis in skeletal muscle cells (H. Sugita, M.K., unpublished observations).

The effects of L-NIL on the expression of IRS-1 and -2 in skeletal muscle and adipose tissue were distinct from those in the liver. In contrast to the preferential improvement in IRS-2 expression in the liver, L-NIL treatment was associated with increased expression of IRS-1, but not of IRS-2, in skeletal muscle. In adipose tissue, L-NIL failed to modulate the expression of either IRS-1 or -2. These results in skeletal muscle are in agreement with our aforementioned unpublished observation that NO donor and iNOS transfection reduced IRS-1 expression but did not affect IRS-2 expression in skeletal muscle cells. Based on the predominant role of hepatic insulin signaling in fasting hyperglycemia, one can reasonably speculate that ameliorated expression of IRS-2 in the liver may be the more important contributor to the reversal of fasting hyperglycemia by L-NIL rather than improved IRS-1 expression in skeletal muscle. It is conceivable, however, that the protective effects of iNOS inhibitor in the skeletal muscle as well as in the liver may contribute in concert to improve whole-body insulin sensitivity in *ob/ob* mice.

The study of Perreault and Marette (12) showed that high-fat diet–induced elevation in iNOS expression was restricted to skeletal muscle and adipose tissue, but not observed in the liver in mice, in contrast to our results in *ob/ob* mice (Fig. 1). This discrepancy might be attributable to the methodological difference to induce obesity, namely leptin mutation in *ob/ob* mice versus high-fat diet. It is also possible that the difference in mouse strains could influence the results. We used *ob/ob* mice that were backcrossed onto C57BL/6J background, while C57BL/6X129SvEv mice were used by Perreault and Marette. Moreover, in contrast to the glucose-lowering effects of iNOS inhibitor (Fig. 2), iNOS disruption did not significantly alter high-fat diet–induced fasting hyperglycemia (12). The lack of iNOS induction in the liver might account for the ineffectiveness of iNOS disruption to mitigate fasting hyperglycemia in high-fat diet–fed mice, although iNOS inhibitor effectively reversed fasting hyperglycemia in *ob/ob* mice in which iNOS expression was elevated in the liver.

Notably, there is a marked difference in the severity of fasting hyperglycemia between *ob/ob* mice and high-fat diet–fed mice (12). In *ob/ob* mice, overt fasting hyperglycemia was associated with increased iNOS expression in the liver. But in mice fed a high-fat diet, fasting hyperglycemia was mild, and iNOS expression did not increase in the liver (12). Thus, iNOS induction in the liver seems to correlate with the magnitude of fasting hyperglycemia. Collectively, one can speculate that increased expression of iNOS in the liver might be a culprit for overt fasting hyperglycemia via suppressing IRS-1 and -2 expression. Further studies will be required to clarify this point.

In summary, the present data show that iNOS inhibitor reversed fasting hyperglycemia in parallel with the concomitant improvement in hepatic IRS-1– and IRS-2–mediated insulin signaling in *ob/ob* mice. The present study

sheds a new light on a therapeutic potential of iNOS inhibitor to improve glycemic control in obesity-related diabetes.

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