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

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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, 1-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.

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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 IKKβ–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). Nit罗斯ative 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.
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 null 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.

**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. 1A). 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. 1B). 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-treated 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
reversed fasting hyperglycemia and significantly ameliorated fasting hyperinsulinemia in ob/ob mice (Fig. 2A 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/L², $P < 0.05$). 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. 2C). Area under the curve analysis in response to the insulin tolerance test also revealed the beneficial effects of L-NIL (Fig. 2D).

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/L², $P < 0.05$). 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. 3A). 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. 3B and online appendix Fig. 3). L-NIL treatment resulted in upregulated mRNA level of IRS-2 under both fasted and fed conditions.
conditions (Fig. 3C 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, p55\(_{H9251}\), and p50\(_{H9251}\) 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. 4D 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. 4F 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
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. 7C) and was reduced by L-NIL treatment in the liver of ob/ob mice under both fasted and fed conditions (Fig. 7D 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...
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.

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.
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 IRS 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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