SUPPLEMENTARY INFORMATION

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

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

Immunoprecipitation and immunoblotting.

Liver lysate was precleared by incubation for 2 h at 4°C with normal rabbit serum and protein A agarose beads (Santa Cruz). Then the precleared samples were incubated with anti-IRβ, IRS-1 or IRS-2 antibodies and protein A agarose beads for 3 h. The immunoabsorbates were washed three times with lysis buffer. After the addition of Laemmli buffer, the immunoprecipitates were boiled for 5 min at 95°C. Immunoprecipitates or equal amounts of protein extracts, as judged by Bradford protein assay, were subjected to SDS-PAGE, and then electrophoretically transferred to nitrocellulose membrane. The nitrocellulose blots were incubated with anti-IRβ, IRS-1, IRS-2, p85 PI3-kinase, iNOS, eNOS, nNOS, phosphorylated Akt (Ser473), Akt or phosphotyrosine antibody, followed by incubation with anti-mouse or rabbit IgG antibody conjugated with horse radish peroxidase (Amersham, Piscataway, NJ). Antigen-antibody complex was visualized using enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA). Densitometric analysis of autoradiograms

was performed using an imaging scanner (PowerLook 1100, UMAX, Dallas, TX) with the NIH image ver. 1.62 software.

Partial purification of iNOS.

1 mg of liver lysate was precleared by incubation at 4°C for 1 h with preswollen control hydrophilic polyacrylamide beads. Then the precleared lysates were incubated at 4°C for 12 h with 5 mg of preswollen 1400W-immobilized beads. After washed three times with lysis buffer, the precipitates with 1400W-immobilized beads were used for immunoblotting with anti-iNOS antibody.

Detection of tyrosine nitration.

Liver tissue was embedded in Tissue Tek OCT compound, frozen in liquid nitrogen, and cut into 5 μm sections using a cryostat microtome. Nitrotyrosine antibody or normal rabbit IgG was diluted at 5 μg/ml in PBS containing 3% BSA and 0.1% triton-X-100, washed 3 times with PBS, and visualized with anti-rabbit IgG antibody conjugated with Alexa Fluora 488 Green (Molecular Probes, Eugene, OR) at a concentration of 1:200 for 60 min in the dark at room temperature. Sections were washed 6 times with PBS, and then stained with 300 nM DAPI (4', 6'-diamidino-2-phenylindole, Molecular Probes) for nuclear staining. Liver sections of both L-NIL- and PBS-treated mice were placed on the same slide and analyzed in triplicate. Immunostaining with normal rabbit IgG served as a negative control. No significant staining was observed in negative control sections.

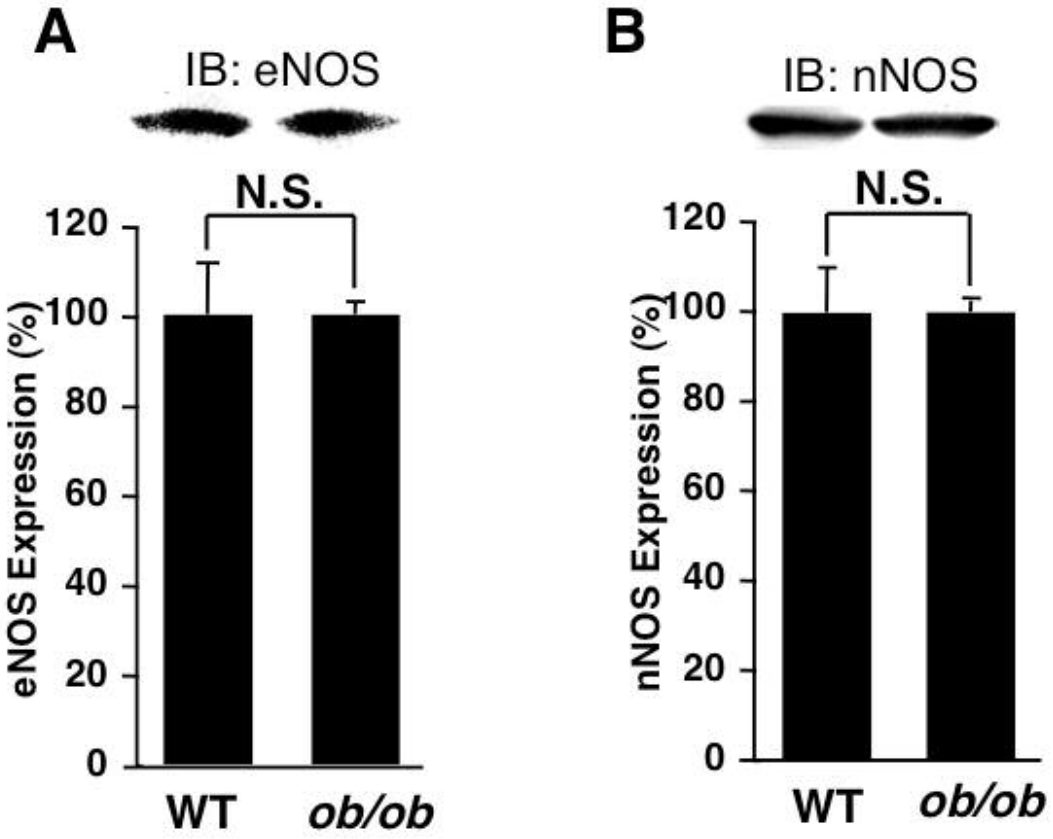
RNase protection assay.

Total RNA was isolated from the liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA fragments for IRS-1, IRS-2 and sterol regulatory element binding protein (SREBP)-1c were generated from mice liver cDNA library (TAKARA BIO, Shiga, Japan) by PCR using the primers of 5'-ccagcctggctatttagctg-3' and 5'-cccaactcaactccaccact-3' for IRS-1, 5'-acaacctatcgtggcacctc-3' and 5'-gacggtggtggtagaggaaa-3' for IRS-2, and 5'-atcggcgcggaagctggggtagcgtc-3' and 5'-actgtcttggttgttgatgagctggagcat-3' for SREBP-1c (Shimomura et al., 1997), which were then subcloned into the pCR2.1 vector (Invitrogen). cDNA fragment for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Ambion.

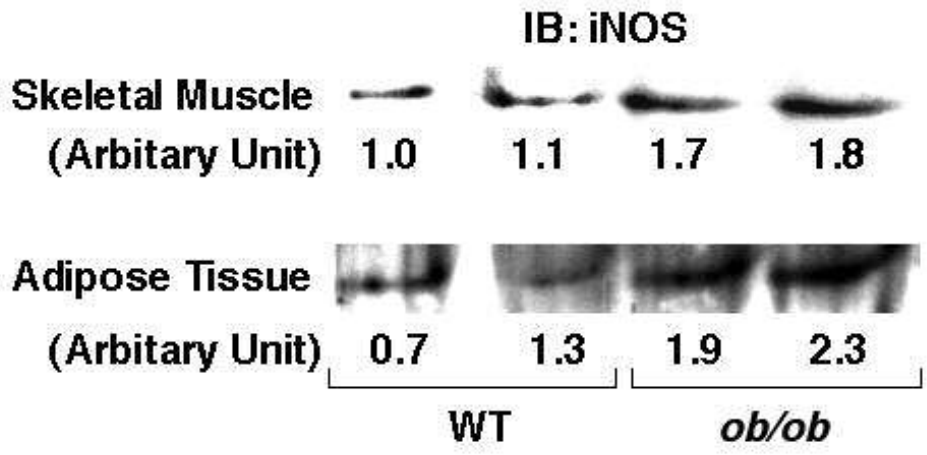
Reference for Supplementary Information

Shimomura I, Shimano H, Horton JD, Goldstein JL, Brown MS: Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J Clin Invest* 99:838-845, 1997

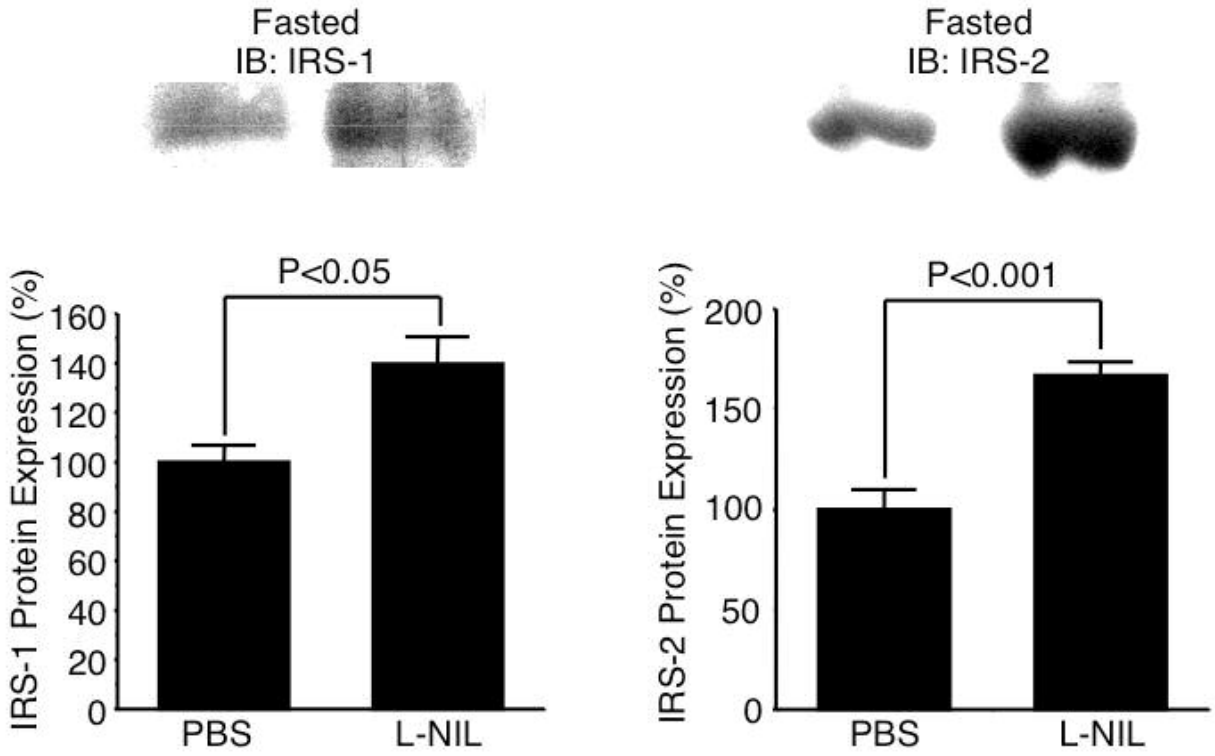
Supplementary Fig. 1. Expression of eNOS and nNOS in the liver of diabetic mice. Immunoblot analysis (IB) revealed that the protein expression of eNOS and nNOS did not differ between diabetic (*ob/ob*) and wild-type (WT) mice.



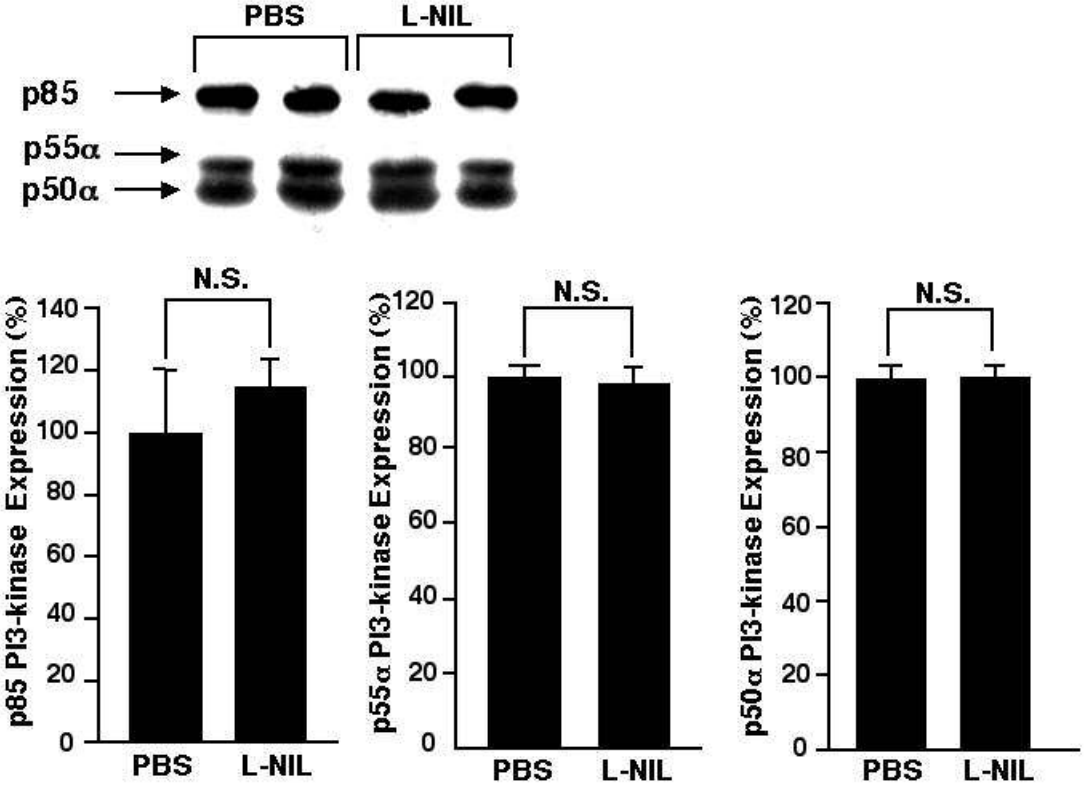
Supplementary Fig. 2. iNOS expression in skeletal muscle and adipose tissue of diabetic mice. Immunoblot analysis (IB) revealed increased iNOS expression in skeletal muscle and adipose tissue of diabetic (*ob/ob*) mice compared with wild-type (WT) mice. iNOS expression is expressed as arbitary unit normalized to the average of that in wild-type mice.



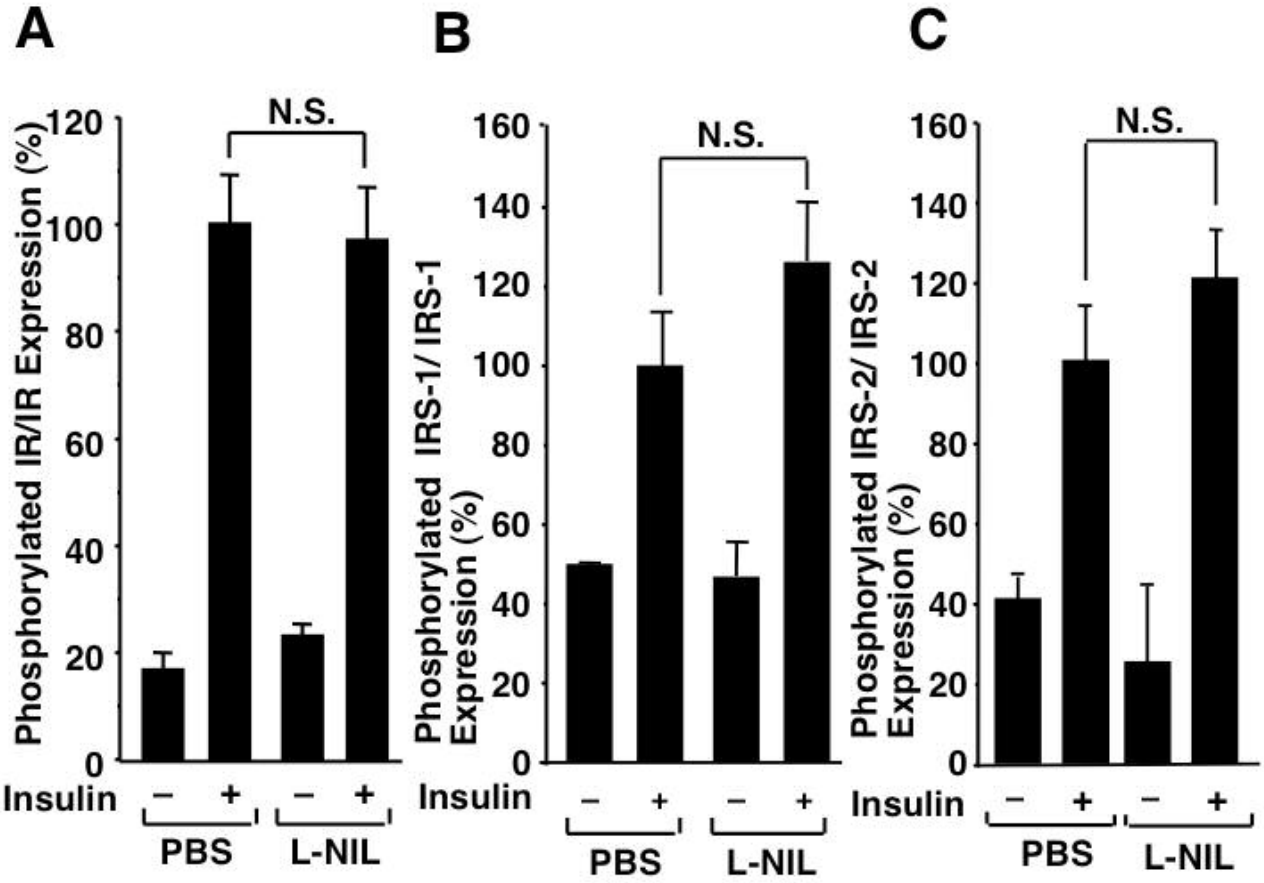
Supplementary Fig. 3. Effects of iNOS inhibitor on IRS-1 and IRS-2 expression in the liver of pair-fed diabetic mice under fasted condition. Immunoblot analysis (IB) revealed that the protein expression of IRS-1 and IRS-2 were increased by L-NIL treatment in the liver of pair-fed diabetic (*ob/ob*) mice under fasted condition, as compared with PBS.



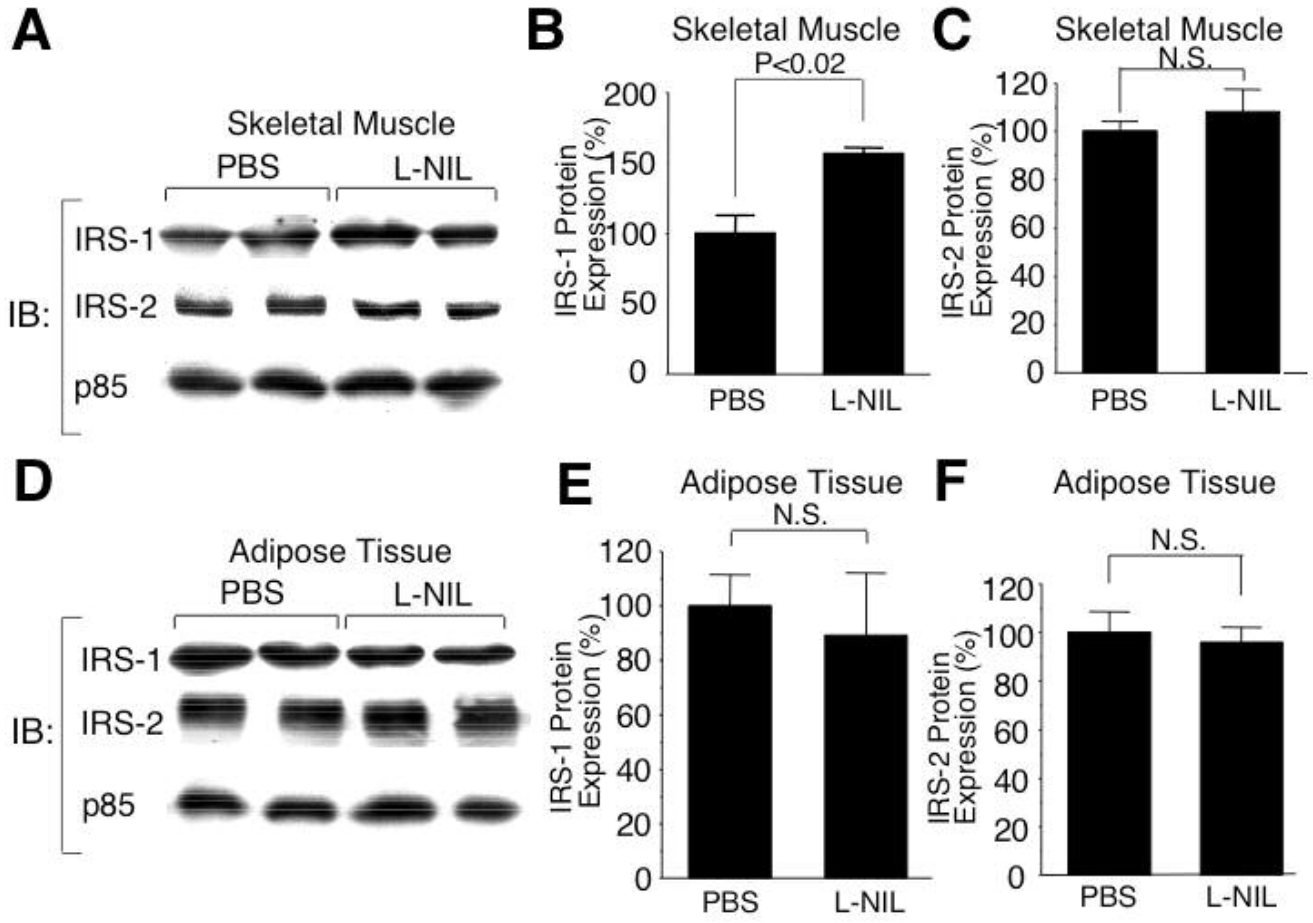
Supplementary Fig. 4. PI3-kinase expression in the liver of L-NIL-treated diabetic mice. The protein expression of p85, p55α and p50α PI3-kinase in the liver did not differ between L-NIL- and PBS-treated diabetic (*ob/ob*) mice.



Supplementary Fig. 5. Ratio of insulin-stimulated tyrosine phosphorylation to protein expression for IR, IRS-1 and IRS-2 in L-NIL-treated diabetic mice. Tyrosine phosphorylation and protein expression were assessed by immunoprecipitation and immunoblotting. L-NIL treatment did not alter the ratio of insulin-stimulated phosphorylation of IR, IRS-1 and IRS-2 to the expression level of the respective protein in the liver of diabetic (*ob/ob*) mice.



Supplementary Fig. 6. IRS-1 and IRS-2 expression in skeletal muscle and adipose tissue of L-NIL-treated diabetic mice. IRS-1 and IRS-2 protein expression was examined by immunoblotting (IB). In skeletal muscle, IRS-1 expression was increased by L-NIL treatment (*A*, *B*) in fasted diabetic (*ob/ob*) mice, whereas IRS-2 expression was unaltered (*A*, *C*). In adipose tissue, there was no difference in IRS-1 and IRS-2 expression under fasted condition between L-NIL and PBS-treated diabetic (*ob/ob*) mice (*D*, *E*, *F*). p85 PI3-kinase expression did not differ between L-NIL- and PBS-treated animals in both skeletal muscle and adipose tissue (*A*, *D*).



Supplementary Fig. 7. Effects of NO donor on IRS-1 and IRS-2 in cultured hepatocytes. Immunoblot analysis (IB) revealed that treatment with NO donor, SIN-1 (0.5 mM) for 24 h reduced the protein expression of IRS-1 and IRS-2 in Hepa1c1c7 cells, while p85 PI3-kinase expression was not altered (*A*). Treatment with GSNO (1 mM) for 24 h also reduced the expression of IRS-1 and IRS-2, but not p85 PI3-kinase, in HepG2 cells (*B*).