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Inducible Nitric-oxide Synthase and Nitric Oxide Donor Decrease Insulin Receptor Substrate-2 Protein Expression by Promoting Proteasome-dependent Degradation in Pancreatic β-Cells

INVOLVEMENT OF GLYCOGEN SYNTHASE KINASE-3β[*][S]

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Insulin receptor substrate-2 (IRS-2) plays a critical role in the survival and function of pancreatic β-cells. Gene disruption of IRS-2 results in failure of the β-cell compensatory mechanism and diabetes. Nonetheless, the regulation of IRS-2 protein expression in β-cells remains largely unknown. Inducible nitric-oxide synthase (iNOS), a major mediator of inflammation, has been implicated in β-cell damage in type 1 and type 2 diabetes. The effects of iNOS on IRS-2 expression have not yet been investigated in β-cells. Here, we show that iNOS and NO donor decreased IRS-2 protein expression in INS-1/832 insulinoma cells and mouse islets, whereas IRS-2 mRNA levels were not altered. Interleukin-1β (IL-1β), alone or in combination with interferon-γ (IFN-γ), reduced IRS-2 protein expression in an iNOS-dependent manner without altering IRS-2 mRNA levels. Proteasome inhibitors, MG132 and lactacystin, blocked the NO donor-induced reduction in IRS-2 protein expression. Treatment with NO donor led to activation of glycogen synthase kinase-3β (GSK-3β) and c-Jun N-terminal kinase (JNK/SAPK) in β-cells. Inhibition of GSK-3β by pharmacological inhibitors or siRNA-mediated knockdown significantly prevented NO donor-induced reduction in IRS-2 expression in β-cells. In contrast, a JNK inhibitor, SP600125, did not effectively block reduced IRS-2 expression in NO donor-treated β-cells. These data indicate that iNOS-derived NO reduces IRS-2 expression by promoting protein degradation, at least in part, through a GSK-3β-dependent mechanism. Our findings suggest that iNOS-mediated decreased IRS-2 expresssion may contribute to the progression and/or exacerbation of β-cell failure in diabetes.

All forms of diabetes develop when pancreatic β-cells can no longer secret a sufficient amount of insulin to maintain normal blood glucose levels. Hence, β-cell failure is the key event in the development of diabetes. Damage, death, and growth arrest of β-cells are, therefore, major contributors to the progression of diabetes.

Insulin receptor substrate-2 (IRS-2) plays a crucial role in functional β-cell mass (1–7). IRS-2 is essential for proliferation and insulin-stimulated activation of phosphatidylinositol 3-kinase and Akt in β-cells (8). Global gene disruption of IRS-2 results in overt diabetes along with β-cell compensation failure (1). IRS-2 is required for adaptive expansion of β-cell mass in response to high fat diet feeding (5). In contrast, IRS-1 knockout mice are insulin-resistant but exhibit normoglycemia because of compensatory hyperinsulinemia associated with adaptive expansion of β-cell mass (9). Moreover, β-cell- or pancreas-specific gene disruption of IRS-2 results in reduced β-cell mass, glucose intolerance, and attenuated glucose-stimulated insulin secretion in mice (2, 10). Conversely, β-cell-specific ectopic expression of IRS-2 prevents high fat diet-induced diabetes (11) and alleviates and/or delays β-cell destruction and diabetes development in nonobese diabetic mice (12) and streptozotocin-treated mice (11). Limited knowledge is, however, available about IRS-2 expression in islets in diabetes.

Of interest, high fat diet reduced IRS-2 protein expression in islets of 90% pancreatectomized rats without alteration in IRS-2 mRNA expression (13). These data raise the possibility that high fat diet feeding might reduce IRS-2 protein expression by enhancing protein degradation.

Interleukin-1β (IL-1β) has been proposed as a common important player in the pathogenesis of type 1 and type 2 diabetes (14). The blockade of IL-1β improves glycemic control and β-cell function in patients with type 2 diabetes (15). A previous study has shown that circulating levels of IL-1 receptor antagonist, an endogenous antagonist of IL-1β, is associated with β-cell capacity in patients with type 1 diabetes (16). In nonobese diabetic mice, gene disruption of IL-1β receptor delays the development of diabetes (17).

Nitric oxide (NO) produced by inducible nitric-oxide synthase (iNOS,[2] also known as NOS2) has been implicated in β-cell damage and death in both type 1 and type 2 diabetes

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: iNOS, inducible nitric-oxide synthase; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal S6 kinase; GSNO, *S*-nitroso-L-glutathione; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; L-NIL, N^6-(1-iminoethyl)-L-lysine; PTIO, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide.

(18–21) and during islet transplantation (22–24). Previous studies have shown that iNOS expression plays an important role in IL-1β-induced dysfunction and death of cultured β-cells (20, 25, 26–28), although other studies have concluded that cytokine induces β-cell damage through both NO-dependent and -independent pathways (29, 30). The expression of iNOS is increased in β-cells and intraislet macrophages of rodent models of type 1 and type 2 diabetes (18, 31–35). Aminoguanidine, an iNOS inhibitor, prevents the development of obesity-induced diabetes in Zucker *fa/fa* rats (36). iNOS depletion and iNOS inhibitor have been shown to block or ameliorate diabetes development in multiple low dose streptozotocin-treated mice and nonobese diabetic mice, murine models of type 1 diabetes (18, 27, 28, 37), although controversial results have been also reported (38, 39). Moreover, β-cell-specific iNOS expression *per se* leads to insulin-dependent diabetes and loss of β-cells without insulitis in mice (41). However, it is not fully understood how NO and iNOS induce and/or exacerbate β-cell damage and loss of functional β-cell mass in diabetes. Here, we show that iNOS and NO donor reduce the protein expression of IRS-2 by promoting proteasome-dependent degradation of IRS-2 in cultured insulinoma cells and mouse islets.

EXPERIMENTAL PROCEDURES

Materials—*S*-Nitroso-L-glutathione (GSNO), *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), L-NIL, and carboxy-PTIO (Cayman Chemical, Ann Arbor, MI); MG132, reduced glutathione (GSH), and oxidized glutathione (GSSG) (Sigma); cycloheximide (Calbiochem); lactacystin (Boston Biochem, Cambridge, MA); SB216763 and SB415268 (Tocris Bioscience, Ellisville, MO); recombinant mouse IFN-γ (R&D Systems, Minneapolis, MN); mouse IL-1β (Roche Applied Science); human tumor necrosis factor-α (TNF-α; Cell Signaling, Beverly, MA); anti-IRS-2, anti-iNOS, and anti-phosphotyrosine (Millipore, Billerica, MA); anti-ubiquitin (Affiniti Research Products, Golden, CO); anti-phospho-glycogen synthase (Novus Biologicals, Littleton, CO); anti-GSK-3β (BD Transduction Laboratories); anti-IRS-2 (EMD Chemicals, Gibbstown, NJ); anti-GAPDH (Trevigen, Gaithersburg, MD); anti-Akt, anti-phospho-Akt (Ser-473); and anti-phospho-GSK-3β (Ser-9), anti-glycogen synthase, anti-c-Jun, anti-phospho-c-Jun (Ser-63), anti-p70 S6 kinase, and anti-phospho-p70 S6 kinase (Thr-389) antibodies (Cell Signaling) were purchased commercially. siRNA oligonucleotides for rat iNOS and GSK-3β were purchased from Invitrogen (iNOS: RSS302394, RSS302395, and RSS351448; GSK-3β: r(CGAUUACACGUCUAGUAUA)dTdT (sense) and r(UAUACUAGACGUGUAAUCG)dTdT (antisense)).

Cell Culture—Rat INS-1/832 insulinoma cells, a kind gift of Dr. C. B. Newgard (Duke University) (42), were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. The mouse βTC-6 insulinoma cell line was obtained from ATCC (Manassas, VA). βTC-6 cells were cultured in Dulbecco's modified Eagle's medium with 15% heat-inactivated FBS, 4 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were treated with GSNO (30–300 μM), SNAP (300 μM), or cytokine (IL-1β (2.5–10 units/ml), IFN-γ (100 ng/ml)) in the presence or absence of insulin (100 nM), L-NIL (200 μM), carboxy-PTIO (300 μM), cycloheximide (1 μg/ml), MG132 (1 μM), lactacystin (1 μM), SB216763 (10 μM), SB415268 (10 μM), SP600125 (10 μM), or rapamycin (1 μM), as indicated in the figure legends.

Cell Transfection—INS-1/832 cells were transfected with siRNA for iNOS, GSK-3β, or control siRNA (43) using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturers' instructions.

Mouse Islet Isolation—Islets were isolated from male wild-type C57BL/6 mice, iNOS knock-out (−/−) mice on C57BL/6 background, wild-type BKS mice, and obese, diabetic (*db*/*db*) mice on BKS background (Jackson Laboratory, Bar Harbor, ME) at 9–12 weeks of age by collagenase digestion followed by centrifugation over a Histopaque (Sigma) gradient, as described previously (44). Upper laparotomy was performed by midline incision, and after clamping the common bile duct at its entrance to the duodenum, 3 ml of M199 medium containing collagenase P (1 mg/ml; Roche Applied Science) was injected into the duct. The swollen pancreas was surgically removed.

Human Islets—Human islets from two non-diabetic subjects (male aged 39 years; female aged 45 years) and two type 2 diabetes patients (male aged 63 years on anti-diabetic medication for 5 years; male age 42 years on anti-diabetic medication for 4 years) were provided by the National Disease Research Interchange (Philadelphia, PA).

Treatment of Islets—Islets isolated from wild-type C57BL/6 mice and from non-diabetic subjects were cultured in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The islets were treated with and without GSNO (400 or 500 μM) for 5 h in the presence or absence of SB216763 (10 μM) or lactacystin (1 μM) or with and without IL-1β (10 units/ml) and IFN-γ (100 ng/ml) or IL-1β (10 units/ml), IFN-γ (100 ng/ml), TNF-α (10 ng/ml), and lipopolysaccharide (LPS, 3 μg/ml; Sigma) as indicated in the figure legends for 20 h in the presence or absence of L-NIL (200 μM).

Western Blot Analysis—Cells or islets were lysed on ice for 30 min in lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Nonidet P-40, 0.4% sodium deoxycholate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Sigma)), as described previously (45). The protein samples were denatured by boiling for 5 min and separated in a 7.5 or 10% polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked in 2% ECL advance blocking agent (GE Healthcare) for 1 h at room temperature and incubated with primary antibody for 2 h at room temperature or overnight at 4 °C. This was followed by incubation with secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The blots were visualized by an enhanced chemiluminescence method using an ECL advance Western blotting detection kit (GE Healthcare). Bands of interest were scanned using an HP Scanjet 4850 and were quantified by NIH ImageJ 1.410 software (National Institutes of Health, Bethesda, MD).

Immunoprecipitation—Lysates were preincubated with 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology,

Inc., Santa Cruz, CA) for 2 h at 4 °C to minimize nonspecific absorption. The supernatants were then incubated with 2 μg of anti-IRS-2 antibody (Santa Cruz Biotechnology, Inc.) and 20 μl of protein A/G-agarose beads at 4 °C overnight. After centrifugation at 1,000 × *g* for 5 min, the pellets were washed five times with Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) and dissolved in 30 μl of SDS-sample buffer.

Evaluation of mRNA Expression Levels—Total RNA was purified using TRIzol reagent (Invitrogen). The first-strand cDNA was synthesized from 1 μg of total RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR reactions were performed using 10 ng of cDNA and TaqMan probes (Applied Biosystems) for IRS-2 (Rn01482270_s1 or Hs0065185_m1) and 18 S ribosomal RNA (Hs99999901_s1), conducted with Mastercycler® ep realplex (Eppendorf, Westbury, NY). Results were normalized to 18 S ribosomal RNA as an endogenous reference gene, and the relative amount of each mRNA was calculated by the comparative *Ct* (threshold cycle) method. iNOS mRNA content in the islets was evaluated by RT-PCR, as described previously (46, 47), using specific primers for mouse and human iNOS (mouse, 5′-ACAGCCTCAGAGTCCTTCAT-3′ and 5′TTGTCACCACCAGCAGTAGT-3′; human, 5′-CAGTACGTTTGGCAATGGAGACTGC-3′ and 5′-GGTCACATTGGAGGTGTAGA GCTTG-3′). RT-PCR products were quantified using a densitometer and image analyzer (Bio-Rad) (46). 36B4 gene expression was used as an internal control (48).

Evaluation of Cell Viability—Cell viability of INS-1/832 cells and islet cells was assessed using Sytox Green (Molecular Probes, Inc., Eugene, OR) and TOX-8 (Sigma) according to the manufacturers' instructions. For Sytox staining, cells were incubated with Sytox Green (1 μM) for 20 min in the dark and observed under a Nikon Eclipse TE2000-5 inverted fluorescence microscope.

Measurement of Nitrite—Nitrite accumulation in culture medium was determined by Griess reagent (Sigma). 50 μl of culture medium was mixed and incubated with 50 μl of Griess reagent for 15 min at room temperature, and absorbance at 540 nm was measured in a microplate reader. Serial dilutions of sodium nitrite were used as standards.

Statistical Analysis—The data were compared using one-way analysis of variance followed by Tukey's least significant difference test or unpaired Student's *t* test. A value of $p < 0.05$ was considered statistically significant. All data are expressed as mean ± S.E.

RESULTS

IL-1β Reduces IRS-2 Protein Expression in an iNOS-dependent Manner in Pancreatic β-Cells—Treatment with IL-1β or with IL-1β plus interferon-γ (IFN-γ) resulted in a time- and dose-dependent induction of iNOS expression and nitrite accumulation in the culture medium in INS-1/832 cells. The induction of iNOS paralleled the reduction in IRS-2 protein expression (Figs. 1 and 2). Treatment with IL-1β for 24 h significantly decreased IRS-2 protein expression starting at a dose of 2.5 units/ml, and the maximum level of the inhibitory effect of IL-1β on IRS-2 was observed at 10 and 25 units/ml (Fig. 1*C*).

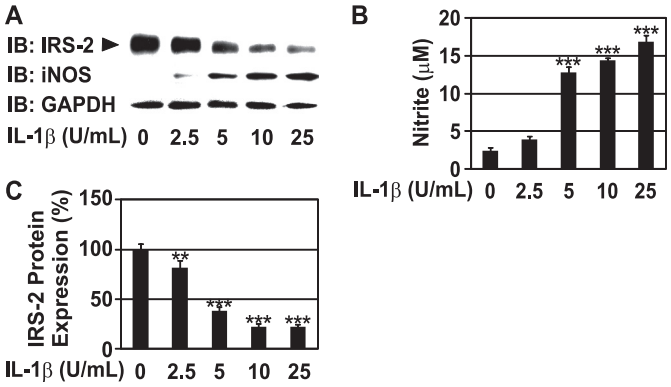

FIGURE 1. **IL-1β decreased IRS-2 protein expression along with the induction of iNOS expression in pancreatic β-cells.** INS-1/832 cells were treated with different doses of IL-1β for 24 h (*A–C*). IL-1β reduced the protein expression of IRS-2 in association with increases in iNOS expression and nitrite concentrations in the culture medium. *IB*, immunoblotting. **, $p < 0.01$; ***, $p < 0.001$ *versus* the cells without IL-1β. *Error bars*, S.E.

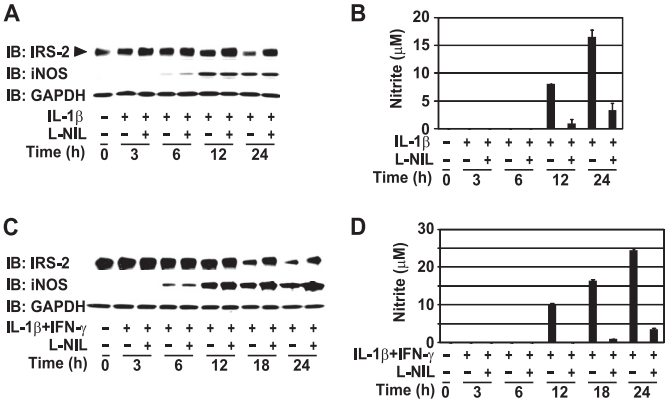

FIGURE 2. **Time-dependent reduction in IRS-2 expression by cytokine and its reversal by iNOS inhibitor in β-cells.** IL-1β (5 units/ml) alone and the combination of IL-1β (10 units/ml) and IFN-γ (100 ng/ml) time-dependently decreased IRS-2 protein expression in parallel with iNOS induction and nitrite accumulation in the culture medium in INS-1/832 cells (*A–D*). iNOS inhibitor, L-NIL (200 μM), blocked the cytokine-induced decreases in IRS-2 expression and nitrite accumulation. Neither IL-1β, IL-1β plus IFN-γ, nor L-NIL altered GAPDH expression. *IB*, immunoblotting. *Error bars*, S.E.

The suppression of IRS-2 protein expression by IL-1β or IL-1β plus IFN-γ was prevented by a specific inhibitor of iNOS, L-NIL, in INS-1/832 cells, along with the reversal of accumulation of nitrite in the culture medium (Figs. 2 and 3). The protein expression of GAPDH was not affected by IL-1β, IL-1β plus IFN-γ, or L-NIL. Likewise, siRNA-mediated knockdown of iNOS inhibited IL-1β-induced decreased protein expression of IRS-2 in INS-1/832 cells (Fig. 3*F*). In contrast to the decreased protein expression of IRS-2, IL-1β did not reduce mRNA expression of IRS-2 in INS-1/832 cells (IRS-2 mRNA level: control, 100 ± 11% (mean ± S.E.); IL-1β, 104 ± 5%. Treatment with neither IL-1β, IL-1β plus IFN-γ, nor L-NIL for up to 24 h increased cell death in INS-1/832 cells and mouse islets, as judged by Sytox staining (data not shown).

In accord with decreased IRS-2 expression by IL-1β, basal and insulin-stimulated phosphorylation of IRS-2 and Akt was attenuated by IL-1β, which was partially prevented by iNOS inhibitor, L-NIL, in INS-1/832 cells (Fig. 4). In the absence of IL-1β, L-NIL did not affect the phosphorylation status of IRS-2

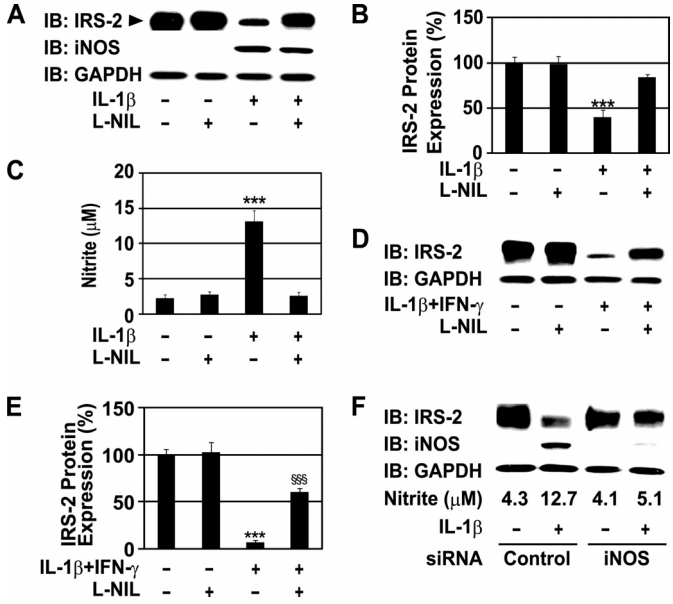

FIGURE 3. **Inhibition of iNOS blocked cytokine-induced reduction in IRS-2 expression in β-cells.** INS-1/832 cells were treated for 24 h with or without IL-1β (5 units/ml) (*A–C* and *F*) or IL-1β (10 units/ml) plus IFN-γ (100 ng/ml) (*D* and *E*) in the presence and absence of iNOS inhibitor, L-NIL (*A–E*), or siRNA to iNOS (*F*). IL-1β alone and IL-1β plus IFN-γ induced iNOS expression and reduced IRS-2 protein expression. L-NIL reverted decreased IRS-2 expression and nitrite accumulation in the culture medium in cytokine-treated cells. Likewise, siRNA-mediated knockdown prevented IL-1β-induced suppression of IRS-2 expression in β-cells in parallel with reversal of elevated nitrite concentration in the medium, as compared with control siRNA. Unless treated with cytokine, L-NIL and iNOS knockdown did not affect IRS-2 expression and nitrite concentrations. GAPDH expression was not altered by cytokine, L-NIL, or iNOS knockdown. *IB*, immunoblotting. ***, $p < 0.001$ *versus* the cells without cytokine and those with cytokine + L-NIL; §§§, $p < 0.001$ *versus* the cells without cytokine. *Error bars*, S.E.

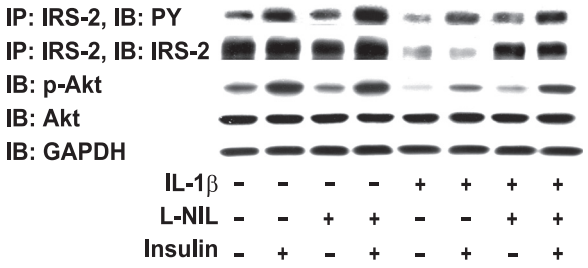

FIGURE 4. **IL-1β-induced attenuation in phosphorylation of IRS-2 and Akt and its restoration by iNOS inhibitor in β-cells.** INS-1/832 cells were treated for 24 h with and without IL-1β (5 units/ml) in the presence or absence of iNOS inhibitor, L-NIL (200 μM). Following 2-h serum deprivation, the cells were incubated with or without insulin (100 nM) for 1 and 10 min for detection of phosphorylation of IRS-2 and Akt, respectively. Immunoprecipitation (*IP*) with anti-IRS-2 antibody followed by immnoblotting (*IB*) with anti-phosphotyrosine (*PY*) or IRS-2 antibody revealed that basal (exogenous insulin-naive) and insulin-stimulated phosphorylation of IRS-2 were decreased by IL-1β treatment along with suppressed expression of IRS-2 in β-cells. L-NIL restored protein expression and tyrosine phosphorylation of IRS-2 in IL-1β-treated cells. Similarly, IL-1β treatment attenuated basal and insulin-stimulated phosphorylation of Akt (*p-Akt*), which was up-regulated by L-NIL. In contrast to IRS-2, neither IL-1β nor L-NIL altered protein expression of Akt and GAPDH. When untreated with IL-1β, L-NIL did not affect phosphorylation of IRS-2 and Akt or protein expression of IRS-2 in β-cells.

and Akt. The protein expression of Akt was not altered by IL-1β or L-NIL.

iNOS-dependent Decrease in IRS-2 Protein Expression in Mouse Islet Cells—Similar to INS-1/832 cells, treatment with IL-1β alone or IL-1β plus IFN-γ decreased IRS-2 protein

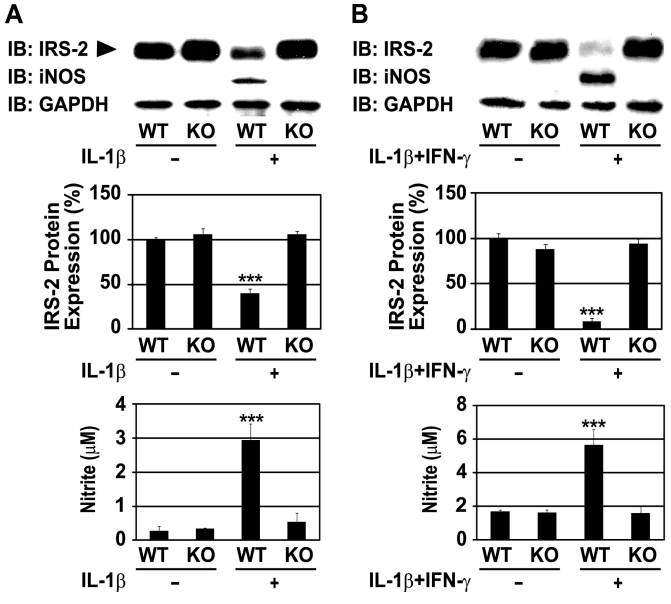

FIGURE 5. **Cytokine treatment decreased IRS-2 protein expression in mouse wild-type, but not iNOS-deficient, islets.** Islets isolated from WT and iNOS KO mice were cultured with and without IL-1β (10 units/ml) alone (*A*) or IL-1β (10 units/ml) plus IFN-γ (100 ng/ml) (*B*) for 20 h. IRS-2 protein expression was decreased by IL-1β and IL-1β plus IFN-γ in wild-type islets along with iNOS induction and increased nitrite concentrations in the culture medium. In contrast, neither IL-1β nor IL-1β plus IFN-γ altered IRS-2 protein expression or nitrite concentration in iNOS-deficient islets. *IB*, immunoblotting. ***, $p < 0.0001$ *versus* the islets without cytokine and KO islets with cytokine. $n = 3$/group. *Error bars*, S.E.

expression in cultured islets isolated from wild-type mice along with induction of iNOS expression (Fig. 5*A*), whereas mRNA content of IRS-2 was not decreased (mouse IRS-2 mRNA level: control, 100 ± 9%; IL-1β, 95 ± 14%) IL-1β plus IFN-γ induced more profound decrease in IRS-2 protein expression in association with greater increases in iNOS expression and nitrite accumulation in wild-type islets (Fig. 5*B*). In islets from iNOS-deficient mice, however, neither IL-1β alone nor IL-1β plus IFN-γ affected IRS-2 expression or increased nitrite concentration in the culture medium (Fig. 5). iNOS inhibitor, L-NIL (200 μM), blocked IL-1β plus IFN-γ-induced decrease in IRS-2 protein expression and nitrite accumulation in wild-type mouse islets (data not shown).

NO Donor Reduces IRS-2 Protein Expression in β-Cells—NO donor, GSNO, decreased the protein expression of IRS-2 in a time-dependent manner in INS-1/832 cells (Fig. 6*A*). GSNO (300 μM) significantly decreased IRS-2 expression starting at 2 h after the addition of the NO donor. Treatment with GSNO for 5 h elicited the inhibitory effects on IRS-2 expression in a dose-dependent manner. A significant reduction in IRS-2 protein expression was observed starting at a dose of 100 μM (Fig. 6*B*). To assess cell viability, we evaluated cell membrane integrity and metabolic cell viability by Sytox staining and TOX-8, respectively. Treatment with GSNO (300 μM) for 5 h did not significantly affect cell viability (data not shown). Another NO donor, SNAP (300 μM), also decreased IRS-2 protein expression in INS-1/832 cells (Fig. 6*C*). GSNO treatment for 5 h dose-dependently decreased IRS-2 protein expression in βTC-6 cells as well (Fig. 6*D*). NO donors did not alter GAPDH protein expression in INS-1/832 and βTC-6 cells.

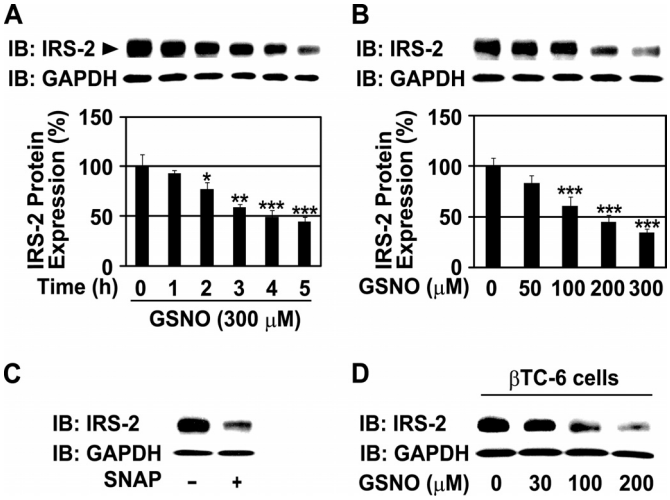

FIGURE 6. **NO donor decreased IRS-2 protein expression in β-cells.** GSNO (300 μM) decreased IRS-2 protein expression in a time-dependent manner in INS-1/832 cells (*A*). 5-h treatment with GSNO exerted dose-dependent inhibitory effects on IRS-2 expression in INS-1/832 cells (*B*) and βTC-6 cells (*D*). Treatment with SNAP (300 μM) for 5 h also resulted in suppressed expression of IRS-2 in INS-1/832 cells (*C*). *IB*, immunoblotting. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* the cells without NO donor. *Error bars*, S.E.

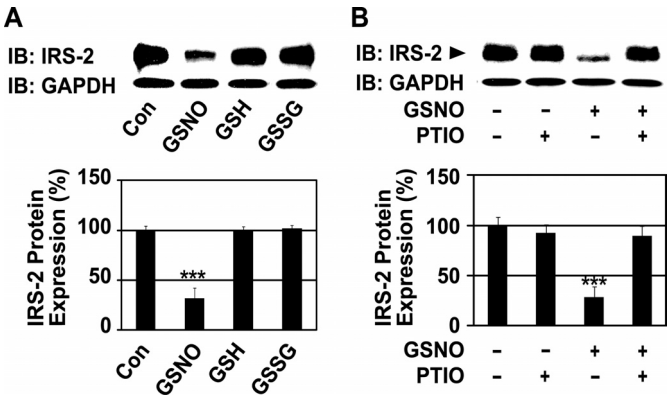

FIGURE 7. **NO-specific inhibitory effect on IRS-2 protein expression in β-cells.** INS-1/832 cells were treated with and without 300 μM GSNO, GSH, or GSSG for 5 h (*A*). ***, $p < 0.001$ *versus* the cells without NO donor (*Con*) and those with GSH and GSSG. Although IRS-2 protein expression was suppressed by GSNO, neither GSH nor GSSG decreased IRS-2 expression. In the presence of carboxy-PTIO (300 μM), treatment with GSNO (300 μM) for 6 h failed to affect IRS-2 expression (*B*). *IB*, immunoblotting. ***, $p < 0.001$ *versus* the cells without GSNO and those with GSNO + PTIO. *Error bars*, S.E.

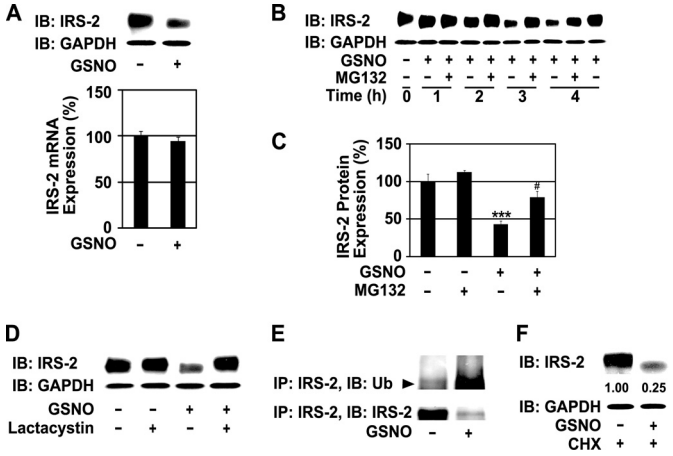

FIGURE 8. **Proteasome-dependent protein degradation of IRS-2 in NO donor-treated β-cells.** INS-1/832 cells were treated with and without GSNO (200 μM) up to 4 h in the presence and absence of proteasome inhibitors, MG132 (1 μM) and lactacystin (1 μM). Treatment with GSNO for 4 h did not alter mRNA level of IRS-2, although IRS-2 protein expression was suppressed at the same time point (*A*). MG132 prevented GSNO-induced time-dependent decrease in IRS-2 protein expression (*B*). MG132 significantly inhibited suppression of IRS-2 expression by 4-h treatment with GSNO (*C*). Lactacystin also reverted suppressed protein expression of IRS-2 in the cells treated with GSNO for 4 h (*D*). Ubiquitination of IRS-2 was evaluated by immunoprecipitation with anti-IRS-2 antibody followed by immunoblotting with anti-ubiquitin (*Ub*) or IRS-2 antibody. 4-h treatment with GSNO (300 μM) resulted in increased ubiquitination of IRS-2 compared with control, although IRS-2 protein expression was decreased (*E*). Consistent with proteasome-dependent degradation of IRS-2, GSNO (300 μM) treatment for 3 h reduced the protein expression of IRS-2 in the presence of cycloheximide (*CHX*) (1 μg/ml), an inhibitor of protein synthesis, as well. The *numbers below* the immunoblot for IRS-2 indicate relative intensities of the bands of IRS-2 normalized to that of the cells untreated with GSNO (*F*). GSNO and the inhibitors did not alter GAPDH protein expression in β-cells. *IP*, immunoprecipitation; *IB*, immunoblotting. ***, $p < 0.001$ *versus* the cells without GSNO and those with GSNO + MG132; #, $p < 0.05$ *versus* the cells without GSNO. *Error bars*, S.E.

As opposed to the inhibitory effects of GSNO, neither reduced (GSH) nor oxidized glutathione (GSSG) affected IRS-2 expression in INS-1/832 cells (Fig. 7*A*). Moreover, the inhibitory effect of GSNO (300 μM) on IRS-2 expression was abrogated by an NO scavenger, carboxy-PTIO (300 μM), in INS-1/832 cells (Fig. 7*B*). These results indicate the specific effect of NO on IRS-2 protein expression.

Similar to the effect of IL-1β, GSNO did not decrease mRNA expression of IRS-2 in INS-1/832 cells (Fig. 8*A*). These findings suggest that NO donor may promote protein degradation of IRS-2 in β-cells. Likewise, GSNO decreased IRS-2 protein expression in both wild-type and iNOS-deficient islets to a similar extent (see Fig. 11, *A* and *B*), whereas IRS-2 mRNA levels were not decreased (IRS-2 mRNA: control (WT), 100 ± 9%; KO, 105 ± 12%; GSNO: WT, 95 ± 14%; KO, 115 ± 10%). Treatment with GSNO for 5 h did not increase cell death in the islets, as judged by Sytox staining (data not shown).

To investigate an involvement of proteasome-dependent protein degradation, we examined the effects of inhibitors for proteasome, MG132 and lactacystin. MG132 (1 μM) and lactacystin (1 μM) inhibited GSNO-induced reduction in IRS-2 protein expression in INS-1/832 cells and mouse islets (Figs. 8 (*B–D*) and 11*C*). GSNO treatment led to increased ubiquitination of IRS-2 protein in INS-1/832 cells (Fig. 8*E*). These findings support a role of ubiquitination-involved proteasome-dependent degradation in NO donor-induced suppression of IRS-2 protein expression in β-cells. Consistent with the role of protein degradation, treatment with GSNO reduced IRS-2 expression in the presence of cycloheximide, an inhibitor of protein synthesis, in INS-1/832 cells (Fig. 8*F*).

Inhibition of GSK-3β Significantly Ameliorated NO Donor-induced Suppression of IRS-2 Expression in β-Cells—To further investigate underlying mechanisms, we examined the effects of inhibitors for glycogen synthase kinase-3β (GSK-3β), c-Jun N-terminal kinase (JNK/SAPK), and mammalian target of rapamycin (mTOR) in NO donor-treated INS-1/832 cells. GSNO treatment resulted in activation of GSK-3β and JNK/SAPK, as indicated by increases in phosphorylation of glycogen synthase and c-Jun, endogenous substrates of GSK-3β and JNK/SAPK, respectively. Increased phosphorylation of glycogen synthase and c-Jun was blocked by SB216763 and SP600125, inhibitors

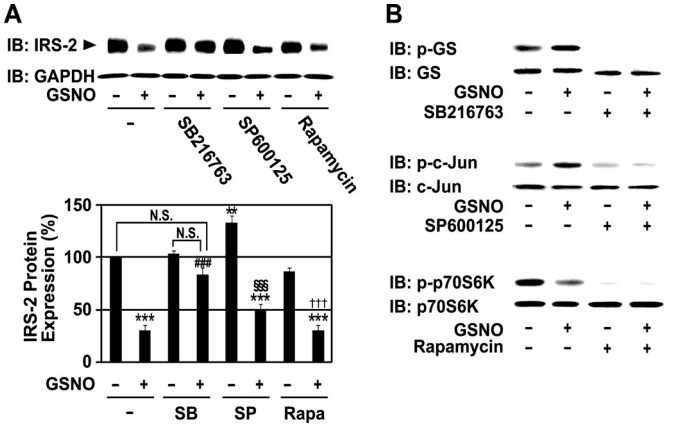

FIGURE 9. **The effects of inhibitors for GSK-3β, JNK/SAPK, and mTOR on IRS-2 protein expression in NO donor-treated β-cells.** INS-1/832 cells were treated with and without GSNO (200 μM) for 5 h in the presence and absence of inhibitors for GSK-3β (SB216763; 10 μM), JNK/SAPK (SP600125; 10 μM), or mTOR (rapamycin; 1 μM). SB216763 inhibited the NO donor-induced decrease in IRS-2 protein expression, whereas SB216763 did not alter IRS-2 expression when untreated with NO donor (*A*). In contrast, there were little if any effects of SP600125 and rapamycin on IRS-2 expression in GSNO-treated β-cells. GSNO treatment resulted in increases in phosphorylation of glycogen synthase (*GS*) and c-Jun, substrates of GSK-3β and JNK/SAPK, respectively (*B*). In contrast, phosphorylation of p70S6K was decreased by GSNO. SB216763, SP600125, and rapamycin inhibited phosphorylation of glycogen synthase, c-Jun, and p70S6K, respectively. GAPDH expression was not affected by GSNO or the inhibitors. *IB*, immunoblotting. **, $p < 0.01$; ***, $p < 0.001$ *versus* the cells with no treatment; ###, $p < 0.001$ *versus* the cells with GSNO alone; §§§, $p < 0.001$ *versus* the cells with SP 600125 alone; †††, $p < 0.001$ *versus* the cells with rapamycin alone. *N.S.*, not significant. *Error bars*, S.E.

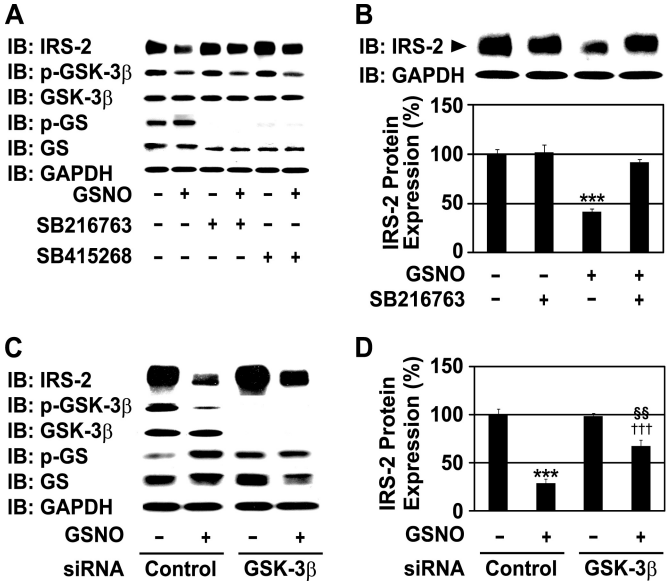

FIGURE 10. **Inhibition of GSK-3β significantly blocked NO donor-induced decrease in IRS-2 expression in β-cells.** We evaluated the effects of GSK-3β inhibitors, SB216763 and SB415268, and siRNA-mediated knockdown of GSK-3β on IRS-2 protein expression in INS-1/832 cells treated with and without GSNO (300 μM) for 5 h. *A*, GSNO decreased IRS-2 expression and phosphorylation of GSK-3β, whereas phosphorylation of glycogen synthase (*GS*) was increased in the absence of GSK-3β inhibitors. SB216763 (10 μM) and SB415268 (10 μM) up-regulated IRS-2 expression in GSNO-treated cells, along with suppression of phosphorylation of glycogen synthase. The inhibitors did not alter phosphorylation of GSK-3β. Neither GSNO nor the inhibitors affected the expression of GSK-3β, glycogen synthase, and GAPDH. *B*, SB216763 (10 μM) blocked suppressed expression of IRS-2 in GSNO-treated cells. When untreated with NO donor, SB216763 did not increase IRS-2 protein expression. ***, $p < 0.001$ *versus* the cells without GSNO and those with GSNO + SB216763. *C* and *D*, siRNA-mediated knockdown of GSK-3β inhibited GSNO-induced decrease in IRS-2 expression, as compared with control siRNA. Knockdown of GSK-3β blocked increased phosphorylation of glycogen synthase by GSNO. GAPDH expression was not altered by GSNO or knockdown of GSK-3β. ***, $p < 0.01$ *versus* the cells without GSNO; §§, $p < 0.01$ *versus* GSK-3β siRNA-transfected cells without GSNO; †††, $p < 0.001$ *versus* control siRNA-transfected cells with GSNO. *IB*, immunoblotting. *Error bars*, S.E.

for GSK-3β and JNK/SAPK, respectively, in GSNO-treated INS-1/832 cells (Fig. 9). To the contrary, mTOR activity was decreased by GSNO, as reflected by decreased phosphorylation of p70S6K, a substrate of mTOR. The protein expression of glycogen synthase, c-Jun, and p70S6K was not altered by GSNO treatment or the inhibitors.

GSK-3β inhibitor, SB216763 (10 μM), significantly prevented GSNO-induced reduction in IRS-2 protein expression in INS-1/832 cells (Fig. 9). In contrast, JNK inhibitor, SP 600125 (10 μM), failed to significantly increase IRS-2 protein expression in GSNO-treated INS-1/832 cells, although it up-regulated slightly but significantly IRS-2 protein expression in NO donor-untreated cells. There was little effect if any of mTOR inhibitor, rapamycin (1 μM), on IRS-2 expression in NO donor-treated β-cells. SP600125 and rapamycin effectively reduced activities of JNK/SAPK and mTOR, as judged by the phosphorylation status of c-Jun and p70S6K.

GSNO treatment resulted in decreased inhibitory phosphorylation of GSK-3β at serine 9 and increased phosphorylation of glycogen synthase, both of which indicate activation of GSK-3β by NO donor, in INS-1/832 cells and mouse islets (Figs. 10 and 11). GSK-3β inhibitors, SB216763 and SB415268, up-regulated IRS-2 protein expression in GSNO-treated INS-1/832 cells and mouse islets, along with suppression of phosphorylation of glycogen synthase. Phosphorylation of GSK-3β was not altered by the GSK-3β inhibitors because they inhibit the kinase activity of GSK-3β independent of phosphorylation status of the kinase. GSK-3β inhibitor, SB216763, did not alter the mRNA level of IRS-2 in GSNO-treated INS-1/832 cells (IRS-2 mRNA: vehicle, 100 ± 5%; SB216763, 102 ± 6%) and mouse islets (IRS-2 mRNA: control, 100%; GSNO, 97%). Similar to the inhibitors, siRNA-mediated knockdown of GSK-3β partially but significantly inhibited GSNO-induced reduction in IRS-2 expression and increase in phosphorylation of glycogen synthase in INS-1/832 cells, as compared with control siRNA (Fig. 10, *C* and *D*). Unless the cells were treated with NO donor, neither GSK-3β inhibitors nor knockdown of GSK-3β altered IRS-2 expression in β-cells.

IRS-2 Protein Expression Was Decreased in Islets of Obese, Diabetic Mice Along with Increased iNOS Expression—To further investigate the biological relevance of iNOS-mediated reduction in IRS-2 expression, we evaluated IRS-2 and iNOS expression in islets isolated from obese, diabetic (*db/db*) mice. IRS-2 protein expression was significantly decreased along with ~4-fold increase in iNOS mRNA expression in islets from *db/db* mice, as compared with wild-type mice (Fig. 11*D*). Unexpectedly, IRS-2 mRNA expression was increased in islets from *db/db* mice compared with lean wild-type mice (IRS-2 mRNA: WT, 100 ± 3%; *db/db*, 218 ± 37%, $p < 0.05$). Blood glucose levels following 4-h fasting and body weight were significantly greater in *db/db* mice than in wild-type mice (blood glucose: *db/db*, 451 ± 24 mg/dl; WT, 135 ± 3 mg/dl, $p < 0.002$; body weight: *db/db*, 43.4 ± 1.7 g; WT, 26.2 ± 1.0 g, $p < 0.002$).

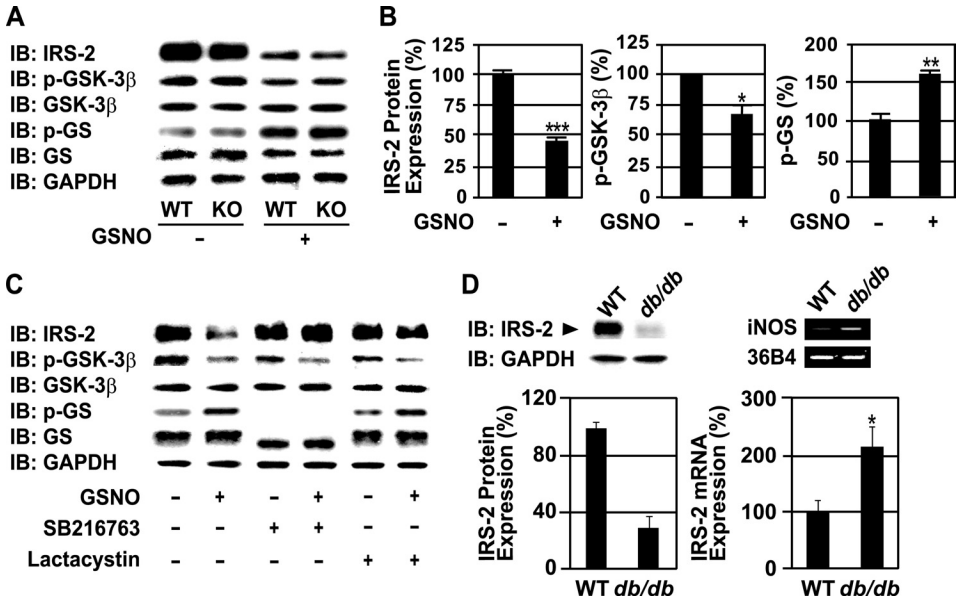

FIGURE 11. **Effects of NO donor on IRS-2 expression in mouse islets and IRS-2 protein expression in islets from diabetic mice.** *A* and *B*, treatment with GSNO (400 μM) for 5 h resulted in decreases in IRS-2 protein expression and phosphorylation of GSK-3β and increase in phosphorylation of glycogen synthase (*GS*) in islets isolated from WT and iNOS KO mice. The protein expression of GSK-3β, glycogen synthase, and GAPDH was not altered by GSNO. *, $p < 0.02$; **, $p < 0.005$; ***, $p < 0.001$ *versus* the cells without GSNO. $n = 3$/group. *C*, GSNO-induced decrease in IRS-2 expression was inhibited by GSK-3β inhibitor, SB 216763 (10 μM), and proteasome inhibitor, lactacystin (1 μM), in mouse islets. SB216763 abrogated phosphorylation of glycogen synthase (*GS*). In contrast, lactocystin did not alter the phosphorylation status of glycogen synthase in mouse islets treated with and without GSNO. *D*, IRS-2 protein expression was decreased in islets isolated from obese, diabetic (*db/db*) mice compared with wild-type mice. The protein expression of GAPDH did not differ between the two groups. iNOS mRNA content was increased in islets from *db/db* mice relative to wild-type mice, whereas 36B4 expression did not differ between the two groups. *, $p < 0.05$; ***, $p < 0.001$ *versus* WT. $n = 4$/group. *Error bars*, S.E.

DISCUSSION

Here, we show that iNOS and NO donor decrease IRS-2 expression in insulinoma cell lines and mouse islet cells by increasing proteasome-dependent protein degradation of IRS-2. Our data clearly indicate that iNOS is required for IL-1β- and IL-1β plus IFN-γ-induced reduction in IRS-2 expression in cultured pancreatic β-cells.

Moreover, the protein, but not mRNA, expression of IRS-2 was decreased in islets isolated from obese, diabetic (*db/db*) mice along with increased iNOS expression, as compared with lean wild-type mice (Fig. 11*D*). Our preliminary results in human islets are consistent with these findings. Treatment with cytokine mixture and GSNO resulted in decreased IRS-2 protein expression along with iNOS induction in human islets (supplemental Fig. 1, *A* and *B*). IRS-2 mRNA expression was not decreased by cytokine mixture (IRS-2 mRNA: control, 100%; IL-1β + IFN-γ + TNF-α, 104%; IL-1β + IFN-γ + TNF-α + LPS, 90%) or GSNO (IRS-2 mRNA: control, 100%; GSNO, 127%). Furthermore, our preliminary data suggest that IRS-2 protein expression may be decreased in islets of type 2 diabetes patients, whereas mRNA levels of IRS-2 and iNOS seem greater in islets from type 2 diabetes patients, as compared with non-diabetic individuals (supplemental Fig. 1*C*).

IRS-2 has a critical role in functional β-cell mass. These findings, therefore, suggest that iNOS may mediate proinflammatory cytokine (*i.e.* IL-1β)-induced damage and reduced mass of β-cells, at least in part, by suppressing IRS-2 expression in β-cells. iNOS expression is observed in β-cells and macrophages in the islets of diabetic rodents (18, 31–35). It is reasonable to speculate that NO derived from iNOS expressed in both intraislet macrophages and β-cells might contribute to decreased IRS-2 protein expression and β-cell dysfunction *in vivo* in diabetes.

Previous studies have revealed the transcriptional regulation of IRS-2 in β-cells. Glucagon-like peptide-1 (GLP-1) and GLP-1 receptor agonists promote the transcription and mRNA level of IRS-2 through cAMP-response element-binding protein-mediated mechanisms (49, 50). Acute exposure to glucose results in a rapid increase in IRS-2 mRNA levels in β-cells (51), although underlying mechanisms remain to be determined. In contrast, neither cytokine nor NO donor altered IRS-2 mRNA levels in INS-1/832 cells and mouse islets (Figs. 4 and 8), whereas the protein expression of IRS-2 was markedly reduced. Our findings highlight a role of ubiquitination-involved proteasome-dependent degradation of IRS-2 in the detrimental effects of proinflammatory cytokines and NO donor in β-cells. In contrast to IRS-2, iNOS and NO donor did not alter the protein expression of other components of the insulin signaling pathway, including insulin receptor, Akt, GSK-3β, mTOR, and p70S6K (Figs. 5, 9, 10, 11) (data not shown). It is conceivable, therefore, that NO-mediated promotion of proteasomal degradation may be specific to IRS-2 rather than a consequence of general activation of the ubiquitin-proteasome pathway.

iNOS and NO donor can induce β-cell death. Previous studies have shown that iNOS mediates β-cell death induced by IL-1β (21, 52). In our experiments, however, treatment with IL-1β (5 units/ml) or IL-1β (10 units/ml) plus IFN-γ (100 ng/ml) for up to 24 h and exposure to GSNO (300 or 400 μM) for 5 h did not affect cell viability of INS-1/832 cells and cultured mouse islets, although marked suppression of IRS-2 expression was observed. These results indicate that under these experimental conditions, the NO-mediated decrease in IRS-2 protein

is not attributable to reduced cell viability of the β-cells treated with cytokine or NO donor. Considering a critical role of IRS-2 in β-cell survival, however, it is possible that reduced IRS-2 expression might facilitate NO-involved β-cell death in diabetes.

Moreover, our results indicate that activation of GSK-3β plays a role in NO donor-induced reduction in IRS-2 protein expression in β-cells. Consistent with a previous study (52), iNOS and NO donor decreased basal and insulin-stimulated phosphorylation of Akt in β-cells (Fig. 5). This was associated with activation of GSK-3β, as indicated by reduction in inhibitory phosphorylation of GSK-3β at serine 9 and elevation in phosphorylation of glycogen synthase, an endogenous substrate of GSK-3β (Figs. 9–11). (Akt inhibits GSK-3β activity by phosphorylating serine 9 in GSK-3β.) The inhibition of GSK-3β by pharmacological inhibitors or siRNA-mediated knockdown significantly prevented NO donor-induced reduction in IRS-2 expression in INS-1/832 cells and cultured mouse islets. NO donor treatment also resulted in increased phosphorylation (activation) of JNK/SAPK in β-cells, as shown previously (52). The inhibition of JNK/SAPK, however, did not block the effects of NO donor on IRS-2 expression in β-cells.

A previous study has shown that mTOR mediates high glucose- and insulin-like growth factor-I (IGF-I)-induced proteasome-dependent degradation of IRS-2 in β-cells (53). However, NO donor treatment led to a decrease in mTOR activity, and rapamycin failed to inhibit NO donor-induced reduction in IRS-2 protein expression in INS-1/832 cells (Fig. 9). These findings indicate that high glucose/IGF-I and NO donor promote IRS-2 protein degradation through distinct signaling pathways.

β-Cell-specific overexpression of constitutively active GSK-3β causes reductions in β-cell mass and proliferation of β-cells (54). Of interest, these authors observed a trend of reduced IRS-2 protein expression in islets of the β-cell-specific GSK-3β transgenic mice compared with wild-type mice. However, the decreased IRS-2 protein expression was not statistically analyzed in their study. Nor was IRS-2 mRNA expression examined (54). Conversely, IRS-2 protein expression is up-regulated in the islets of β-cell-specific GSK-3β knock-out mice relative to wild-type mice (55). Importantly, GSK-3β inhibitors, SB216763 and LiCl, increased IRS-2 protein expression in cultured isolated islets and mouse MIN 6 insulinoma cells under serum deprivation (55), a condition that leads to activation of GSK-3β. These observations seem consistent with our findings, although these authors did not evaluate IRS-2 expression at the mRNA level. Together, our results support the notion that activation of GSK-3β results in decreased protein expression of IRS-2 in β-cells.

β-Cell-specific gene disruption of GSK-3β rescues the development of diabetes and reduced β-cell mass in IRS-2 knock-out mice (40). These results argue that GSK-3β can function as a downstream effector in mediation of the deleterious effects of IRS-2 deficiency in β-cells. Our results support an important role of GSK-3β in β-cell damage. Of note, our data indicate that GSK-3β may function as an upstream negative regulator of IRS-2 as well. Reduced IRS-2 expression leads to attenuated Akt activity in β-cells, which, in turn, results in activation of GSK-3β via decreased phosphorylation of serine 9 in GSK-3β. One can speculate, therefore, that activation of GSK-3β and reduction in IRS-2 expression might comprise a vicious cycle through a positive feedback mechanism, which contributes to progression of β-cell damage and diabetes. Further studies are required to clarify this point.

In conclusion, IL-1β alone and in combination with IFN-γ reduces IRS-2 protein expression by promoting protein degradation in pancreatic β-cells. iNOS is required for the inhibitory effect of IL-1β and IL-1β plus IFN-γ on IRS-2 protein expression. Our results indicate that GSK-3β plays an important role in NO-mediated suppression of IRS-2 protein expression in β-cells.

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