Nitric Oxide Inhibits the Proliferation and Invasion of Pancreatic Cancer Cells through Degradation of Insulin Receptor Substrate-1 Protein

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Abstract

Nitric oxide (NO), which plays a role in the posttranslational modification of proteins, exhibits tumoricidal activity. However, the mechanism remains largely unclear. We investigated whether the regulation of insulin receptor substrate (IRS)-1 protein expression and insulin/insulin-like growth factor (IGF) signaling by NO is involved in the proliferation and invasion of pancreatic cancer cells. NO donor inhibited insulin/IGF-I–stimulated phosphorylation of insulin receptor/IGF-I receptor, IRS-1, Akt/PKB, and glycogen synthase kinase-3β along with decreased expression of IRS-1 protein in MIAPaCa-2 cells, whereas NO donor enhanced the phosphorylation of extracellular signal-regulated kinase 1/2. In contrast, a selective inducible nitric oxide synthase inhibitor, 1400W, upregulated the expression of IRS-1 protein and the phosphorylation of IRS-1, Akt/PKB, and glycogen synthase kinase-3β, along with enhanced proliferation and invasion of Panc-1 cells expressing inducible nitric oxide synthase protein. NO donor induced IRS-1 protein reduction through increased ubiquitination and degradation. For the detection of the site responsible for NO-induced ubiquitination, IRS-1 deletion mutant genes were transfected and overexpressed in MIAPaCa-2 cells. The results indicate that the COOH terminus of the IRS-1 protein is required for NO donor–induced ubiquitination and protein degradation. Cells stably transfected with COOH-terminal deletion mutants of IRS-1 exhibited reduced IGF signaling and cell proliferation compared with vectoralone–transfected cells, with no influence of NO on IGF signaling and invasion, although stable transfectants with full-length IRS-1 protein exhibited remarkable NO-induced reduction in IGF signaling, cell proliferation, and invasion. These findings indicate that NO inhibits the proliferation and invasion of pancreatic cancer cells, at least in part, through upregulation of IRS-1 protein degradation and resultant downregulation of the insulin/IGF-I-Akt pathway. Mol Cancer Res; 8(8): 1152–63. ©2010 AACR.

Introduction

Insulin/insulin-like growth factor (IGF) signals play a key role in cancer proliferation and invasion (1–3). Insulin/IGF-I and IGF-II bind to insulin/IGF-I receptors and then phosphorylate the tyrosine of the cognate receptors. Insulin receptor substrate (IRS)–1, an adaptor protein, exists mainly in the cytosol and binds to phosphorylated insulin receptor and IGF-I receptor (IGF-IR), resulting in the phosphorylation and activation of IRS-1. IRS-1 transduces phosphatidylinositol-3 kinase (PI3K), which in turn activates further downstream components, including Akt/PKB and glycogen synthase kinase-3β (GSK-3β). Alternatively, phosphorylated and activated IRS-1 can also bind to another adaptor protein, Grb-2, which activates mitogen-activated protein kinase, another major insulin/IGF signal cascade parallel to the PI3K-Akt/PKB pathway (4, 5). IRS-1 protein expression was detected in several types of cancer, including pancreatic cancer, breast cancer, and hepatic cell carcinoma (6, 7). Thus, insulin/IGF signal has been considered to play a major role not only in metabolic actions, including stimulation of glucose uptake and synthesis of glycogen and protein, but also in cancer viability including proliferation and invasion. IRS-1 is a key molecule in insulin/IGF signaling, which transduces a signal from the insulin receptor/IGF-IR to the PI3K and mitogen-activated protein kinase pathways (8). However, the mechanism of the regulation of IRS-1 expression and insulin/IGF signals in cancer cells remains unclear.

Recent studies have shown that nitric oxide (NO) plays a role in the posttranslational modification of proteins (9–12). NO is produced by three distinct genes: neuronal and endothelial nitric oxide synthases (nNOS and eNOS) and...
inducible nitric oxide synthases (iNOS; refs. 13, 14). In contrast to the activities of nNOS and eNOS that are tightly regulated by calcium-dependent calmodulin binding, iNOS does not require calcium ion or posttranslational modification for its activity. Therefore, iNOS expression is associated with prolonged, exaggerated NO generation up to > 1,000-fold compared with nNOS and eNOS (15, 16). Although iNOS expression is increased in macrophages and endothelial cells by various stimuli, including acute inflammation, recent studies revealed that iNOS is expressed even in normal conditions in many tissues, including skeletal muscle and cancer (17, 18). In the skeletal muscle of diabetic mice, NO was found to play a key role for insulin resistance (17, 19). Controversial results have been reported about the roles of NO in cancer. Recent articles reported that endogenous NO promotes oncogenesis and angiogenesis in various cancers (20, 21). In contrast, other studies have shown that NO inhibits cell proliferation and induces apoptosis in various cells including cancer cells, in vitro and in vivo (22-28). These studies suggest that NO can act either as a tumor suppressor or as a tumor enhancer depending on cell type and the level of NO in the cells. However, the molecular mechanism underlying the inhibitory effects of NO on cancer viability remains unclear.

In this study, we show that NO induces proteasome-dependent IRS-1 protein degradation, and that the regulation of IRS-1 expression and insulin/IGF signaling by NO is involved in NO donor–mediated inhibition of the proliferative and invasive activities of pancreatic cancer cells. These data provide new insight into the molecular basis underlying the regulation of cancer viability by NO.

Materials and Methods

Materials
MG132, S-nitrosoglutathione (GSNO), S-nitroso-N-acetylpenicillamine (SNAP), and L-NAME were purchased from Calbiochem. A highly selective NO inhibitor, 1400W, was purchased from Cayman Chemical. Recombinant IGF-1 was purchased from Peprotech. Insulin and anti-Flag antibody were purchased from Sigma. Anti-phospho-Tyr 1135/1136 IGF-IRβ, anti-phospho-Ser 573 Akt/PKB, anti-phospho-Ser 9/11 GSK-3β, anti-phospho-extracellular signal-regulated kinase (Erk)-1/2, anti-Akt/PKB, anti-GSK-3β, and anti-Erk-1/2 antibodies were purchased from Cell Signaling Technology. Anti-phosphotyrosine and anti-ubiquitin antibodies were purchased from Santa Cruz Biotechnology. Anti-iNOS antibody was purchased from Becton Dickinson. Geneticin (G-418) was purchased from Life Technologies, Inc.

Cell culture
MIAPaCa-2, Panc-1, MCF-7, MB 468, and HepG2 cells were obtained from the American Type Culture Collection and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C under a humidified atmosphere of 5% CO2.

Nitrite assay
The concentration of nitrite was determined using a 2,3-diaminonaphthalene kit (Dojindo Laboratories; ref. 29). Briefly, MIAPaCa-2 cells and Panc-1 cells were incubated in phenol red–free DMEM. The medium was centrifuged at 1,000 × g for 15 minutes and the supernatant was placed on 96-well microplates. 2,3-Diaminonaphthalene was added to each well and incubated at room temperature for 15 minutes, and then stop solution was added. The fluorescence intensity of each well at 450 nm (excitation 360 nm) was measured with a fluorescence microplate reader. The concentrations of nitrite were calculated by reference to a standard curve of nitrite.

Constructs
cDNA construct of full-length human IRS-1 (hIRS) wild-type (WT) was produced by reverse transcription-PCR. ssDNA was used as a template, which was produced by reverse transcription using oligo-dT primer from mRNA isolated from HepG2 cells, and then PCR of the single-stranded hIRS-1 WT DNA was performed. For the cDNA construct of the full-length hIRS-1 WT gene, the sense primer was 5′-ATGGGAGGCCATCCGAGAGCGAT-3′ and the antisense primer was 5′-CTGACGCTCTTGCTGGTCTCTGGAAAC-3′. The PCR was begun with denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 1 minute, and extension at 72°C each for 3 minutes 30 seconds with a final extension at 72°C for 10 minutes. AccuPrime Pfx (Invitrogen) was used as the DNA polymerase.

For the production of deletion mutants (DM), PCR was performed using full-length hIRS-1 as a template. The primers used were as follows: DM1, 5′-ATGGGCCGAGCCCATCCGAGGAGCGAT-3′ (sense) and 5′-GTCCCCCACGTGTGACATGTTCA-3′ (antisense); DM2, 5′-ATGGGAGGCCATCCGAGAGCGAT-3′ (sense) and 5′-CTCATACATCATGGCAGAGACGATGCTCTGGAAAC-3′ (antisense); and DM3, 5′-ATGGTCAACACACGACGCCACCTCCGAGGAGCGAT-3′ (sense) and 5′-CTGACGCTCTTGCTGGTCTCTGGAAAC-3′ (antisense). The PCR was begun with denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 1 minute, and extension at 72°C each for 3 minutes 30 seconds with a final extension at 72°C for 10 minutes. AccuPrime Pfx (Invitrogen) was used as the DNA polymerase.

All PCR products were treated with Taq DNA polymerase and then subcloned into pCR2.1-TOPO vector (Invitrogen). Subcloned pCR2.1-TOPO vector was digested by EcoRI and then genes were inserted into pCMV Tag 4A vector (Stratagene), which is a mammalian expression vector, and then treated with EcoRI and CIAP. An illustration of the PCR products of full-length IRS-1, DM1, DM2, and DM3 is provided (see Fig. 2C).
Cell transfection

MIAPaCa-2 cells were transfected with pCMV Tag 4A/IRS-1 (full-length), pCMV Tag 4A/IRS-1 DM1, pCMV Tag 4A/IRS-1 DM2 (as a dominant negative), pCMV Tag 4A/IRS-1 DM3, and pCMV Tag 4A vector alone using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were used for the assay of IRS-1 protein degradation and detection of ubiquitination. In addition, the cells transfected with protein expression vectors were incubated with G418 for the selection of transfected gene–expressing cells for 2 weeks and then cloned. The clone that highly expresses the transfected gene was selected and then used for the assays.

Cell lysis

Cell lysates were obtained as previously described (19). Briefly, cells were lysed with cell lysis buffer [50 mmol/L

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**FIGURE 1.** A and B, concentration of nitrite in medium. A, MIAPaCa-2 cells were cultured with GSNO (1 mmol/L) or SNAP (1 mmol/L) for 0, 6, and 24 h. The concentration of nitrite in supernatant was determined. *, P < 0.05, compared with control. B, Panc-1 cells were cultured with 1400W (100 μmol/L) for 0, 3, 6, and 24 h. The concentration of nitrite in supernatant was determined. *, P < 0.05, compared with 0 h. **, P < 0.05, compared with control. C to E, regulation of insulin/IGF signal by NO donor in MIAPaCa-2 cells. MIAPaCa-2 cells, which were incubated in DMEM with 10% FBS overnight and grown to 80% confluence, were used. After incubation with SNAP (1 mmol/L) or GSNO (500 μmol/L) under serum starvation for 6 h, the cells were incubated with insulin (100 nmol/L) or IGF-I (25 nmol/L) for 5 min and then harvested. Cell lysates were subjected to immunoprecipitation (IP) associated with immunoblotting (IB) or immunoblotting. All experiments were repeated three times and the same results were obtained.

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FIGURE 2. NO donor–induced reduction of IRS-1 protein expression through proteasome-mediated degradation in MIAPaCa-2 cells. A, MB 468, MIAPaCa-2, Panc-1, and MCF-7 cells were incubated under various concentrations of GSNO in DMEM with 10% FBS for 24 h. After incubation, cells were harvested and cell lysates were subjected to immunoblotting for detection of IRS-1 protein expression. All experiments were repeated three times and the same results were obtained. B, MIAPaCa-2 cells were incubated in DMEM with 10% FBS overnight. Subsequently, MG132 (3.3 μmol/L), a proteasome inhibitor, was added 30 min before (a) GSNO (500 μmol/L) or (b) SNAP (1 mmol/L) administration, followed by incubation for 24 h. Cell lysates were subjected to immunoblotting for detection of IRS-1 protein expression. Data shown are the results of triplicate experiments. Bars, SEM. *, P < 0.05, compared with control; ++, P < 0.05, compared with GSNO only. C, schematic representation of the functional domains of hIRS-1 protein and the structural organization of the deletion mutants. PH and PTB indicate pleckstrin homology and phosphotyrosine binding regions, respectively. The various hIRS-1 deletion mutants contain a FLAG epitope (DYKDDDDK) at the COOH terminus. D, MIAPaCa-2 cells were transfected with full-length IRS-1, IRS-1 DM1, IRS-1 DM2, and IRS-1 DM3 using Lipofectamine 2000, followed by incubation with GSNO (500 μmol/L) for 24 h. Each overexpressed protein was detected by immunoblotting using anti-Flag antibody. E, MIAPaCa-2 cells were transfected with full-length IRS-1, IRS-1 DM1, IRS-1 DM2, and IRS-1 DM3. MIAPaCa-2 cells were incubated in DMEM with 10% FBS overnight, and then MG132 (3.3 μmol/L), a proteasome inhibitor, was added 30 min before SNAP (1 mmol/L) administration, followed by incubation for 4 h. For the detection of ubiquitination, cell lysates were subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-ubiquitin. All experiments were repeated three times and the same results were obtained.
Tris-HCl (pH 7.6), 150 mmol/L NaCl, 10 mmol/L sodium fluoride, 2 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L DTT, and 1% NP40. Following incubation on ice for 30 minutes, lysate samples were centrifuged at 13,000 × g for 30 minutes. Aliquots of the supernatant containing equal amounts of protein, determined using the Lowry assay, were subjected to immunoprecipitation followed by SDS-PAGE.

**Immunoprecipitation and immunoblotting**

Immunoprecipitation was done by incubating the lysates with antibody at 4°C for 18 hours. The immune complexes were collected by incubation with protein A/G-agarose beads for 1.5 hours at 4°C, washed three times with wash buffer [50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 10 mmol/L sodium fluoride, 2 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L DTT, and 0.1% NP40], and boiled in Laemmli sample buffer.

Cell lysates containing equal amounts of either protein or immunoprecipitates were subjected to SDS-PAGE. Following electrophoretically onto nitrocellulose membrane (Bio-Rad), the membranes were blocked in 5% nonfat dried milk for 2 hours at room temperature and incubated with primary antibody for 2 hours at room temperature or overnight at 4°C. This was followed by incubation with secondary antibody conjugated with horseradish peroxidase for 1 hour at 4°C. Western blotting chemiluminescence luminal reagent (Perkin-Elmer) was used to visualize the blots. Bands of interest were scanned by using Power Look (UMAX Technologies) and were quantified by using NIH Image 1.62 software (NTIS).

**Cell proliferation assay**

To assess cell growth, MIAPaCa-2 and Panc-1 cells were seeded in 24-well plates (2 × 10^4 per well) in triplicates. The cells were preincubated for 24 hours in DMEM in the presence of 1% glutamine, 10% FBS, and antibiotics (1% penicillin and streptomycin sulfate) at 37°C under a humidified atmosphere of 5% CO₂ and were then exposed to various reagents for 24, 48, and 72 hours. The cells were collected and counted after being stained with 0.4% trypan blue (Sigma-Aldrich).

**Cell invasion assay**

The in vitro invasive potential of MIAPaCa-2 cells and Panc-1 cells was determined using BioCoat Matrigel Invasion Chambers (Becton Dickinson). According to the manufacturer’s instructions, the Matrigel was hydrated with 0.5 mL of DMEM (serum-free) and incubated for 2 hours in humidified tissue culture incubator at 37°C under a humidified atmosphere of 5% CO₂.
of cell migration used control inserts with 8.0-μm pores in 24-well plates. After detaching the cells with 0.25% trypsin and counting, they were diluted to 1.0 × 10^5 cells/mL in medium containing 1% FBS. A total of 0.5 × 10^5 cells per well were placed on the top chamber of the insert above the medium containing chemoattractants, which was placed in the lower chamber. After incubation for 22 hours under a humidified atmosphere of 5% CO₂, the cells on the upper surface of the membrane were removed with a cotton swab and the migrated cells on the lower side of the membrane were fixed and stained using Diff-Quick (American Scientific Products) and counted in 10 random fields at ×100 magnification (30).

Statistical analysis
Data were compared using one-way ANOVA followed by Fisher’s protected least significant difference test. \( P < 0.05 \) was considered statistically significant. All values were expressed as mean ± SEM.

Results

NO donors increase the concentration of nitrite (NO₂⁻) and 1400W inhibits nitrite production in the medium in which cancer cells are cultured

NO donors added to the culture medium provided NO to cancer cells. Cancer cells expressing iNOS protein can produce NO in normal conditions. We determined the concentration of nitrite, a metabolite of NO, in the culture medium. Both GSNO and SNAP, NO donors, augmented the concentration of nitrite in the culture medium in which MIAPaCa-2 cells were cultured for 6 and 24 hours (Fig. 1A). The concentration of nitrite was elevated with time in the medium in which Panc-1 cells were cultured. 1400W, a highly selective iNOS inhibitor, inhibited nitrite production in the culture medium at 3 and 6 hours (Fig. 1B).

NO influences insulin/IGF signals in MIAPaCa-2 cells
The stimulation of insulin or IGF-I resulted in remarkable tyrosine phosphorylation of insulin receptor, IGF-1R,
and IRS-1; phosphorylation of Akt/PKB at Ser\textsuperscript{473} and GSK-3β at Ser\textsuperscript{β}; phosphorylation of insulin receptor and IRS-1, phosphorylation of Akt/PKB at Ser\textsuperscript{473}, and phosphorylation of GSK-3β at Ser\textsuperscript{β}. In addition, SNAP inhibited IGF-I-stimulated tyrosine phosphorylation of IGF-IR and IRS-1, phosphorylation of Akt/PKB at Ser\textsuperscript{473}, and phosphorylation of GSK-3β at Ser\textsuperscript{β}. Furthermore, SNAP reduced IRS-1 protein expression, although this did not alter the expression of other proteins, including IGF-IR, Akt/PKB, GSK-3β, and Erk-1/2, in downstream of IGF signaling, as well as β-actin expression. On the other hand, SNAP induced the phosphorylation of Erk-1/2 without stimulation of insulin/IGF-I and enhanced insulin/IGF-I-stimulated phosphorylation of Erk-1/2; however, SNAP did not influence Erk-1/2 protein expression in MIAPaCa-2 cells (Fig. 1C and D). GSNO, a NO donor, inhibited IGF-I-stimulated phosphorylation of IRS-1, phosphorylation of Akt/PKB, IRS-1, and Akt/PKB, but enhanced the phosphorylation of Erk-1/2 as well as SNAP (Fig. 1E).

**NO downregulates IRS-1 protein expression through proteasome-mediated degradation in MIAPaCa-2 cells**

GSNO inhibited IRS-1 protein expression in MCF-7 as well as MIAPaCa-2 cells in a dose-dependent manner, but did not influence IRS-1 protein expression in MB 468 and Panc-1 cells, which exhibited less IRS-1 protein expression (Fig. 2A). The proteasome inhibitor MG132 completely reversed the reduction of IRS-1 protein expression by NO donors (GSNO and SNAP) in MIAPaCa-2 cells. Neither GSNO nor MG132 influenced GSK-3β and β-actin protein expression (Fig. 2B).

To further investigate IRS-1 protein degradation induced by NO donor, cDNA constructs of full-length IRS-1, IRS-1 DM1, IRS-1 DM2, and IRS-1 DM3 were produced and subcloned in mammalian expression vectors (Fig. 2C). MIAPaCa-2 cells were transfected with these expression vectors. GSNO reduced full-length IRS-1, IRS-1 DM1, and IRS-1 DM3 protein expression, although GSNO did not alter IRS-1 DM2 and β-actin protein expression (Fig. 2D). Ubiquitination of WT and mutated IRS-1 was detected by immunoprecipitation using anti-Flag antibody followed by immunoblotting with anti-ubiquitin. SNAP induced the ubiquitination of full-length IRS-1, IRS-1 DM1, and IRS-1 DM3, but did not induce the ubiquitination of IRS-1 DM2 (Fig. 2E). These results indicate that NO donor is capable of inducing ubiquitination at multiple sites in the COOH terminus of the IRS-1 protein.

**iNOS protein is expressed in Panc-1 cells and 1400W upregulates IRS-1 protein expression and the IRS-1-Akt pathway in Panc-1 cells**

iNOS protein was detected by immunoblotting in Panc-1 cells. IRS-1 protein expression was significantly increased by 1400W in a dose-dependent manner. However, Akt/PKB, β-actin, and Erk-1/2 protein expression was not altered in Panc-1 cells (Fig. 3A). 1-NAME, a nonselective NOS inhibitor, increased IRS-1 expression in Panc-1 cells, but it was not remarkable compared with the increase by 1400W (Fig. 3B). GSNO inhibited IRS-1 protein expression, upregulated by 1400W in Panc-1 cells (Fig. 3C). On the other hand, 1400W did not alter IRS-1 protein expression in MCF-7, which expresses high level of the protein (Supplementary Figure). 1400W enhanced IGF-I-stimulated tyrosine phosphorylation of IRS-1, phosphorylation of Akt/PKB at Ser\textsuperscript{473}, and phosphorylation of GSK-3β at Ser\textsuperscript{β} in Panc-1 cells. In contrast, 1400W did not alter IGF-I-stimulated phosphorylation of Erk-1/2 (Fig. 3D). These results indicate that endogenous NO produced by iNOS plays a role in insulin/IGF-I signaling.

**NO inhibits the proliferation of cancer cell lines and IRS-1 protein expression is associated with cancer cell proliferation**

Cell proliferation assay revealed that 100 and 500 μmol/L GSNO inhibited the proliferation of MIAPaCa-2 and MCF-7 cells in culture medium containing 10% FBS (Fig. 4A). Mammalian cell expression vectors, pCMV Tag 4/IRS-1 (full-length), pCMV Tag 4/IRS-1 DM2, and pCMV Tag 4A vector alone, were transfected into MIAPaCa-2 and incubated with G418 for the selection of protein-expressing cells for more than 14 days. Subsequently, the cells expressing high levels of full-length IRS-1 protein or IRS-1 DM2 protein were cloned. Phosphorylation of Akt/PKB at Ser\textsuperscript{473} and of GSK-3β at Ser\textsuperscript{β} by stimulation of IGF-I were reduced in IRS-1 DM2-transfected cells in comparison with vector alone--or full-length IRS-1--transfected cells. SNAP failed to reduce the phosphorylation of Akt/PKB at Ser\textsuperscript{473} and GSK-3β at Ser\textsuperscript{β} by stimulation of IGF-I in IRS-1 DM2--transfected cells, but reduced their phosphorylation in vector alone--or full-length IRS-1--transfected cells. No difference was observed between these cell lines in terms of Akt/PKB and GSK-3β protein expression (Fig. 4B).

Proliferation of MIAPaCa-2 cells was elevated in culture medium containing serum or IGF-I, whereas no proliferation was observed in culture medium without serum or IGF-I. Proliferation of full-length IRS-1--transfected cells was greater than that of the vector alone--transfected cells in the culture medium containing 10% FBS. On the other hand, the proliferation of IRS-1 DM2--transfected cells was attenuated compared with full-length IRS-1 and vector alone (Fig. 4C). The proliferation of full-length IRS-1--transfected cells was greater than that of vector alone--transfected cells in the culture medium containing 100 nmol/L IGF-I without 10% FBS, whereas IGF-I--stimulated proliferation of IRS-1 DM2--transfected cells was not observed (Fig. 4D). GSNO (200 μmol/L) significantly reduced the proliferation of vector alone--full-length IRS-1-- and IRS-1 DM2--transfected cells in culture medium containing 10% FBS or IGF-I. In the culture medium containing serum or IGF-I, the reduction rate of proliferation of full-length IRS-1--transfected cells was the greatest, whereas it was the least in IRS-1 DM2--transfected cells.
Proliferation of Panc-1 cells was not observed in the presence or absence of 1400W (100 μmol/L) when cultured without serum or IGF-I (Fig. 5A). 1400W significantly enhanced the proliferation of Panc-1 cells when cultured with 10% FBS (Fig. 5B). To further investigate the role of iNOS in IGF-I-stimulated proliferation, we evaluated the effects of the selective iNOS inhibitor 1400W in Panc-1 cells cultured with IGF-I in the absence of FBS. In the absence of 1400W, IGF-I failed to increase the cell numbers of Panc-1. The combination of IGF-I and 1400W, however, increased the number of Panc-1 cells (Fig. 5C). These results provide further evidence for the involvement of the downregulation of IGF-I signaling in NO-induced inhibition of cancer cell proliferation.

Sensitivity of invasion to NO is dependent on IGF signaling

In the invasion assay, there was no difference in invasion among vector alone–, full-length IRS-1–, and IRS-1 DM2–transfected MIAPaCa-2 cells in the absence of the NO donor. The addition of 200 μmol/L GSNO remarkably reduced the invasion of vector alone– and full-length IRS-1–transfected MIAPaCa-2 cells, but did not alter the invasion of IRS-1 DM2–transfected MIAPaCa-2 cells (Fig. 6A and C). The invasion of Panc-1 cells incubated with 1400W (5 and 100 μmol/L) was significantly greater than that of Panc-1 cells incubated without 1400W (Fig. 6B and D).

Discussion

Here, we showed that NO donor reduced IRS-1 protein expression via proteasome-dependent degradation and inhibited insulin/IGF-I–stimulated phosphorylation of Akt/PKB and GSK-3β, but enhanced the phosphorylation of Erk-1/2 in pancreatic cancer cells. In this study, the COOH-terminal deletion mutants of IRS-1 (DM2) worked as a dominant negative, as previously reported (4). NO donor inhibited IGF-I–induced phosphorylation...
of Akt/PKB and GSK-3β in MIAPaCa-2 cells transfected with IRS-1 WT or vector, but not in cells transfected with IRS-1 DM2, thus revealing the importance of IRS-1 in the inhibition of insulin/IGF signal by NO. IRS-1 expression and IGF-I signaling have important roles in the proliferation and invasion of MIAPaCa-2 cells and Panc-1 cells, consistent with previous reports on other cancer cells (3, 31-33). NO donor inhibited the IGF-I signaling, proliferation, and invasion of MIAPaCa-2 cells transfected with full-length IRS-1 or vector. In contrast, the selective iNOS inhibitor upregulated IRS-1 protein expression and insulin/IGF signal, resulting in enhanced proliferation and invasion activity in Panc-1 cells. These results indicate that the expression of IRS-1 protein is regulated by endogenous NO production by iNOS as well as exogenous NO, resulting in downregulation of IGF-I signaling and inhibition of the proliferation and invasion of MIAPaCa-2 and Panc-1 cancer cells.

Furthermore, we identified the COOH terminus as the site responsible for IRS-1 protein degradation by NO, which is located in the SH2-containing molecule binding site next to the phosphotyrosine binding (PTB) domain (Fig. 2C). The observation of the ubiquitination and degradation of both IRS-1 DM1 and IRS-1 DM3 indicates the possibility that there may be at least two sites responsible for NO donor–induced ubiquitination in the IRS-1 protein.

The mechanism explaining the degradation of the IRS-1 protein was previously reported (19, 34). Yu et al. showed that activated R-Ras induces degradation of IRS-1 protein associated with suppression of estrogen action in MCF-7 cells (34). However, in contrast to H-Ras and N-Ras, both of which contain Cys118 residue in the conserved region, R-Ras not containing a Cys residue in the region is not modified by NO (35, 36). In addition, they described that R-Ras–dependent IRS-1 protein degradation was ubiquitin independent. Thus, R-Ras does not seem to contribute to NO-mediated IRS-1 degradation. We previously reported that PI3K, mammalian target of rapamycin, and c-jun NH2-terminal kinase/stress-activated protein kinase inhibitors failed to block NO-induced IRS-1 protein reduction, despite having these inhibitors attenuated insulin-induced IRS-1 protein reduction in mouse C2C12 cells (19). Therefore, further investigation will be necessary to clarify the mechanism of NO-mediated ubiquitination and degradation of IRS-1.

In contrast to NO-induced downregulation of the IRS-1-PI3K-Akt pathway, NO donor upregulates the phosphorylation of Erk-1/2 in MIAPaCa-2 cells. These results are in accordance with previous studies reporting that NO modifies H-Ras and N-Ras directly by S-nitrosylation, resulting in the activation of the signaling (35, 37). Lim et al. showed that the PI3K-Akt pathway phosphorylates and
activates eNOS, resulting in the activity of H-Ras and N-Ras and tumor growth (36). In addition, other previous studies reported NO-induced cell proliferation and cell invasion (38, 39). The authors of these reports argue that NO-induced activation of Erk-1/2 leads to cell survival, in opposition to our data indicating the tumoricidal effects of NO. However, in light of this discrepancy, we hypothesize that the outcome of cancer cells treated with NO may depend on the balance of the PI3K-Akt and Ras-Erk pathways. For instance, the rate of NO-induced inhibition of proliferation was highest in MIAPaCa-2 cells overexpressing full-length IRS-1, but minimal in cells expressing

**FIGURE 6.** NO donor inhibits the invasion of MIAPaCa-2 cells and 1400W enhances the invasion of Panc-1 cells. Cell invasion was determined using BioCoat Matrigel Invasion Chambers. Stable transfected MIAPaCa-2 cells (A and C) or Panc-1 cells (B and D) were placed in the upper chambers. Conditioned medium of NIH 3T3 cells was used as a chemoattractant. Data shown are the results of triplicate experiments (A and B). Bars, SEM. C and D, representative staining of migrated cancer cells. *, P < 0.01, compared with control.
IRS-1 DM2. This means that MIAPaCa-2 cells overexpressing IRS-1 may be dependent on the IRS-1-Akt pathway, whereas MIAPaCa-2 cells overexpressing IRS-1 DM2 may be independent of the pathway.

NO donor exhibited minimal inhibition in the proliferation of stable IRS-1 DM2–transfected MIAPaCa-2 cells, although NO donor failed to reduce the invasion of the cells. These results indicate that NO may downregulate the proliferation of MIAPaCa-2 cells partially through mechanisms other than the reduction of IRS-1 protein expression and phosphorylation. Namely, as previously reported, NO donor inhibits the activity of Akt/PKB through S-nitrosylation (12), indicating the possibility that it may be one of several mechanisms. Furthermore, NO induced DNA damage, and the activation of p53 and mitogen-activated protein kinase may be related to the phenomenon, as reported previously by Hofseth et al. (40).

This study shows that GSNO and SNAP provide high concentration of nitrite in the culture medium at 6 hours after administration. Furthermore, Panc-1 cells produced nitrite, a metabolite of NO, in culture medium. The level of NO in the cells is an important factor that influences the pathophysiologic activity of NO. iNOS can provide high concentrations of NO in Panc-1 cells, and NO derived from 100 to 500 μmol/L GSNO and 1 mmol/L SNAP may result in a high NO concentration in the cytosol of cancer cells. High levels of NO have been shown to exhibit cytotoxicity in various cells (41). Because the half time in which GSNO provides NO is longer than that of SNAP, we intentionally used SNAP for short-term experiments and GSNO for long-term experiments (42, 43).

GSNO inhibited IRS-1 protein expression at dosages of 100 to 200 μmol/L. These dosages of GSNO also inhibited cell proliferation and invasion. These findings suggest that NO-mediated IRS-1 degradation contributes to the inhibition of cell proliferation and invasion by NO donor. GSNO reduces IRS-1 protein expression, which is upregulated by 1400W in Panc-1 cells (Fig. 3C). These results suggest that endogenous NO is sufficient to reduce IRS-1 expression; therefore, GSNO does not reduce IRS-1 expression in Panc-1 cells without 1400W.

The usefulness of cancer therapy using NO, including iNOS gene therapy and administration of NO donor, was recently confirmed in animal models (44–46). Consequently, NO therapy has been focused on and is currently undergoing clinical evaluation for cancer prevention (47). However, the molecular mechanism still remains unclear. Our data suggest that NO-induced downregulation of the insulin/IGF-IRS-1-Akt pathway may play an important role in the proliferation and invasion of pancreatic cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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