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Nitric Oxide Regulates Growth Factor Signaling in Pancreatic Cancer Cells

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1. Introduction

Growth factor signaling plays a critical role in cancer proliferation and invasion. Therefore, molecules, involved in growth factor signaling have become the targets of cancer therapy, and many drugs targeting growth factor signaling pathways have been developed. Some of these drugs have been used clinically, while many more are being tested in clinical trials. However, to date, molecule-targeted therapies for pancreatic cancer have not been developed.

Nitric oxide (NO) was discovered two decades ago and was initially identified as an endothelial relaxing factor. Subsequently, NO has been shown to play key roles in the post-translational modification of proteins and the regulation of protein enzymatic activity. In this paper, we present evidence indicating that NO influences cancer proliferation and invasion, and discuss the mechanisms trough which NO is thought to exert these effects.

2. Production of NO in cells and tissues

NO is produced by three distinct genes products: neuronal and endothelial nitric-oxide synthases (nNOS and eNOS) and inducible nitric-oxide synthases (iNOS) (Palmer *et al.*, 1987). The activities of nNOS and eNOS are tightly regulated by calcium-dependent calmodulin binding, whereas iNOS does not require calcium ion or posttranslational modification for its activity. As a result, iNOS expression is associated with prolonged, exaggerated NO generation of up to > 1,000-fold greater than that generated by nNOS and eNOS. Although iNOS expression is increased in macrophages and endothelial cells by various stimuli, including acute inflammation, recent studies have revealed that iNOS is expressed even in normal conditions in many tissues, including skeletal muscle and cancer (Perreault and Marette, 2001; Xie and Fidler, 1998). The expression of iNOS protein has been reported in pancreatic cancer cells, colon cancer cells, gastric cancer, breast cancer, hepatocellular carcinoma, glioma cells, melanoma cells, and laryngeal squamous cell carcinoma.

3. NO-donors

Several types of reagents called NO-donors, are capable of releasing NO constitutively (Table 1). *S*-Nitrosothiols (RSNO), which break down to form NO and the corresponding disulphide (RSSR), are an important class of NO -donor drugs. NO-donors containing R-NO (S-NO), are unstable and release NO upon breakdown. Decomposition of these compounds is catalysed by Cu+ ions, which themselves can be formed by reduction of Cu^{2+} ions by thiols. Breakdown is accelerated by light at ultraviolet and optical wavelength and is influenced by PH.

Organic nitrates such as nitroglycerin, isosorbide dinitrate and mononitrate, which have long been used as vasodilators for the treatment of angina pectoris, release NO via both enzymatic and non-enzymatic pathways. Iron-nitrosyl complexes such as sodium nitroprusside (SNP), sydnonimine, and amine NONOate, all demonstrate NO donating capacity. NO -donating non-steroidal anti-inflammatory drugs (NO-NSAIDs), which were developed recently, are potential anti-cancer drugs (Gao *et al*., 2005). NO-NSAIDs consist of a conventional NSAID to which an NO-releasing moiety is attached covalently. Glutathione S-transferase -activated NO-donors such as JS-K, have shown some therapeutic promise in cancer without hypotension (Weiss *et al*.).

NO-donor reagents offer a convenient source of NO for *in vitro* and *in vivo* experiments. Researchers can thereby avoid use NO gas but must consider intrinsic half-life, metabolites, and other activities derived from the unique moiety in choosing a NO-donor reagent.

S-Nitrosothiols
S-nitrosoglutathione (GSNO)
S-nitroso-N-acetylpenicillamine (SNAP)
Organ nitrates
Nitroglycerin (NTG)
isosorbide dinitrate (ISDN)
Iron-nitrosyl complex
sodium nitroprusside (SNP)
Sydnonimine
3-morpholino-sydnonimine (SIN-1)
Molsidomine
Diazeniumdiolate (NONOate)
Angeli's salt
Diethylamine
O2-(2,4-Dinitrophenyl)
1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K)
NO-donating NSAIDs
Nitric oxide-donating aspirin (NO-ASA)
NO-naproxen
NONO-ASA

Table 1. NO donors

4. Actions of NO *in vivo*

4.1 cGMP-dependent actions

Guanylyl cyclases (GC) are expressed in the cytoplasm of almost all mammalian cells and mediate a wide range of important physiological functions, including inhibition of platelet aggregation, relaxation of smooth muscle, vasodilation, neuronal signal transduction, and immunomodulation (Collier and Vallance, 1989). GCs have evolved to synthesize cGMP in response to diverse signals, such as NO. NO activates GC by binding directly to heme to form a ferrous-nitrosyl-heme complex. Endogenous and exogenous compounds, including autocoids, hormones, neurotransmitters, and toxins, produce cellular responses through cGMP. The specificity of cellular responses to cGMP is dictated by cGMP-binding motifs in target proteins PKA (cAMP-dependent protein kinase) and PKG (cGMP-dependent protein kinase) (Francis and Corbin, 1999), cyclic nucleotide-gated cation channels (Biel *et al.*, 1999; Kaupp, 1995) and cGMP-regulated phosphodiesterases (Beavo, 1995).

4.2 cGMP-independent actions

The major cGMP-independent actions of NO are nitrosative post-translational modifications, including protein *S*-nitrosylation and tyrosine nitration. Post-translational modification of proteins by S-nitrosylation, attachment of nitrosonium ion (NO) to cysteine sulfhydryls, is a major mode of signaling in mammalian cells

Indeed, critical signaling molecules and transcription factors are primary targets of NO (Stamler *et al.*, 2001). To date, over 100 proteins have been shown to be *S*-nitrosylated both *in vitro* and *in vivo*. In many of these proteins, *S*-nitrosylation leads to functional alterations. Signaling proteins that are directly modified by S-nitrosylation include Ras, Akt, JNK, PTEN, IκB kinase, and Bcl2. (Azad *et al.*; Lander *et al.*, 1997; Numajiri *et al.*; Park *et al.*, 2000; Reynaert *et al.*, 2004; Yasukawa *et al.*, 2005)

5. Roles for NO in cancer

Conflicting results have been reported regarding the roles of NO in cancer. Recent papers reported that endogenous NO promotes oncogenesis and angiogenesis in various cancers (Ambs *et al.*, 1998; Camp *et al.*, 2006). 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* (Chawla-Sarkar *et al.*, 2003; Jarry *et al.*, 2004; Kalivendi *et al.*, 2001; Kotamraju *et al.*, 2007; Notas *et al.*, 2006; Peshes-Yaloz *et al.*, 2007; Wang *et al.*, 2003). These studies suggest that NO can act either as a tumor suppressor or 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.

5.1 Roles in carcinogenesis and cancer promotion

NO and reactive nitrogen species (RNOS) induce the formation of nitrosamines, which can cause cancers in a wide variety of animal species. Nitrosation of nucleic acid bases leads to deamination which in turn results in mutagenic or carcinogenic conversion cytosine to uracil, guanine to xanthine, methylcytosine to thymine and adenine to hypoxanthine (Caulfield *et al.*, 1998; Wink *et al.*, 1991). RNOS can cause both single- and double- strand breaks in DNA.

Furthermore, NO inhibits DNA repair proteins and poly- (ADP-ribose) polymerase (PARP), which regulates DNA repair and apoptosis (Sidorkina *et al.*, 2003). Thus, NO induces DNA damage that can lead to carcinogenesis.

NO can promote cancer by enhancing vascularization, which favors growth and metastasis , and by inhibiting apoptosis. NO induces the expression of VEGF in carcinoma cells and suppresses angiostatin and thrombospondin-1, inhibitors of angiogenesis, resulting in promotion of tumor vascularization (Cooke and Losordo, 2002; Dulak *et al.*, 2000). The growth of xenografted murine mammary adenocarcinoma which expresses iNOS is reduced by treatment with iNOS inhibitor (Thomsen *et al.*, 1997). Nitric oxide (NO)-mediated S-nitrosylation of Bcl-2 prevents its ubiquitination and subsequent proteasomal degradation, leading to inhibition of apoptosis. NO-mediated S-nitrosylation and stabilization of Bcl-2 protein was the primary mechanism involved in the malignant transformation of nontumorigenic lung epithelial cells in response to long-term carcinogen exposure (Azad *et al.*).

5.2 Anti-cancer effects

In contrast to the aforementioned effects of NO, other studies have shown that NO inhibits cell proliferation and induces apoptosis in various cells including cancer cells, *in vitro* and *in vivo* (Chawla-Sarkar *et al.*, 2003; Jarry *et al.*, 2004; Kalivendi *et al.*, 2001; Kotamraju *et al.*, 2007; Notas *et al.*, 2006; Peshes-Yaloz *et al.*, 2007; Wang *et al.*, 2003). Nitrosylcobalamin (NO-Cbl), an analog of vitamin B12 that delivers nitric oxide (NO) and xhibits anti-tumor activity; NO-Cbl increases the expression of tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) and its receptors, resulting in apoptosis of human tumors. (Chawla-Sarkar *et al.*, 2003). The tumor suppressor P53 participates in numerous critical cellular functions including gene transcription, DNA repair, cell cycle control, genomic stability, and apoptosis (Gottlieb and Oren, 1996; Harris, 1996) . DNA damage, especially DNA double strand breaks caused by ionizing radiation or other exogenous mutagens, induces p53 protein accumulation and activation, leading to cell cycle arrest during G1/S transition (Huang *et al.*, 1996). High concentrations of nitric oxide (NO), inducing DNA damages, also triggers wild-type p53 protein accumulation and apoptosis (Messmer *et al.*, 1994). In addition, nitric oxide induces death of colon cancer cells through down-regulation of beta-catenin via proteasome-independent degradation (Prevotat *et al.*, 2006). Some report document specific effects of NO in pancreatic cancer. Decker et al. reported that human pancreatic cancer cells engineered to overexpress eNOS show down-regulation of liver metastasis and tumor growth in mice (Decker *et al.*, 2008). Wang et al. established a role of NOS2 in pancreatic cancer growth and metastasis in an animal model. They demonstrated that pancreatic cancer clones expressing low levels of NOS 2 produced tumors in the pancreas which metastasized to the liver, whereas those expressing high levels of NOS 2 did not (Wang *et al.*, 2003).

6. Growth signaling in cancer

Insulin/insulin-like growth factor (IGF) signals play a key role in cancer proliferation and invasion (Bergmann *et al.*, 1995; Furukawa *et al.*, 2005; Kim *et al.*, 2007). Insulin/IGF-I and IGF-II bind to insulin/IGF-I receptors, leading to tyrosine phosphorylation of the cognate

receptors. Insulin receptor substrate (IRS)-1, an adaptor protein, exists mainly in the cytosol, and binds to phosphorylated insulin receptor (IR) and IGF-I receptor (IGF-IR), resulting in the phosphorylation and activation of IRS-1. IRS-1 activates phosphatidylinositol-3 kinase (PI3K), which in turn activates further down-stream components, including Akt/PKB and glycogen synthase kinase (GSK)-3β. Alternatively, phosphorylated and activated IRS-1 can also bind to another adaptor protein, Grb-2, which activates mitogen-activated protein kinase (MAPK), another major insulin/IGF signaling cascade parallel to the PI3K-Akt/PKB pathway (Ito *et al*., 1996; Tanaka and Wands, 1996). IRS-1 protein expression is detected in several types of cancer, including pancreatic cancer, breast cancer, and hepatic cell carcinoma (Asano *et al*., 2005; Chang *et al*., 2002). Thus, insulin/IGF signaling is thought to play a major role in not only 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 that transduces a signal from IR/IGF-IR to both PI3K and MAPK pathways (Asano *et al*., 2005).

Epidermal growth factor (EGF) signaling also plays a key role in cancer proliferation and invasion. EGF binds to EGF receptor (EGFR) and triggers tyrosine phosphorylation of the receptor. Phosphorylated EGFR activates phosphatidylinositol-3-kinase (PI3K), which activates further down-stream components, including Akt. Alternatively, phosphorylated EGFR can also activate the Ras/MEK/ERK pathway, another major EGF signaling cascade parallel to the PI3K/Akt pathway.

In the section to follow, we present our data showing effects of nitric oxide on growth factor signaling.

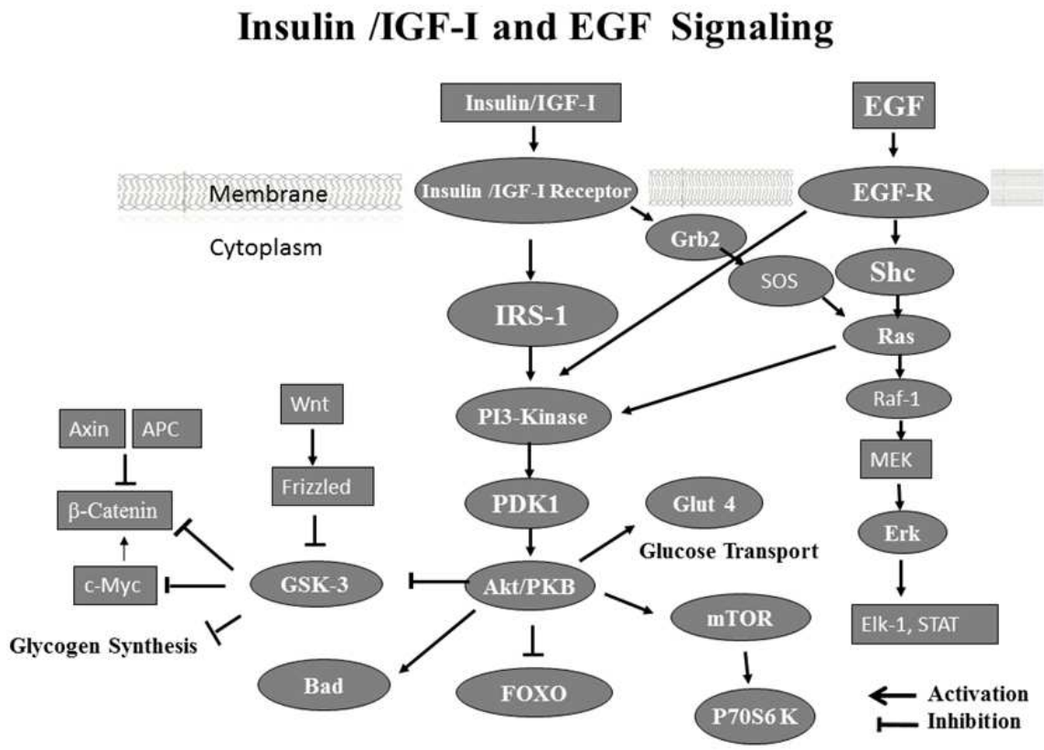

Fig. 1. Insulin/IGF-I and EGF signaling

7. NO regulates growth signaling

7.1 NO regulates insulin signaling in skeletal muscle

Expression of iNOS is elevated in skeletal muscle of patients with type 2 diabetes (Torres *et al.*, 2004) and in high fat diet-induced diabetic mice. Perreault and Marette showed that disruption of the iNOS gene protects against high fat diet-induced insulin resistance in mice (Perreault and Marette, 2001). Furthermore, we demonstrated that disruption of the iNOS gene reverses IRS-1 protein reduction in skeletal muscles of leptin deficient obese mice and NO-donor treatment induces proteasome-dependent IRS-1 degradation in skeletal muscle cells (Sugita *et al.*, 2005). Thus, NO inhibits insulin signaling and is associated with IRS-1 protein degradation, resulting in insulin resistance. This may explain the occurrence of insulin resistance in patients with inflammation or diabetes.

7.2 NO influences insulin/IGF signals in MIAPaCa-2 cells

We examined whether NO influences on insulin/IGF-I signaling in MIAPaCa-2 cells, a pancreatic cancer cell line. Protein expression and phosphorylation were detected by immunoblotting using specific antibodies. SNAP, a NO -donor, inhibited insulin-stimulated tyrosine phosphorylation of IR, IRS-1, the phosphorylation of Akt/PKB at Ser ^{473}, and GSK-3β at Ser ^{9}. In addition, SNAP inhibited IGF-I-stimulated tyrosine phosphorylation of IGF-IR and IRS-1, phosphorylation of Akt/PKB at Ser ^{473}, and GSK-3β at Ser ^{9}. Furthermore, SNAP reduced IRS-1 protein expression, although this did not alter the expression of other IGF signaling proteins, including IGF-IR, Akt/PKB, GSK-3β and Erk 1/2 or of β-actin protein. SNAP induced phosphorylation of Erk 1/2 without stimulation by insulin/IGF-I, and enhanced the insulin/IGF-1-stimulated phosphorylation of Erk 1/2; however, SNAP did not influence Erk 1/2 protein expression in MIAPaCa-2 cells (Figure 2A and B).

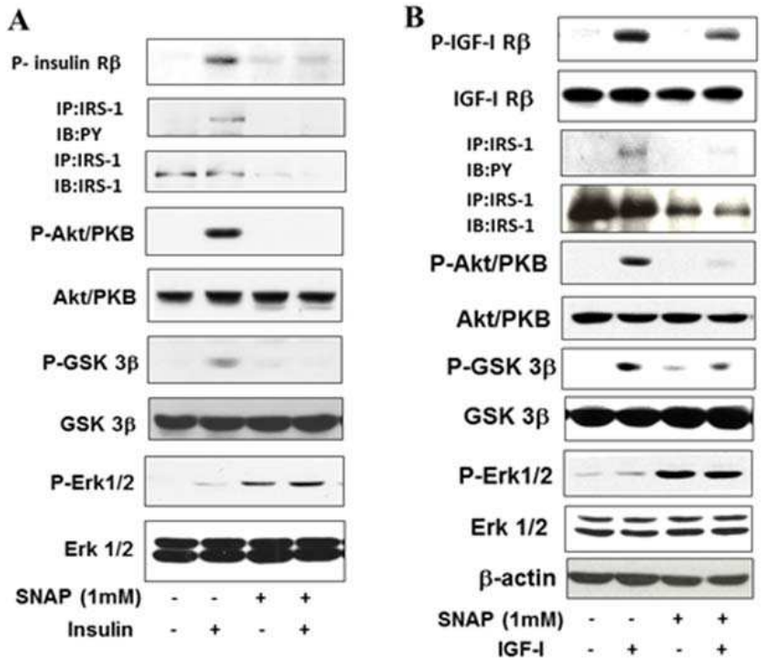

Fig. 2. NO influences IGF signals in MIAPaCa-2 cells

GSNO, a NO-donor, 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 (Figure 3A). The proteasome inhibitor, MG132, completely reversed the reduction of IRS-1 protein expression by NO-donors in MIAPaCa-2 cells. Neither GSNO nor MG132 influenced GSK-3β and β-actin protein expression (Figure 3B). To further investigate IRS-1 protein degradation induced by NO-donor, cDNA constructs of IRS-1 full-length, IRS-1 DM1, IRS-1 DM2, and IRS-1 DM3 were produced and sub-cloned into mammalian expression vectors (Figure 3C). MIAPaCa-2 cells were transfected with these expression vectors. GSNO reduced IRS-1 full-length, IRS-1 DM1, and IRS-1 DM3 protein expression, although GSNO did not alter IRS-1 DM2 and β-actin protein expression (Figure 3D). Ubiquitination of wild-type and mutant IRS-1 was detected by immunoprecipitation using anti-Flag antibody followed by immunoblotting with anti-ubiquitin. SNAP induced the ubiquitination of IRS-1 full-length, IRS-1 DM1, and IRS-1 DM3, but did not induce the ubiquitination of IRS-1 DM2 (Figure 3E). These results indicate that NO-donor is capable of inducing ubiquitination at multiple sites in the carboxy-terminus of the IRS-1 protein.

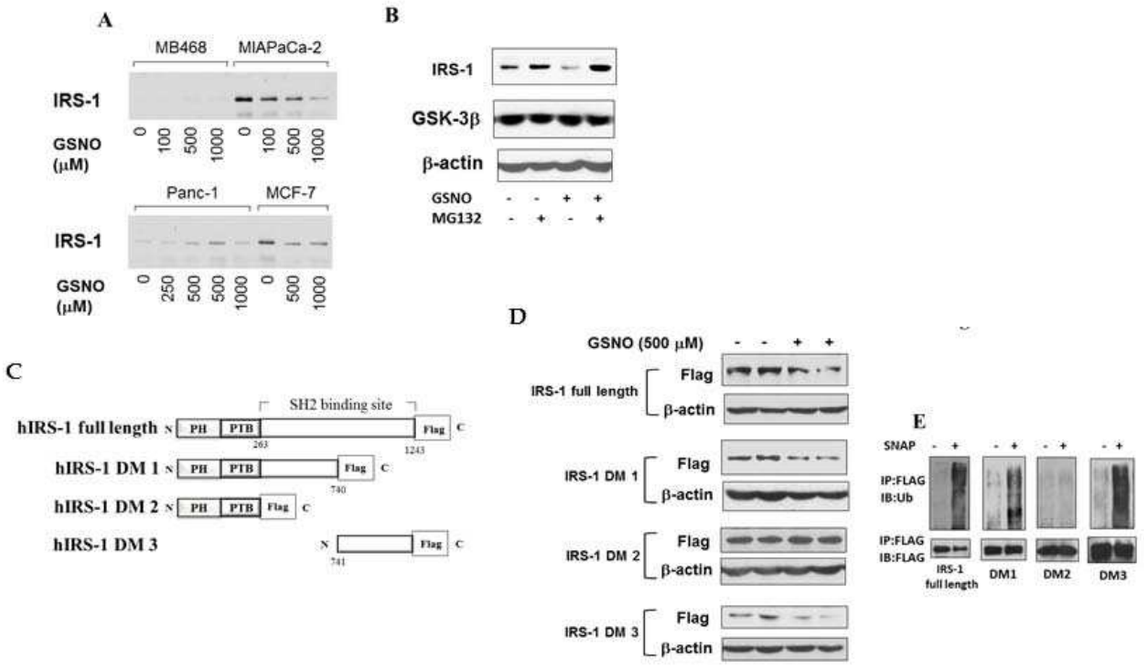

Fig. 3. NO donor downregulates IRS-1 protein expression through proteasome-mediated degradation in MIAPaCa-2 cells

iNOS protein was detected by immunoblotting in Panc-1 cells, a pancreatic cancer -derived cell line. IRS-1 protein expression was significantly increased by 1400 W, an iNOS specific inhibitor, in a dose-dependent manner. Expression of Akt/PKB, β-actin, and Erk 1/2 protein was unaffected by treatment (Figure 4A). GSNO inhibited IRS-1 protein expression, upregulated by 1400W (Figure 4B). Treatment of 1400W enhanced IGF-I-stimulated tyrosine phosphorylation of IRS-1, phosphorylation of Akt/PKB at Ser ^{473}, and GSK-3β at Ser ^{9} in

Panc-1 cells. In contrast, 1400 W did not alter IGF-I-stimulated phosphorylation of Erk 1/2 (Figure 4C). These results indicate that endogenous NO produced by iNOS plays a role in insulin/IGF-I signaling.

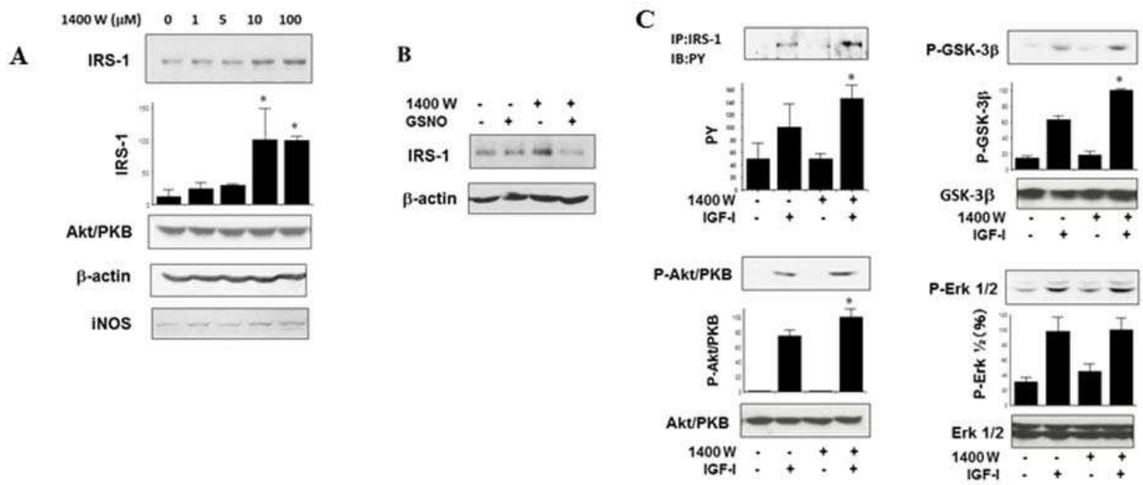

Fig. 4. iNOS inhibitor 1400W upregulates IRS-1 protein expression and IRS-1/Akt pathway in Panc-1 cells, a pancreatic cancer cell line

Mammalian 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 IRS-1 full-length protein or IRS-1 DM2 protein were cloned.

Proliferation of MIAPaCa-2 cells was elevated in a culture medium containing serum or IGF-I, while no proliferation was observed in a culture medium without serum or IGF-I. Proliferation of cells overexpressing full-length IRS-1 was greater than that of vector alone-transefected cells in the culture medium containing 10 % FBS. By contrast, the proliferation of cells expressing IRS-1 DM2 was attenuated compared to cells transfected with full-length IRS-1 or vector alone (Figure 5). The proliferation of cells transfected with full- length- IRS-1 was greater compared to that of vector alone-transfected cells in the culture medium containing 100 nM IGF-I without 10% FBS, while IGF-I-stimulated proliferation of IRS-1 DM2- transfected cell was not observed (Figure 5). GSNO (200 μM) significantly reduced the proliferation of vector alone-, IRS-1 full- length-, and IRS-1 DM2- transfected cells in culture medium containing 10 % FBS or IGF-I. To further investigate the role of iNOS in IGF-I-stimulated proliferation, we evaluated the effects of selective iNOS inhibitor, 1400W, in Panc-1 cells cultured with IGF-I in the absence of FBS. Proliferation in Panc-1 cells was not observed in the presence and absence of 1400W (100 μM), when cultured without serum or IGF-1 (Figure 6A). 1400W significantly enhanced the proliferation of Panc-1 cells when cultured with 10% FBS (Figure 6B). 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 (Figure 6C). These results provide further evidence for the involvement of downregulation of IGF-I signaling in NO-induced inhibition of cancer cell proliferation.

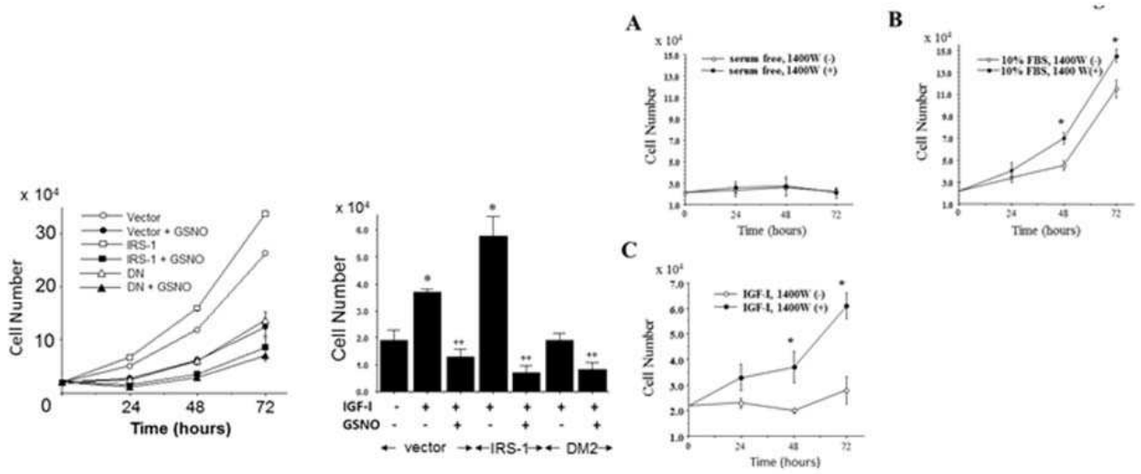

Fig. 5. and 6. NO inhibited the proliferation of cancer cell lines and IRS-1 protein expression was associated with cancer cell proliferation

In vitro invasive potential of MIAPaCa-2 cells and Panc-1 cells was determined using BioCoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA). There was no difference between the invasion of vector alone-, IRS-1 full-length-, and IRS-1 DM2-transfected MIAPaCa-2 cells in the absence of the NO -donor. The addition of 200 μM GSNO markedly reduced invasion in vector alone- and IRS-1 full-length-transfected MIAPaCa-2 cells but did not alter invasion in IRS-1 DM2-transfected MIAPaCa-2 cells (Figure 7A). Invasion in Panc-1 cells incubated with 1400W (5 and 100 μM) was significantly greater than that of untreated cells (Figure 7B).

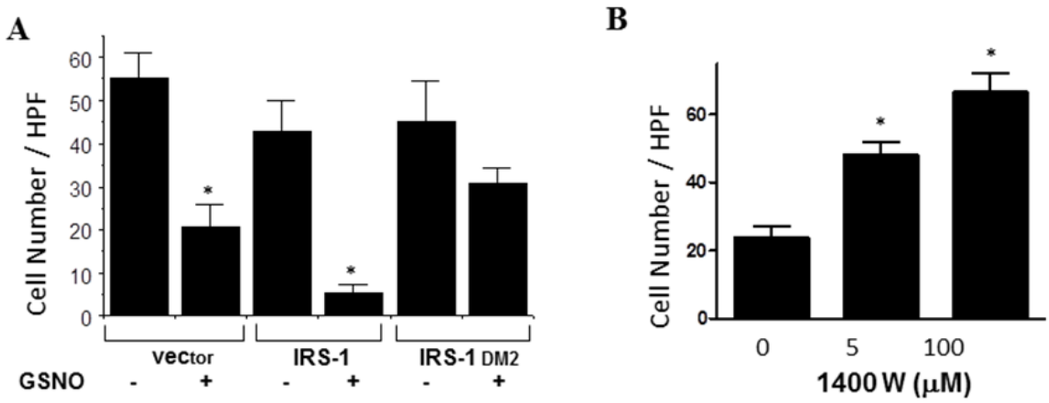

Fig. 7. Sensitivity of NO to invasion was dependent on IGF signaling

NO-donor treatment leads to several effects on insulin/IGF signaling in pancreatic cancer cells. NO-donor treatment reduced IRS-1 protein expression via proteasome-dependent degradation, and inhibited insulin/IGF-I-stimulated phosphorylation of Akt/PKB and GSK-3β, while enhancing phosphorylation of Erk 1/2 in pancreatic cancer cells (Figure 2, 3, 4). NO-donor inhibited IGF-I-induced phosphorylation of Akt/PKB and GSK-3β in MIAPaCa-2 cells transfected with IRS-1 wild-type or vector, but not in cells transfected with a dominant

negative carboxy-terminal deletion mutants (Tanaka and Wands, 1996) of IRS-1. This indicates 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 (Kim *et al.*, 2007; Shi *et al.*, 2007; Tanaka and Wands, 1996). NO donor inhibited IGF-I signaling, proliferation, and invasion in MIAPaCa-2 cells transfected with IRS-1 full-length or vector. In contrast, treatment with a selective iNOS inhibitor upregulated IRS-1 protein expression and insulin/IGF signaling, 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 by exogenous NO, resulting in the downregulation of IGF-I signaling and the inhibition of cancer proliferation and invasion in MIAPaCa-2 and Panc-1 cells (Figure 4, 5, 6).

Furthermore, the carboxy-terminus as the site responsible for IRS-1 protein degradation by NO, which is located in SH2-containing molecule binding site next the phosphotyrosine binding (PTB) domain was detected. The observation of the ubiquitination and degradation of IRS-1 deletion mutants indicates the possibility that there may be at least two sites responsible for NO donor-induced ubiquitination in the IRS-1 protein. These data had been published in 2010 (Sugita *et al.*)

In addition, NO inhibits Akt activity directly through post-translational modification, (Yasukawa *et al.*, 2005), which seems to contribute to NO-induced cancer inhibition.

Furthermore, we confirmed that NO-donors down-regulate EGF-stimulated phosphorylation of EGFR and Akt in colon cancer cells (data not shown).

8. Therapeutic prospects

The usefulness of cancer therapy using NO, including iNOS gene therapy and administration of NO-donor, was recently confirmed in animal models (Adams *et al.*, 2008; Kiziltepe *et al.*, 2007; Wang *et al.*, 2004). Consequently, NO therapy has been focused on, and is currently undergoing clinical evaluation for cancer prevention (Ma *et al.*, 2007). This should leads to clinical trials using NO -donors in the near future. Nitroglycerin, a NO-donor, has long been used as a vasodilating, and the safety of nitroglycerin therapies is well established. Nitroglycerin treatment on non-small cell lung cancer is currently planned as a phase II clinical trial. A promising novel class of drugs, nitric oxide-donating NSAIDs (NO-NSAIDs), has been found to be more active than classical NSAIDs against cancer (Rigas and Williams, 2008). The effects of the NO-donating aspirin derivative, NCX 4040, on three human pancreatic adenocarcinoma cell lines were recently described (Capan-2, MIA PaCa-2 and T3M4)(Rosetti *et al.*, 2006). Clinical trials using NO-donors or NO-donating aspirin derivatives are urgently required.

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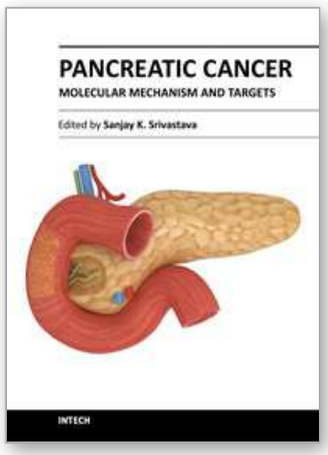

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This book provides the reader with an overall understanding of the biology of pancreatic cancer, hereditary, complex signaling pathways and alternative therapies. The book explains nutrigenomics and epigenetics mechanisms such as DNA methylation, which may explain the etiology or progression of pancreatic cancer. Book also summarizes the molecular control of oncogenic pathways such as K-Ras and KLF4. Since pancreatic cancer metastasizes to vital organs resulting in poor prognosis, special emphasis is given to the mechanism of tumor cell invasion and metastasis. Role of nitric oxide and Syk kinase in tumor metastasis is discussed in detail. Prevention strategies for pancreatic cancer are also described. The molecular mechanisms of the anti-cancer effects of curcumin, benzyl isothiocyante and vitamin D are discussed in detail. Furthermore, this book covers the basic mechanisms of resistance of pancreatic cancer to chemotherapy drugs such as gemcitabine and 5-flourouracil.

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