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Inhibitory Effects of Nitric Oxide on Invasion of Human Cancer Cells

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Abstract

Hypoxia increased the ability of two human cancer cell lines, PC-3M and T24, to invade through Matrigel, while sodium nitroprusside (SNP), a nitric oxide (NO) donor, strongly inhibited this invasion, along with down-regulating HIF-1 α . SNP also inhibited the function of mitochondria in PC-3M cells, and mitochondrion-specific inhibitors reduced the invasion of these cells. Furthermore, knocking down either Rieske iron-sulfur protein (Fe-S) of mitochondrial complex III or HIF-1 β in these cells decreased their invasive potential. Our findings suggest that NO inhibits invasion of cancer cells via both inhibition of HIF-1, and impairment of mitochondria.

Keywords

nitric oxide (NO); sodium nitroprusside (SNP); mitochondria; Hypoxia Inducible Factor 1 (HIF-1); invasion

Introduction

Nitric oxide (NO) is a highly reactive gas molecule that can either function as a beneficial physiological agent utilized for essential functions such as vasodilation, ischemia protection, and neurotransmission, or as a pathological agent that causes or exacerbates diseases, such as septic shock, cardiac hypertrophy and diseases of the central nervous system. Whether NO is helpful or harmful depends on a variety of factors, such as the cellular environment in which NO is released, and the dose released [1,2]. Recently, a number of studies have shown that levels of total nitric oxide synthase activity are significantly elevated in many tumors, providing impetus for investigations on the role of NO in tumor growth, metastasis, and angiogenesis

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[3,4,5]. However, in contrast, it has been reported that NO at low concentrations inhibits cancer cell invasion via a cGMP-dependent pathway [6]. Furthermore, a recent study performed by Le and coworkers showed that nitric oxide synthase II suppresses the growth and metastasis of human cancer [7].

HIF-1 activates the transcription of many genes encoding proteins involved in angiogenesis, glucose metabolism, cell proliferation/survival and invasion/metastasis. HIF-1 α is overexpressed in many human cancers as a result of intratumoral hypoxia as well as genetic alterations, and significant associations between HIF-1 α overexpression and patient mortality have been observed in a number of human cancers [8]. HIF-1 α , therefore, represents a novel potential target for cancer therapy. In this regard, it is of interest that NO generated from various NO-donors has been shown to inhibit the expression of HIF-1 α , via activating the prolyl hydroxylase-domain proteins [9,10].

NO also impairs the function of mitochondria by inhibiting the mitochondrial electron transport chain at multiple sites [11]. Damage to mitochondria is known to alter the expression of nucleus-encoded genes [12]. On one hand, it has been reported that damage to mitochondria can induce tumor progression and invasion [13]. On the other hand, chemotherapeutic drugs for cancer that destroy mitochondria can induce apoptosis in tumor cells [14].

It is conceivable that NO donors may serve as cancer chemotherapeutic agents. In the current study, we tested the potential inhibitory effect of NO on cancer cell invasion, investigated the possible mechanisms involved, and established a role for mitochondria as well as HIF-1 in this process.

Materials and Methods

Chemicals

SNP, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), oligomycin, myxothiazol, and antimycin A were purchased from Sigma.

Measurement of Oxygen Consumption

The cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) in the presence or absence of 100 μ M of SNP for 24 hours before trypsinization. 2.5×10^7 of the cells were then suspended in 2 ml of cell respiration buffer (118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1 mM CaCl_2 , 20 mM glucose, and 25 mM Hepes, pH 7.5) and the rate of endogenous oxygen consumption of the cells were measured with a fiberoptic spectrofluorimeter at room temperature. The partial pressure of O_2 in the buffer was continuously recorded by a fiber-optic oxygen sensor (Foxy AI-300; Ocean Optics, Dunedin, FL).

RNA Interference

Retroviral vectors expressing small hairpin RNAs for Fe-S (pSIREN-siFe-S#1) and Drosophila HIF-1 α (pSIREN-D-HIF) were kind gifts from Dr. N. S. Chandel. Virus preparation and infection of cells were carried out as described [15]. The infected cells were selected with puromycin and cultured in RPMI 1640 containing pyruvate (1 mM). A RNA hairpin oligo designed to target the 5'-CCCCGAAAUGACAUCAGAU-3' sequence of HIF-1 β and a scrambled sequence (UUCUCCGAACGUGUCACGU), were cloned into the Bbs I and EcoR I restriction sites of pRVGP (a retroviral vector kindly provided by Dr. S. T. Smale,) and were designated as pRVGP-siHIF-1 β and pRVGP-SCX, respectively. The virus preparation and infection of the cells were performed as described [16]. The infected cells were selected with puromycin (3 μ g/ml) in RPMI 1640.

Cell Culture and Invasion Assay

PC-3M and T24 cells were cultured in RPMI 1640 (Invitrogen) containing 10% FBS (Omega), fungizone, and penicillin-streptomycin (Invitrogen) at 37 °C and 5% CO₂. The invasion assays were performed using 24-well Matrigel invasion chambers (BD Biosciences), with the protocol provided by the manufacturer. For PC-3M cells, 1.5×10^4 cells were seeded in each chamber containing RPMI 1640 supplemented with 10% FBS, and for T24 cells, 1.25×10^4 cells were seeded in each chamber containing RPMI 1640 supplemented with 5% FBS. For hypoxia treatment, the chambers were placed in an airtight cell culture incubator equilibrated and maintained in 1% O₂, 5% CO₂ and 94% N₂ for 24 hours (Model 3130, Thermo). The cells were further cultured under normoxic condition for 24 hours before measurement of cell invasion.

Immunoblotting

Whole-cell lysates were prepared using lysis buffer (25 mM Hepes, PH 7.4, 1 mM EDTA, 400 mM NaCl, 1 mM DTT, and 1% Triton X-100). The cell lysates (50 µg) were resolved on a 8% (for HIF-1 α and -1 β) or 10% SDS polyacrylamide gel (for Fe-S). The HIF-1 α protein was detected using a 1:200 dilution of a HIF-1 α antibody (BD Transduction Laboratories), and HIF-1 β was detected using a 1:1000 dilution of HIF-1 β antibody made in our laboratory. Fe-S was detected using a 1:500 dilution of a Fe-S antibody (Molecular Probes). The β -actin was detected using a 1:1000 dilution of a β -actin antibody (clone AC-15, Sigma-Aldrich, St. Louis, MO) served as loading controls.

Cell proliferation rate

PC-3M and T24 cells (1×10^5) were plated into each well of 12-well culture plates and cultured under 1% O₂ or 21% O₂ at 37 °C for 24 hours in the presence or absence of SNP, or SNP and PTIO. For the treatment of cells with the mitochondrion-specific inhibitors, the cells were pre-treated with these chemicals for 2 hours, then washed with PBS before the hypoxia treatment. The concentrations of these chemicals are the same as used in invasion assay. The cells were then cultured under 21% O₂ for further 24 hours before trypsinization. The cells were then suspended in an equal volume of medium and subjected to counting with a hemocytometer.

Transient Transfections and Reporter Gene Assay

A luciferase reporter driven by six concatenated hypoxia-responsive element (HRE) and a minimal SV-40 promoter (6 X HRE-SV40-luc) [17] was transfected into PC-3M and T24 cells cultured in 12 well-plates using the FuGene 6 (Roche Molecular Biochemicals, Indianapolis, IN.) transfection reagent. After 24 h, some of the plates were exposed to hypoxia (1% O₂, 94% N₂ and 5% CO₂) for 24 h. Cells were then harvested and lysed in Passive lysis buffer (Promega). Luciferase activities were measured using the Dual-luciferase system (Promega) with the protocol recommended by the manufacturer. All transfection experiments were performed in triplicate.

Results

Inhibitory Effect of SNP, a NO Donor, on Cancer Cell Invasion

Given the important role of HIF-1 in cancer progression and the inhibitory effect of NO on the activity of HIF-1, we hypothesized that NO would inhibit cancer cell invasion. To test this, we performed *in vitro* invasion assays using two highly invasive human cancer cell lines, the prostate cancer cell line PC-3M, and the bladder cancer cell line T24. As shown in Fig. 1A, hypoxia treatment significantly increased the invasive potential of both cell lines, and sodium nitroprusside (SNP), a NO donor, strongly inhibited the invasion of the cells. Under our experimental conditions, SNP did not show any cytotoxicity to T24 and PC-3M cells as

demonstrated by measuring their cell proliferation rates (Fig. 1 in electronic supplemental materials), excluding the possibility that its inhibitory effect on invasion resulted from any inhibitory effect on cell proliferation under these circumstances. When the cells were treated with PTIO, a scavenger of NO, and SNP simultaneously, the inhibitory effect of SNP on invasion was largely negated (Fig. 1B), indicating that the inhibitory effect of SNP on invasion is dependent on the NO released from this compound.

SNP Inhibited the Expression of HIF-1 α in the Cancer Cells

We carried out immunoblotting assay to investigate whether SNP inhibits the expression of HIF-1 α in the above cells under our experimental conditions. As shown in Fig. 2, SNP inhibited the expression of HIF-1 α in PC-3M and T24 cells at 100 μ M. These data suggest that SNP inhibits cancer cell invasion at least partially via inhibiting the expression of HIF-1 α .

Effect of knocking down HIF-1 on Cancer Cell Invasion

It has been reported that HIF-1 promotes human colon carcinoma cells invasion [18]. To investigate whether HIF-1 is generally involved in cancer cell invasion, and to provide further evidence for our hypothesis that the inhibitory effect of NO on invasion is mediated by a HIF-1-related mechanism, we knocked down HIF-1 β using shRNA in PC-3M and T24 cells. Given that HIF-1 is composed of two subunits, HIF-1 α and HIF-1 β , deficiency in either should diminish the function of HIF-1. As shown in Fig. 3A, the HIF-1 β protein was down-regulated in cells infected with a retroviral vector expressing a shRNA targeting HIF-1 β , compared with cells infected by the retroviral vector expressing a scrambled RNA sequence. The hypoxic induction of a luciferase reporter gene driven by six concatenated hypoxia-responsive element (HRE) and a minimal SV-40 promoter was inhibited in the cells deficient for HIF-1 β , indicating that the transcriptional activity of HIF-1 was abrogated in these cells (Fig. 3B). The activities of the reporter were inhibited even in the cells deficient for HIF-1 β cultured under normoxia, indicating that HIF-1 is still active in the two wild-type cell lines under normoxia. The invasive potential of the cells deficient for HIF-1 β was reduced, and SNP further decreased the invasion of these cells, indicating that HIF-1 plays a role in cancer cell invasion, and that SNP inhibits cancer cell invasion, at least partially, but not only, via a HIF-1 mediated mechanism (Fig. 3C and D).

Role of Mitochondria in Cancer Cell Invasion

Mitochondria have been reported to be involved in cell migration and cancer progression [13,19], and NO has been reported to be an inhibitor of mitochondrial function [11]. To explore the role of mitochondria in the invasive process, we tested the effects of mitochondrion-specific inhibitors on cell invasion. As shown in Fig. 4A, antimycin A, oligomycin and myxothiazol significantly inhibited the invasion of PC-3M cells. We did not observe any inhibition of proliferation or induction of apoptosis by any of these compounds for PC-3M cells under these experimental conditions (data not shown). Since mitochondria has been reported to be involved in regulation of the expression of HIF-1 α [15,20,21,22,23], it is important to note that these compounds did not inhibit expression of HIF-1 α in the cells under these experimental conditions (Fig. 4B), indicating that the inhibitory effects of these chemicals on invasion are not mediated by the HIF-1 pathway, and thus, that the effects of HIF-1 and mitochondria on invasion represent independent mechanisms. To further investigate the role of mitochondria in cancer cell invasion, we knocked down Fe-S, a subunit of complex III of the mitochondrion in PC-3M and T24 cells, using shRNA. As shown in Fig. 5A, Fe-S protein levels were down-regulated in cells infected with pSIREN-siFe-S#1, a retroviral vector expressing a shRNA targeting Fe-S. The invasive activities of the cells deficient for Fe-S were significantly reduced as compared with the control cells infected with pSIREN-D-HIF, a retroviral vector expressing a shRNA targeting *Drosophila* HIF-1 α that does not affect human HIF-1 α expression (Fig. 5B).

To further confirm the notion that the effects of mitochondria and HIF-1 are independent in this study, we tested the hypoxic induction of expression of HIF-1 α in the cells defective in mitochondrial function and found that the expression levels of HIF-1 α were comparable in cells with and without functional mitochondria under our hypoxic condition (1% O₂) (Fig. 5C). In summary, these data has provided direct evidence that functional mitochondria are required for cancer cell invasion.

Inhibitory Effect of SNP on Mitochondria

It is known that NO is an inhibitor of mitochondria. To confirm that SNP inhibited the function of mitochondria in the cells we used in this study, we treated the cells with SNP or solvent for 24 hours, and measured the rate of oxygen consumption of the cells. As shown in Fig. 6, SNP attenuated cell respiration of PC-3M and T24 cells, consistent with the notion that the inhibition of cell invasion by SNP is at least partially mediated by impairment of mitochondrial function.

Discussion

Solid tumors are generally in a hypoxic state because of poor vascularity. Hypoxia is associated with resistance of cancer cells to radiation therapy and chemotherapy, metastasis and poor outcome of cancers [24]. During invasion, cancer cells produce proteases, such as the urokinase-type plasminogen-activator receptor (uPAR) and matrix metalloproteinase-2 (MMP2), which digest the basement membrane/extracellular matrix (ECM), break cell-cell contacts, and digest the basement membrane. MMP-2 and uPAR have been shown to be target genes of HIF-1 [8].

It has been shown that NO inhibits the expression of HIF-1 α via activating HIF-1-prolyl hydroxylases [9,10]. In this study, we first tested our hypothesis that NO can inhibit invasion via inhibiting HIF-1. We found that SNP did indeed strongly inhibit invasion by two highly invasive human cancer cell lines in culture, accompanied by down-regulating the HIF-1 α protein. We also found that the invasive ability of the cells deficient for HIF-1 β was compromised, suggesting that the inhibitory effect of NO on invasion may be ascribed to its inhibitory activity against HIF-1. Our finding is consistent with the study by Krishnamachary and coworkers [18], in which they knocked down HIF-1 α in a human colon carcinoma cell line, HCT116, and observed a decreased invasive potential for cells deficient in HIF-1 α . Postovit and coworkers [6] found that at low concentrations (1 pM-0.1 μ M), several NO donors inhibited MDA-MB-231 cell invasion via a cGMP-dependent pathway. In our study, we observed a much stronger inhibitory effect of SNP at relatively high concentration (100 μ M) on invasion of human cancer cell lines. Our results also suggest that the inhibitory effect of SNP on invasion is not restrained to a certain specific type of cancer cell.

We doubted that the inhibitory effect of NO on invasion is mediated only by a HIF-1-related mechanism, because several of our findings could not be fully explained in this way. Firstly, though SNP did not affect the protein level of HIF-1 α in the cells cultured under normoxia, it still inhibited the invasion of the cells under these conditions. Secondly, the inhibitory effect on invasion by elimination of HIF-1 β by shRNA in the cells was much less than that of treatment with SNP. Finally, the invasive potentials of PC-3M and T24 cells deficient for HIF-1 β were further inhibited by SNP.

Mitochondria participate in numerous functions in the cell, including the production of energy and the generation of movement [19,25,26,27]. NO impairs the function of mitochondria by inhibiting the mitochondrial electron transport chain at multiple sites [11]. We therefore hypothesized that NO might inhibit invasion via impairing the function of mitochondria. We performed three experiments to address this hypothesis. Firstly, we treated PC-3M cells with mitochondrion-specific inhibitors, and found that they significantly inhibited the invasion of

the cells. Interestingly, the inhibitors did not inhibit expression of HIF-1 α under this experimental condition, indicating that the effects of HIF-1 and mitochondria on invasion represent independent mechanisms. Since other investigators have proposed that the hypoxia-induced HIF-1 α expression is dependent on functional mitochondria [15,20,21,22,23], we further investigated if the hypoxia-induced expression of HIF-1 α is reduced in cells knocked down for Fe-S, and found that HIF-1 α is expressed normally in these cells cultured under 1% O₂. We believe that the discrepancy between our results and those cited above results from the more severe hypoxia conditions used in our experiments (1.0% O₂ vs 1.5% O₂), and/or from the use of different cell types by other investigators and ourselves. This interpretation is supported by the observations of Vaux and coworkers in which they show that HIF-1 α is expressed normally in a variety of mutant cells that lacked mitochondria DNA (rho0 cells) [28], and also by our observations that reduction in mitochondrial function dose lead to a diminution in HIF-1 α expression in PC-3M cells cultured under less severe hypoxic condition (3% O₂) (Fig. 2 in electronic supplemental materials). Secondly, we measured the oxygen consumption of the cells treated with SNP and found that SNP inhibited the respiration rate of the T24 and PC-3M lines, thus confirming that this NO donor negatively affects mitochondrial function. Finally, we found that the invasive ability of the cells deficient for Fe-S were significantly reduced.

At present, we do not know why impairment of function of mitochondria reduces the invasive potential of the cells. We speculate that two mechanisms may be involved: firstly, impairment of function of mitochondria may reduce the energy supply to such a degree that it is insufficient for cell invasion; secondly, the impairment of mitochondrial function may trigger a change in the expression patterns of proteins encoded by nuclear DNA that play roles in cell invasion via mitochondria-to-nuclear communication, thereby resulting in inhibition of invasion.

In summary, our findings established a strong inhibitory effect of SNP on cancer cell invasion, and a novel role for mitochondria in cancer cell invasion. Our data also suggest that NO inhibition of invasion of cancer cells occurs via at least two mechanisms: impairment of mitochondria, and inhibition of HIF-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Inhibitory effect of SNP on invasion of human cancer cells. A, the human bladder cancer cell line, T24, and the human prostate cancer cell line, PC-3M, were seeded into Matrigel-coated invasion chambers and cultured under hypoxia (1% O₂) or normoxia (21% O₂) for 24 hours in the presence or absence of SNP at the indicated concentrations. The cells were then cultured under normoxia for a further 24 hours before counting the invaded cells on the underside of the filters. For each condition, data are presented as the mean cell number per view field, from fifteen fields in three chambers, along with the standard deviation (SD). B, the invasion assay was performed as described in A, except for one group of cells were cultured in the presence of SNP and PTIO.

Figure 2. Immunoblot for HIF-1α. The cells were treated with hypoxia or normoxia for 6 hours in the presence or absence of SNP at the indicated concentrations. Proteins were extracted from the whole cells and subjected to immunoblot analysis using an anti- HIF-1α monoclonal antibody. P- HIF-1α: phosphorylated HIF-1α.

a **b** **c** **d** **Figure 3.**

Effect of depleting HIF-1 on invasion of human cancer cells. A, whole cell extracts were prepared from the cells infected with either (pRVGP-SCX), a retroviral vector expressing a short hairpin RNA (shRNA) for a scrambled sequence (SCX), or (pRVGP-siHIF-1β), a retroviral vector expressing a shRNA targeting HIF-1β, and subjected to immunoblot analysis

for HIF-1 β . 0.25 μ g of a luciferase reporter gene driven by six concatenated hypoxia-responsive element (HRE) and a minimal SV-40 promoter (6 X HRE-SV40-luc) together with 25 ng of the Renilla luciferase reporter driven by the Herpes simplex virus thymidine kinase promoter were transfected into PC-3M and T24 cells cultured in 12 well-plates using the FuGene 6 transfection reagent. After 24 h, some of the plates were exposed to hypoxia for 24 hours. All transfection experiments were performed in triplicate and the firefly luciferase activities were normalized to the *Renilla* luciferase activities. RLU, relative luciferase units. C and D, invasion assays were performed for the infected cells cultured under hypoxia (1% O₂) in the presence or absence of SNP. ★, ▲: P<0.05.

a **b** **Figure 4.**

Inhibitory effects of mitochondrion-specific inhibitors on invasion of human cancer cells. A, PC-3M cells were treated with each of the mitochondrion-specific inhibitors at the indicated concentrations for 2 hours, and then seeded into the invasion chambers for the invasion assay as described in Fig 1. B, the cells were treated with the indicated compounds for 2 hours and then cultured under hypoxia (1% O₂) or normoxia (21% O₂) for 6 hours. Proteins were then extracted from the whole cells, and aliquots (50 μg) were loaded on SDS polyacrylamide gel and subjected to immunoblot assay for HIF-1α. P- HIF-1α: phosphorylated HIF-1α. * : 35 μg of proteins from PC-3M cells were loaded.

Figure 5.

Effect of inhibiting the respiratory chain of mitochondria on the invasion of human cancer cells. A, the cells were infected with either pSIREN-D-HIF, a retroviral vector expressing a shRNA for Drosophila HIF-1 α , as a control, or pSIREN-siFe-S#1, a retroviral vector expressing a shRNA targeting Fe-S. Proteins were extracted from the whole infected cells and subjected to immunoblot assay for Fe-S. B, the invasion assay was performed for these cells cultured under normoxia (21% O₂). C, proteins were extracted from the infected cells cultured under normoxia or hypoxia for 6 hours, and subjected to immunoblot for HIF-1 α .

Figure 6.

SNP inhibits cell respiration. The cells were treated with 100 μ M SNP for 24 hours, and then trypsinized and suspended in cell respiration buffer. The O₂ consumption of the cells was continuously recorded.