Oxygen-mediated Regulation of Tumor Cell Invasiveness

INVOLVEMENT OF A NITRIC OXIDE SIGNALING PATHWAY*

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Tumor hypoxia is associated with a poor prognosis for patients with various cancers, often resulting in an increase in metastasis. Moreover, exposure to hypoxia increases the ability of breast carcinoma cells to invade the extracellular matrix, an important aspect of metastasis. Here, we demonstrate that the hypoxic up-regulation of invasiveness is linked to reduced nitric oxide signaling. Incubation of human breast carcinoma cells in 0.5% versus 20% oxygen increased their in vitro invasiveness and their expression of the urokinase receptor, an invasion-associated molecule. These effects of hypoxia were inhibited by nitric oxide mimetic drugs; and in a manner similar to hypoxia, pharmacological inhibition of nitric oxide synthesis increased urokinase receptor expression. The nitric oxide signaling pathway involves activation of soluble guanylyl cyclase (sGC) and the subsequent activation of protein kinase G (PKG). Culture of tumor cells under hypoxic conditions (0.5% versus 20% oxygen) resulted in lower cGMP levels, an effect that could be prevented by incubation with glyceryl trinitrate. Inhibition of sGC activity with a selective blocker or with the heme biosynthesis inhibitor desferrioxamine increased urokinase receptor expression. These compounds also prevented the glyceryl trinitrate-mediated suppression of urokinase receptor expression in cells incubated under hypoxic conditions. In contrast, direct activation of PKG using 8-bromo-cGMP prevented the hypoxia- and desferrioxamine-induced increases in urokinase receptor expression as well as the hypoxia-mediated enhanced invasiveness. Further involvement of PKG in the regulation of invasion-associated phenotypes was established using a selective PKG inhibitor, which alone increased urokinase receptor expression. These findings reveal that an important mechanism by which hypoxia increases tumor cell invasiveness (and possibly metastasis) requires inhibition of the nitric oxide signaling pathway involving sGC and PKG activation.

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Hypoxia in cancers is associated with resistance to therapy and with increased tumor growth and metastatic potential. Several studies have demonstrated that tumor cells exposed to hypoxia exhibit reduced sensitivity to radiation and drug therapy (1–3), increased ability to invade the extracellular matrix in vitro (4, 5), and greater in vivo metastatic potential (6, 7). Exposure of human MDA-MB-231 breast carcinoma cells to hypoxia enhances their ability to invade the extracellular matrix (Matrigel), and this effect of hypoxia is linked to increased expression of the cell-surface urokinase plasminogen activator receptor (uPAR) (4). Furthermore, hypoxia has been shown to increase metastasis of human melanoma cells transplanted into nude mice by up-regulating uPAR expression (8).

Recently, it was shown that a causal link between hypoxia and the acquisition of resistance to chemotherapeutic agents is a reduction in the production of endogenous nitric oxide (NO) by tumor cells (2). In that study, the increase in drug resistance caused by hypoxia was prevented by low concentrations of NO-mimetic drugs; and in a manner similar to hypoxia, pharmacological inhibition of endogenous NO production with an NO synthase inhibitor led to a drug resistance phenotype. Those findings suggested that NO may play a function in the regulation of tumor cell adaptive responses to alterations in local oxygenation levels.

Nitric oxide is produced endogenously by the enzyme NO synthase (9), and has been implicated in several biological processes such as gene regulation. For example, NO has been shown to activate AP-1 (activator protein-1)-regulated genes via a pathway dependent on cGMP production (10). Nitric oxide also modulates hypoxic gene expression. Studies have revealed that NO inhibits the hypoxic induction of erythropoietin, vascular endothelial growth factor, and hypoxia-inducible factor-1 (HIF-1) (11–13).

Soluble guanylyl cyclase (sGC) is a well characterized receptor for NO. This heterodimeric protein catalyzes the conversion of GTP to cGMP. Nitric oxide binds to the heme moiety of sGC, thereby inducing conformational changes that result in sGC activation. cGMP is, in turn, a second messenger that amplifies NO signals to downstream effectors (14). Elevated levels of cGMP have been negatively correlated with vascular smooth muscle growth and have been shown to prevent platelet aggregation as well as the adherence of neutrophils to endothelial...
cells (15–17). Furthermore, there is also evidence that cGMP can prevent the hypoxic up-regulation of vascular endothelial growth factor expression (12).

Several proteins interact with cGMP and potentially regulate gene expression and cell phenotype at various levels of the signaling cascade. These include protein kinase G (PKG), cGMP-activated phosphodiesterases, and cGMP-gated ion channels. Of these, it is thought that PKG is responsible for the majority of the cellular effects of cGMP. PKG is a serine/threonine kinase that is activated following cGMP binding (18). Upon activation, PKG phosphorylates many intracellular targets, often resulting in alterations in gene expression. Based on the previous knowledge that NO plays a role in the regulation of cellular adaptive responses to hypoxia and given the importance of cGMP-dependent signaling in the actions of NO, we sought to investigate the role of sGC, cGMP, and PKG in the NO-mediated regulation of tumor cell invasion.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glyceryl trinitrate (GTN; Sabex, Boucherville, Quebec, Canada) and sodium nitroprusside (SNP; Sigma) were used as NO-mimetic drugs. N-Monomethyl-L-arginine (L-NMMA; Calbiochem-Novabiochem) was used to inhibit endogenous NO production. 3-Isobutyl-1-methylxanthine (Sigma) was used to inhibit phosphodiesterase activity. Desferrioxamine mesylate (DFO; Sigma) was used as an iron chelator and inhibitor of heme biosynthesis. 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Sigma) was used to selectively inhibit sGC. 8-Bromo-cGMP (8-Br-cGMP; Sigma) was used to activate PKG, and KT5823 (Calbiochem) was used to selectively inhibit PKG.

**Cells**—The MDA-MB-231 cell line was maintained in monolayer culture in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen) in a standard Sanyo CO2 incubator (5% CO2 in air at 37°C; Esbe Scientific, Markham, Ontario, Canada). This is a metastatic breast cancer cell line that was initially isolated in 1973 from a pleural effusion obtained from a 51-year-old patient (19).

**Culture Conditions**—To establish hypoxic conditions, cells were placed in airtight chambers (BellCo Biotechnology, Vineland, NJ) that were flushed with a gas mixture of 5% CO2 and 95% N2. Oxygen FIG. 1. Effect of GTN and SNP on the hypoxic up-regulation of in vitro invasion by MDA-MB-231 cells. NO-mimetic drugs were added to the cells at the beginning of the 24-h assay, and the invasion index for each treatment was calculated after counting the cells that penetrated the membrane. Bars represent the mean normalized invasion indices ± S.D. *, significantly different from the invasion index of untreated cells incubated in 20% O2 (n = 6). p values are indicated under “Results.”

**FIG. 2.** uPAR expression in MDA-MB-231 breast cancer cells. Shown are the results from Western blot analysis of uPAR protein expression in cells incubated in 1% O2 in the presence of various concentrations of GTN (n = 3) (A) or in 20% O2 in the absence or presence of the NO synthase antagonist L-NMMA (0.5 μM) alone or in combination with GTN (0.1 μM) (n = 6) (B). The effect of GTN (1 μM) on uPAR mRNA levels was also examined by Northern blot analysis of cells cultured under conditions similar to those for the Western blots (n = 3 and 6, respectively) (C and D). In all cases, GTN was added at the beginning of the 24-h incubation period. 18S rRNA was used to assess uniformity of RNA loading in the Northern blots. Bars represent the mean relative densities ± S.D. *, statistically significant differences compared with control (20% O2) values. All p values are indicated under “Results.”
**Figure 3. Effect of hypoxia on sGC activity and expression in MDA-MB-231 breast carcinoma cells.** A, total cellular cGMP accumulated in MDA-MB-231 breast carcinoma cells cultured for 6 h in 20 or 0.5% O2 in the presence or absence of GTN (1 μM). cGMP levels were measured and normalized to total protein levels. Values represent mean accumulated cGMP ± S.D. (n = 3). B, Western blot analysis of sGC expression in cells incubated for 24 h in 20 or 0.5% O2 (n = 3). Bars represent the mean densities ± S.D.*, statistically significant differences compared with control (20% O2) density values, p values for each condition are indicated under “Results.”

In **In Vitro Invasion Assays**—To determine the effect of hypoxia and the NO signaling pathway on the invasion of MDA-MB-231 cells, we used a previously described assay (4) that employs reconstituted basement membrane (Matrigel®, Collaborative Biomedical Products, Bedford, MA) as the substrate for invasion. Briefly, Costar Transwell® plastic inserts with a 6.5-mm diameter polycarbonate membrane (8-μm pore; Corning Costar Corp., Cambridge, MA) were inserted into the wells of a 24-well tissue culture plate, and allowed to air-dry for ~12 h. After reconstituting the membrane with serum-free medium, 5.0 × 104 cells in serum-containing medium were added to the insert. Following a 24-h incubation under either hypoxic or standard conditions, the invasion was assayed by counting using a microscope, the total number of stained cells on the underside of the polycarbonate membranes. In a pilot study, we determined that the rate of MDA-MB-231 cell proliferation is identical at 1% versus 20% O2 for at least 48 h, thereby indicating that differences in cell numbers on the membranes at the end of the invasion assay are reflective of altered invasive ability alone.

**Northern Blot Analysis**—Following incubation, total cellular RNA from cells was isolated using a Gentra Purescript® RNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). The isolated RNA was subsequently separated by electrophoresis, transferred to a charged nylon membrane (Micron Separations, Westborough, MA), and fixed with ultraviolet radiation using a UV cross-linker (Bio-Rad). The membranes were prehybridized at 42 °C in a hybridization incubator for ~1 h using prewarmed ULTRAhyb® hybridization buffer (Ambion Inc., Austin, TX). They were then hybridized overnight at 42 °C with a cDNA probe that was cloned in a Bluescript plasmid vector and labeled with [32P]dCTP using an Amersham Biosciences Oligolabelling kit. Following serial washes, the membranes were used to expose Kodak X-Omat film. After 1–4 days, the film was developed and analyzed. The density of the rRNA bands was used to normalize the amount of total RNA loaded in each well.

**Measurement of Guanylyl Cyclase Activity**—To determine sGC activity, cellular cGMP levels were measured using a commercially available enzyme-linked immunosorbent assay (STI-Signal Transduction Products, San Clemente, CA). Briefly, cells were cultured for 6 h in 20% or 0.5% O2 in the presence or absence of GTN (1 μM). 3-Isobutyl-1-methyloxanthine (500 μM) was included in the culture medium to inhibit phosphodiesterase activity, thereby allowing for a measurable accumulation of cGMP. Cells were subsequently extracted over ice in 1 ml of 6% trichloroacetic acid (BDH Laboratory Supplies, Poole, England). The homogenate was then centrifuged at 13,000 × g for 10 min. The supernatant fraction was then extracted five times with 2 ml of water-saturated diethyl ether (BDH Laboratory Supplies). The cGMP contained in this fraction was subsequently acetylated and measured using the enzyme-linked immunosorbent assay kit.

**Western Blot Analysis of uPAR and sGC**—Following incubation, cells were lysed in 40 mM HEPES (pH 7.2), 100 mM NaCl, 0.1 mM EDTA (pH 8.0), 0.2% Triton X-100, 1 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride. The lysates were homogenized, followed by DNA shearing (10 times with a 25-gauge needle), boiling (5 min), and centrifugation (14,000 × g for 15 min). The supernatant was collected and stored at ~80 °C until used. Samples were subjected to SDS-PAGE, and the resolved proteins were transferred onto an Immobilon-P membrane (Millipore Corp., Bedford, MA) using a wet transfer apparatus (Bio-Rad). The membranes were blocked overnight at 4 °C in a solution containing 1% phosphate-buffered saline and 0.01% Tween 20 (PBS-T) and 5% dry milk powder. The blots were subsequently incubated for 1.5 h with a monoclonal anti-uPAR antibody (2 μg/ml; monoclonal antibody 3937, American Diagnostica Inc., Greenwich, CT) or polyclonal anti-sGC antiserum (0.5 μg/ml; Cayman Chemical Co., Ann Arbor, MI), followed by six 5-min washes with PBS-T. The membranes incubated with the anti-uPAR antibody were incubated for 1 h with a horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody (1:7500 dilution; Bio-Rad), and the membranes incubated with the anti-sGC antibody were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:15000 dilution; Vector Laboratories, Inc., Burlingame, CA). Following six additional 5-min washes with PBS-T, secondary antibodies were detected by enhanced chemiluminescence (Amersham Biosciences) and exposure on Kodak X-Omat Blue film.

**Data Analysis and Statistical Methods**—X-ray films of Northern and Western blot experiments were scanned and analyzed using a Sigma Gel densitometry software package (SPSS Inc., Chicago, IL). Data are presented as means ± S.D. Statistical analyses were performed using the StatView statistical software package (Abacus Concepts, Inc., Berkeley, CA). Statistical significance was determined using one-way analysis of variance, followed by Fisher’s post hoc analysis. Student’s t test was used when only two sets of data were compared. All statistical tests were two-sided, and differences were considered statistically significant at p < 0.05.

**RESULTS**

**Effect of NO-mimetic Drugs on the Hypoxic Up-regulation of sGC Activity**—In Fig. 1, although hypoxia increased the in vitro invasive ability of MDA-MB-231 breast carcinoma cells by >5-fold, this effect of hypoxia was prevented by concomitant treatment with low concentrations of two different NO-mimetic drugs, GTN (1 pM and 0.1 μM; p < 0.002 and 0.001, respectively; one-way analysis of variance, followed by Fisher’s test) and SNP (0.1 nM; p < 0.001), administered at the beginning of the 24-h invasion assay.
In our previous study (4), we showed that the hypoxic up-regulation of invasiveness is accompanied by increased uPAR expression and that a blocking anti-uPAR antibody could prevent the hypoxic up-regulation of invasiveness, thereby establishing a causal link between uPAR expression and hypoxia-induced invasion. In the present study, culture of MDA-MB-231 cells in 0.5% O2 for 24 h also resulted in up to 3.5-fold increases (p < 0.0001) in uPAR protein levels (Figs. 2A and 5B) and up to 5-fold increases (p < 0.0002) in uPAR mRNA levels (Figs. 2C, 4A, and 5A). Moreover, administration of single doses of GTN (1 μM, 1 nM, and 0.1 μM) to cells incubated for 24 h in 0.5% O2 was sufficient to prevent the up-regulation of uPAR protein expression (p < 0.004, 0.005, and 0.001 for each concentration of GTN, respectively) (Fig. 2A). Similarly, the hypoxia-mediated increase in uPAR mRNA levels was also inhibited when a low concentration of GTN (1 μM) was used (p < 0.001) (Fig. 2C).

**Effect of NO Synthase Inhibition on uPAR Protein and mRNA Levels**—As demonstrated in a previous study, MDA-MB-231 cells express all three isoforms of NO synthase (2). To assess whether endogenous NO inhibits uPAR expression, NO synthesis in MDA-MB-231 cells was blocked by incubation with the NO synthase inhibitor L-NMMA (0.5 mM). In a manner characteristic of cells exposed to hypoxia, a 24-h incubation with a single dose of L-NMMA resulted in an overall 50% increase (p < 0.004) in uPAR protein levels (Fig. 2B) and a 2.8-fold increase (p < 0.04) in uPAR mRNA levels (Fig. 2D) in MDA-MB-231 cells even when cultured in 20% O2. In contrast, compared with uPAR expression in cells incubated in 20% O2 alone, no significant increase in uPAR protein (p = 0.89) or transcript (p = 0.35) levels was observed in cells incubated with a combination of L-NMMA (0.5 mM) and GTN (0.1 mM) in 20% O2 (Fig. 2, B and D).

**Effect of Hypoxia on sGC Signaling**—The results obtained using the enzyme-linked immunosorbent assay for cGMP revealed that, compared with cells incubated in 20% O2, MDA-MB-231 breast carcinoma cells incubated in 0.5% O2 for 6 h in the presence of 3-isobutyl-1-methylxanthine (500 μM) exhibited a 50% reduction in accumulated cGMP levels (p < 0.002) (Fig. 3A). This effect of hypoxia on cGMP levels was prevented by co-incubation with the NO-mimetic drug GTN (1 μM).

Western blot analysis was conducted to determine the effects of hypoxia on sGC protein levels. sGC is a heterodimeric protein consisting mainly of an α1- and a β1-subunit. Culture under hypoxic conditions for 24 h resulted in a 44% decrease in the levels of the β1-subunit (p < 0.0001). In contrast, culture under hypoxic conditions resulted in a 2.3-fold increase (p < 0.02) in the levels of the α1-subunit (Fig. 3B). Although the ratio of the α1- and β1-subunits was altered during hypoxia, the total amount of sGC was not significantly changed.

**Role of sGC in the NO-mediated Inhibition of uPAR Expression**—Fig. 4A shows that, compared with incubation of cells
under control conditions (20% O₂ alone), incubation of MDA-MB-231 cells for 24 h with the selective sGC blocker ODQ (0.5 μM) resulted in a 2.7-fold increase (p < 0.05) in the levels of uPAR mRNA (Fig. 4A). Although the presence of GTN (1 μM) prevented the hypoxic up-regulation of uPAR mRNA expression, GTN was unable to block the effect of hypoxia when ODQ was also present in the medium (Fig. 4A).

sGC is a heme-containing enzyme that requires ferrous iron for its biosynthesis and activity. Therefore, to further assess the participation of this enzyme in the regulation of uPAR expression by the NO signaling pathway, we cultured MDA-MB-231 cells in the presence of DFO (100 μM), an iron chelator and inhibitor of heme biosynthesis. The results showed that, in a manner similar to culture under hypoxic conditions or after pharmacological inhibition of NO synthase, culture in the presence of DFO resulted in a 4-fold increase (p < 0.007) in the levels of uPAR mRNA (Figs. 4B and 5C). In contrast to hypoxia, the effect of DFO on uPAR mRNA expression was not prevented by 1 μM GTN.

Furthermore, the up-regulation of uPAR mRNA and protein expression by hypoxia was significantly reduced (p < 0.0001) in a dose-dependent manner (up to 100%) by the presence of 8-Br-cGMP (0.1–10 μM, 24 h), a non-hydrolyzable analog of cGMP (Fig. 5, A and B). Interestingly, the presence of 8-Br-cGMP (1 μM) also resulted in the complete inhibition (p < 0.01) of the DFO-induced up-regulation of uPAR mRNA expression.
These results indicate that a major component of the hypoxia- and DFO-mediated stimulation of uPAR expression is the inhibition of sGC.

Role of PKG in the NO-mediated Inhibition of uPAR Expression

As indicated earlier, cGMP-mediated activation of PKG is an important component of the NO signaling pathway. To further elucidate the role of NO signaling in the regulation of uPAR expression, MDA-MB-231 cells were incubated for 6 h with the PKG inhibitor KT5823 (10 μM). Northern and Western blot analyses revealed that selective inhibition of PKG, even in 20% O₂, resulted in a 1.8-fold increase (p < 0.05) in uPAR protein and mRNA expression (Fig. 6, A and B). These results demonstrate that PKG activation by cGMP is necessary for the inhibition of uPAR expression by NO.

Effect of 8-Br-cGMP on Hypoxia-induced Invasion—Results from the in vitro invasion assay using Matrigel as a substrate for invasion revealed that, compared with the invasiveness of cells incubated in 20% O₂, hypoxia stimulated the invasiveness of MDA-MB-231 cells by 3.9-fold (p < 0.0001) (Fig. 7). This effect of hypoxia on invasiveness was completely inhibited by the presence of various concentrations of 8-Br-cGMP (0.1 μM to 1 mM) (Fig. 7).

DISCUSSION

The major finding of this study is that NO signaling plays an important role in the regulation of hypoxia-induced invasiveness of human MDA-MB-231 breast carcinoma cells. Furthermore, our results strongly suggest that the mechanisms by which cells adapt to hypoxia and reduced NO activity involve convergent processes.

This study also presents a novel role for cGMP-dependent signaling in the regulation of cellular invasiveness. Specifically, it was shown that the NO-mediated inhibition of uPAR expression is dependent on the sequential activation of sGC and PKG. Furthermore, it was determined that the hypoxic up-regulation of uPAR expression and the concomitant enhancement of the in vitro invasiveness are associated with reduced levels of sGC and PKG signaling. Because invasion of the extracellular matrix is an essential component of the metastatic process, these results suggest that perturbations in the cGMP-dependent signaling pathway could lead to increases in metastatic potential.

Our results show that the effects of hypoxia on sGC activity and uPAR expression can be prevented by low concentrations of GTN. These findings point to a mechanism of oxygen sensing and gene regulation whereby phenotypes are modified in response to a decrease in NO-mediated signaling. As shown in Fig. 8, we propose that this phenomenon is due to a reduction in endogenous NO synthesis. Molecular oxygen is obligatory for the conversion of L-arginine into NO and L-citrulline by the enzyme NO synthase (20). Indeed, exposure of cells to low levels of O₂ (1–3%) inhibits NO production by up to 90% (21). Due to the reduced NO levels associated with hypoxia, there is...
Inhibition of Invasiveness by NO

![Diagram of the NO signaling pathway](image)

**Fig. 8. Proposed model for the NO signaling pathway leading to inhibition of uPAR expression and invasiveness.** Activation of sGC by NO leads to increased production of cGMP, which in turn activates PKG. The latter phosphorylates target proteins and leads to inhibition of uPAR expression and cellular invasiveness. The components of the pathway following PKG activation specific to inhibition of invasion remain to be elucidated. However, interference with this pathway at the level of NO production (hypoxia, NO synthase inhibition), sGC activation (ODQ, DFO), cGMP depletion (phosphodiesterases), and PKG activation (KT5823) leads to increased uPAR expression, cellular invasiveness, and possibly metastasis. 8-Br-cGMP can directly activate PKG and thereby lead to inhibition of invasion.

A decrease in guanylyl cyclase activity and a consequential reduction in cGMP levels (Fig. 8). Supporting this concept, Taylor et al. (22) showed that culturing intestinal epithelial cells under hypoxic conditions (1% O₂) results in a significant decrease in basal and stimulated cGMP levels. Here, we have similarly shown that low O₂ levels decrease cGMP generation. Furthermore, we have demonstrated that the decrease in cGMP signaling is correlated with an enhancement of uPAR expression as well as with increased invasiveness. To strengthen the concept that uPAR expression is regulated through sGC activation, this study showed that GTN was unable to inhibit uPAR expression when sGC activity was directly blocked with either ODQ or DFO (Fig. 4).

Previous studies have shown that treatment with DFO results in cellular adaptive responses similar to those induced by hypoxia (13, 23–25). This has led to the hypothesis that a hemeprotein or changes in the redox status of cells are responsible for the adaptive responses to hypoxia (26). In the present study, the DFO-mediated up-regulation of uPAR expression was inhibited by 8-Br-cGMP (Fig. 5). This indicates that a major component of the DFO-mediated stimulation of uPAR expression is inhibition of guanylyl cyclase activity and that DFO does not interfere with signals acting downstream of guanylyl cyclase.

There are many potential targets that could be phosphorylated by PKG and therefore serve as downstream effectors in the NO signaling pathway. One possible mechanism by which PKG regulates cellular adaptations to changes in oxygenation involves a perturbation of the MAPK pathway. This pathway is activated by hypoxia (27), and studies have shown that NO can prevent the phosphorylation of ERK through a PKG-mediated interference of Ras/Raf (28). This concept is supported by the study of Mitani et al. (29), who showed that NO donors and cGMP-mimetic drugs reduce elastase expression by suppressing ERK phosphorylation. This leads to a subsequent reduction in the activation and DNA-binding capacity of AML1B (the transcription factor for elastase). It is possible that cGMP-dependent NO signaling similarly inhibits hypoxia-induced ERK phosphorylation, thereby decreasing the activation of the transcription factors responsible for the up-regulation of uPAR expression.

The promoter region of uPAR contains binding sites for transcription factors such as AP-1, Sp1/3, and nuclear factor-κB (30). HIF-1 levels may also contribute to the transcriptional activation of the uPAR gene, as previous examination of the sequences upstream of the uPAR initiation codon revealed the presence of potential HIF-1-binding sites (31). It has also been shown that HIF-1 accumulation and transcriptional activity can be reduced by relatively high concentrations (2.5–500 μM) of NO-mimetic drugs such as SNP, S-nitroso-L-glutathione, and 3-morpholinosydnonimine (12, 13). Preliminary studies in our laboratory have confirmed that high concentrations (0.1–1 mM) of GTN and SNP inhibit HIF-1 accumulation and transactivating activity. The invasive potential of a cell is dependent upon its ability to break down components of the extracellular matrix and basement membranes. This process requires the participation of several proteolytic enzyme systems, among which the urokinase plasminogen activator system figures prominently (32). In vitro invasiveness has been strongly correlated with the levels of cell-surface uPAR expression (4, 33–35). For example, using an in vitro assay similar to the one in this study, Kariko et al. (33) showed that human osteosarcoma cells transfected with a human uPAR cDNA are four times more invasive than control cells. Xing and Rabbani (34) obtained similar results using a rat mammary carcinoma cell line. We have previously demonstrated that uPAR availability for urokinase plasminogen activator binding is essential for the hypoxic stimulation of invasiveness (4, 35). There is also clinical and experimental evidence that increased uPAR expression is associated with metastasis of prostate, colon, and breast carcinomas (34, 36, 37). Furthermore, using human melanoma cells transplanted into nude mice, Rofstad et al. (8) recently demonstrated that hypoxia-induced metastasis is dependent on uPAR up-regulation. Thus, we postulate that the cGMP-dependent inhibition of invasiveness observed in the present study was partially due to down-regulation of uPAR expression. Although the role of uPAR in invasion and tumor progression has been studied extensively, the mechanisms governing its expression are not fully understood. In characterizing the contribution of the plasminogen activator system to the regulation of invasiveness and metastasis, the present study confirmed that uPAR message and protein are up-regulated during hypoxia. In addition, we demonstrated that the NO signaling pathway involving sGC and PKG activation is an integral component of the mechanism that regulates uPAR expression as well as invasiveness.

In summary, this study specifically links NO-mediated activation of sGC and PKG to the regulation of tumor cell invasiveness. Furthermore, our results suggest that sGC and PKG may be useful pharmacological targets for the prevention of cancer invasion and metastasis.

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Inhibition of Invasiveness by NO

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