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Prostaglandin E_2 Induces Hypoxia-inducible Factor-1α Stabilization and Nuclear Localization in a Human Prostate Cancer Cell Line*

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Hypoxia-induced up-regulation of vascular endothelial growth factor (VEGF) expression is a critical event leading to tumor neovascularization. Hypoxia stimulates hypoxia-inducible factor-1α (HIF-1α), a transcriptional activator of VEGF. Cyclooxygenase (COX)-2, an inducible enzyme that catalyzes the formation of prostaglandins (PGs) from arachidonic acid, is also induced by hypoxia. We reported previously that COX-2 inhibition prevents hypoxic up-regulation of VEGF in human prostate cancer cells and that prostaglandin E_2 (PGE_2) restores hypoxic effects on VEGF. We hypothesized that PGE_2 mediates hypoxic effects on VEGF by modulating HIF-1α expression. Addition of PGE_2 to PC-3ML human prostate cancer cells had no effect on HIF-1α mRNA levels. However, PGE_2 significantly increased HIF-1α protein levels, particularly in the nucleus. This effect of PGE_2 largely results from the promotion of HIF-1α translocation from the cytosol to the nucleus. PGE_2 addition to PC-3 ML cells transfected with a GFP-HIF-1α vector induced a time-dependent nuclear accumulation of the HIF-1α protein. Two selective COX-2 inhibitors, meloxicam and NS398, decreased HIF-1α levels and nuclear localization, under both normoxic and hypoxic conditions. Of several prostaglandins tested, only PGE_2 reversed the effects of a COX-2 inhibitor in hypoxic cells. Finally, PGE_2 effects on HIF-1α were specifically inhibited by PD98059 (a MAPK inhibitor). These data demonstrate that PGE_2 production via COX-2-catalyzed pathway plays a critical role in HIF-1α regulation by hypoxia and imply that COX-2 inhibitors can prevent hypoxic induction of HIF-mediated gene transcription in cancer cells.

It is well established that all tumors require the growth of new blood vessels, a process termed angiogenesis, in order to grow beyond 1–2 mm, invade, and metastasize (1). One of the major regulatory factors involved in neovascularization is vascular endothelial growth factor (VEGF).[1] Intratumoral hypoxia is a potent VEGF inducer in solid tumors. Hypoxic regulation of VEGF is mediated by hypoxia-inducible factor-1α (HIF-1α), a key transcription factor that regulates cellular responses to physiological and pathological hypoxia (2). The VEGF gene contains a number of HIF-1α-binding sites in its regulatory region, and HIF-1α is able to activate the VEGF promoter (3). Deletion of the HIF-1α gene or disruption of HIF-1α transcription results in the lack of VEGF secretion by tumor cells, suppression of angiogenesis, and inhibition of solid tumor growth (4, 5). In response to hypoxia, HIF-1α protein accumulates in the cytosol and translocates to the nucleus (6), where it activates hypoxia-sensitive genes, like VEGF, by binding to their promoter/enhancer regions (2). HIF-1α-mediated up-regulation of VEGF, therefore, has been proposed as an angiogenic switch during tumorigenesis (7).

Hypoxic effects on HIF-1α occur mainly at the post-translational level, as HIF-1α mRNA levels are not significantly modified by hypoxia (2). Under normoxic conditions, HIF-1α protein is rapidly degraded via the von Hippel-Lindau (VHL)-ubiquitin-proteasome pathway (8). The VHL protein recognizes the oxygen degradation domain of HIF-1α protein only under normoxic conditions (9). In addition to hypoxia, a variety of factors have recently been demonstrated to be regulators of HIF-1α expression, including reactive oxygen species, nitric oxide, cytokines, and growth factors (10). The regulation of HIF-1α by hypoxia as well as these other factors involves the activation of phosphoinositol 3-kinase (PI3K)/AKT pathway (10) and/or phosphorylation by p42/p44 MAP kinase (10–13).

Cyclooxygenase (COX), also referred to as prostaglandin endoperoxide synthase, is a key enzyme in the conversion of arachidonic acid to prostaglandins (PGs) and other eicosanoids. Two isoforms of COX have been identified. COX-1 is expressed constitutively in many tissues and cell types, whereas COX-2 is inducible by a variety of factors, including cytokines, growth factors, and tumor promoters. COX-2 is highly expressed in a number of human cancers and cancer cell lines, including prostate cancer (14–16). Recent reports (17) have demonstrated that COX-2 expression and activity are induced by hypoxia in human umbilical vein endothelial cells, and this induction is not mediated by HIF-1α but rather by the nuclear factor κB transcription factor. Forced overexpression of COX-2 in a colon cancer cell line results in the overproduction of several proangiogenic factors, including VEGF (18). In addition, prostaglandin E_2 (PGE_2), a major end product of the COX-2-catalyzed reaction, is reported to be a stimulator of angiogenesis (19).

We demonstrated that inhibition of COX-2 suppresses prostate cancer growth and angiogenesis *in vivo*. Tumors treated with a COX-2 inhibitor were smaller, with increased apoptosis, decreased microvessel density, and decreased tumor VEGF

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; COX, cyclooxygenase; PG, prostaglandin; HIF, hypoxia-inducible factor; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; GFP, green fluorescent protein; PI3K, phosphoinositol 3-kinase; PBS, phosphate-buffered saline; Rt, reverse transcriptase; VHL, von Hippel-Lindau; RT, reverse transcriptase.

levels (20). Our *in vitro* studies demonstrated that the administration of a selective COX-2 inhibitor, NS398, prevented the effects of cobalt chloride-simulated hypoxia on VEGF up-regulation in PC-3ML human prostate cancer cells. In that same report, PGE_2 administration restored the effects of cobalt chloride on VEGF in the presence of the COX-2 inhibitor (21). Similar results have recently been observed when the same cell line was exposed to true hypoxia (1% O_2).[2]

We concluded that COX-2 and PGE_2 are involved in the hypoxic induction of VEGF. We hypothesized that the effects of COX-2 and PGE_2 on hypoxia-induced VEGF are mediated by the regulation of HIF-1α expression and activation in PC-3ML human prostate tumor cells. In this report, we demonstrate that PGE_2 has no effect on HIF-1α mRNA expression. However, PGE_2, under normoxic conditions, promotes HIF-1α protein stabilization, particularly in the nucleus. This effect of PGE_2 largely results from the promotion of HIF-1α protein translocation from the cytosol to the nucleus. In addition, we demonstrate that hypoxia-induced HIF-1α accumulation is suppressed by inhibition of COX-2 activity and restored by the addition of exogenous PGE_2. Finally, we provide evidence that the effects of PGE_2 are mediated, primarily, via the MAP kinase pathway. Our findings demonstrate a critical role for COX-2 and PGE_2 in the regulation of HIF-1α and VEGF.

EXPERIMENTAL PROCEDURES

Cell Line and Cell Culture—The PC-3ML human prostate cancer cell line, a subline of the PC-3 cell line, was a generous gift from Mark Stearns (Department of Pathology, MCP-Hahnemann University, Philadelphia). It has been characterized as a cell line with a highly invasive and bone-targeting metastatic phenotype (22). Cells were cultured under normoxic conditions (20% O_2, 5% CO_2, 75% N_2) in a humidified Napco incubator at 37 °C. Hypoxic stimulation was produced with an ambient oxygen concentration of 1% (using a controlled incubator with CO_2/O_2 monitoring and CO_2/N_2 gas sources). PC-3ML cells were incubated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Before the treatments with various compounds or hypoxia, cells were washed with PBS, and serum-free medium was replaced overnight. In order to prevent reoxygenation of hypoxic cells, the medium or lysis buffers were pre-equilibrated to the experimental oxygen conditions overnight and added to cells on ice. For reagents, NS398, butaprost, sulprostone, and PGE_1 alcohol were purchased from Cayman Chemical Co. (Ann Arbor, MI); meloxicam is a product of Biomol Inc. (Plymouth Meeting, PA); PD98059, LY294002, and staurosporine were obtained from Calbiochem.

Preparation of Proteins from the Cytosolic or Nuclear Fractions and Immunoblotting—Proteins from the cytosolic and nuclear fractions of the PC-3ML cells were isolated using a commercial kit purchased from Pierce, according to the manufacturer's instructions. The samples were electrophoresed on a 7.5% SDS-polyacrylamide gel, electrophoretically transferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences), and incubated with a monoclonal anti-HIF-1α antibody (Transduction Laboratories, Lexington, KY) overnight at 4 °C. Secondary horseradish peroxidase-linked donkey anti-mouse IgG (Amersham Biosciences) was used. Filters were developed by the enhanced chemiluminescence system (Amersham Biosciences).

RT-PCR—Cells were incubated in serum-free medium. Total RNA was extracted with Trizol Reagent (Invitrogen). cDNA was prepared by incubating 1 μg of total RNA in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and RNase inhibitors with 250 units of reverse transcriptase, 1 μM of each dNTP, and random primers (0.05 μM, Invitrogen) for 60 min at 37 °C. The fragment was amplified by PCR using specific primers for HIF-1α, sense (bp 184–207), 5′-CGGCGCGAACGACAAGAAAAAGAT-3′ and antisense (bp 1327–1350), 5′-TCGTTGGGTGAGGGGAGCATTACA-3′. A set of specific PCR primers for EP receptor subtypes has been prepared as reported by Sheng *et al.* (45): EP_1 fragment, forward (5′-ACCGACCTGGCGGGCCACGTGA-3′; 321–342) and reverse (5′-CGCTGAGCGTGTTGCACACCAG-3′; 750–729); EP_2 fragment, forward (5′-TCCAATGACTCCCAGTCTGAGG-3′; 750–729) and reverse (5′-TGCATAGATGACAGGCAGCACG-3′); EP_3 fragment, forward (5′-GATCACCATGCTGCTCACTG-3′; 396–415) and reverse (5′-AGTTATGCGAAGAGC-TAGTCC-3′; 904–884); EP_4 fragment forward (5′-GGGCTGGCTGTCACCGACCTG-3′; 565–585) and reverse (5′-GGTGCGGCGCAT-GAACTGGCG-3′; 1050–1030). Primers for β-actin: forward (5′-GAAGAGCTACGAGCTGCC-3′; 2376–2393) and reverse (5′-TGATCCACATCTGCTGGA-3′; 2927–2944). PCR was initiated in a thermal cycle programmed at 95 °C for 5 min, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and amplified with 28 cycles for HIF-1α and β-actin, and 35 cycles for EP receptor subtypes. The amplified products were visualized on 1.5% agarose gels.

Construction of GFP-tagged HIF-1α Vector—The vector expressing GFP-tagged HIF-1α (pEGFP-HIF-1α) was prepared by fusing HIF-1α cDNA to the pEGFP (Clontech, Palo Alto, CA). The complete human HIF-1α cDNA sequence was obtained from GenBank™ (accession number U22431) and amplified by PCR using primers that create the restriction enzyme sites for *Kpn*I and *Bam*HI ends, as described by Wang *et al.* (2). The resulting fragment was subcloned into the *Kpn*I and *Bam*HI site of pEGFP-N1 to generate the GFP-HIF-1α fusion protein vector.

Transient Transfection and Confocal Laser Scanning Microscopy—PC-3ML cells were cultured in Lab-Tek II chamber slides (Nalge Nunc Co., Naperville, IL) and transiently transfected with either the parental GFP construct (pEGFP, Clontech) or the chimeric pEGFP-HIF-1α construct using LipofectAMINE 2000 reagent (Invitrogen). After transfection, cells were treated with either vehicle as control or 1 μM PGE_2, 50 μM NS398, or hypoxia (1% O_2) for various times. Transfected cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature. Samples were then washed with PBS and covered with mounting medium (Vector Laboratories, Burlingame, CA) to avoid fading. The intensity and the subcellular distribution of fluorescent activity were examined by confocal laser scanning microscopy (Leica Lasertechnik, Heidelberg, Germany).

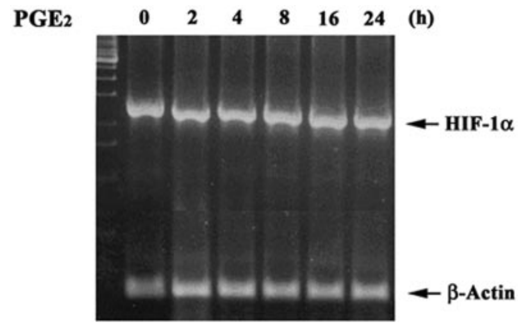

FIG. 1. **Effect of PGE_2 on HIF-1α mRNA expression.** PC-3ML cells were incubated in serum-free medium and treated with either vehicle or 1 μM PGE_2 for various times, as indicated. One μg of total RNA extracted from treated and non-treated cells was reverse-transcribed. The fragment was amplified by specific primers for HIF-1α or β-actin. The amplified products were visualized on 1.5% agarose gels.

RESULTS

PGE_2 Has No Effect on HIF-1α mRNA Expression—Previous reports (21) have suggested that PGE_2 plays a role in hypoxia-induced VEGF expression. To determine whether this process is mediated by the regulation of HIF-1α, we initially examined the effect of PGE_2 on HIF-1α mRNA expression. RT-PCR, as shown in Fig. 1, revealed that HIF-1α mRNA expression is not modulated by the addition of PGE_2 in PC-3ML cells.

PGE_2 Stabilizes HIF-1α Protein Under Both Normoxic and Hypoxic Conditions—HIF-1α is a short lived protein with a half-life of under 5 min (2, 23). HIF-1α is ubiquitinated and subjected to proteasomal degradation in non-hypoxic cells (8). Under hypoxic conditions, HIF-1α ubiquitination and proteasomal degradation are significantly decreased, leading to increased protein levels. We examined HIF-1α protein expression in PC-3ML cells under normoxic and hypoxic conditions. Western blot analysis with protein samples extracted either from the cytosol or the nucleus revealed that PC-3ML cells expressed a low level of HIF-1α protein in the cytosol, and a relatively higher level in the nucleus under normoxic conditions. Hypoxia (12 h) did not have a significant effect on the

[2] X. H. Liu, A. Kirschenbaum, M. Lu, S. Yao, A. Dosoretz, J. F. Holland, and A. C. Levine, unpublished data.

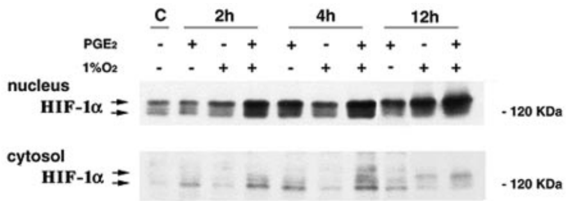

FIG. 2. **PGE_2-induced HIF-1α protein stabilization.** PC-3ML cells were cultured in serum-free medium and treated with either 1 μM PGE_2, or 1% O_2, or combination of PGE_2 (1 μM) and 1% O_2 for various times as indicated. Proteins in the nuclear and cytosolic fractions were isolated and subjected to Western blot analysis. Twenty μg of proteins were loaded in each lane. *C*, control.

total cytosolic protein levels but induced a band shift from low molecular weight to high molecular weight, suggesting an effect on the post-translational modification of HIF-1α protein in the cytosol. Hypoxia induced a significant increase in HIF-1α protein levels in the nuclear fraction, suggesting that hypoxia induced HIF-1α protein translocation from the cytosol to the nucleus in this cell line (Fig. 2).

We next examined the effect of PGE_2 on HIF-1α protein expression. Fig. 2 demonstrates that PGE_2 up-regulated HIF-1α levels in both the cytosolic and nuclear fractions in normoxic cells. The PGE_2-induced HIF-1α protein expression in the cytosol was first noted 2 h after treatment which was prior to the induction observed in the nuclear fraction (4 h). The combination of PGE_2 and hypoxia resulted in greater increases in HIF-1α protein levels, particularly in the nuclear fraction, than those seen with either treatment alone. Of note, the combination of hypoxia and 1 μM PGE_2 induced a band shift in the cytosolic fraction, which, again, may represent an effect on the post-translational modification of the protein (Fig. 2). These data indicate that PGE_2 and hypoxia act both independently and synergistically to increase HIF-1α protein levels and nuclear localization in PC-3 ML human prostate cancer cells.

PGE_2-induced Nuclear Localization of HIF-1α Protein—Previous studies (6) have determined that endogenous HIF-1α translocates to the nucleus in hypoxic cells. Nuclear translocation of HIF-1α is necessary for its activation and its transcriptional activation of a variety of hypoxia-regulated genes, including VEGF. In addition, the translocation of HIF-1α protein to the nucleus has been proposed recently (24) as a regulatory step involved in its stabilization. Our data from Western blotting indicated that both hypoxia and PGE_2 significantly increased HIF-1α protein levels, particularly in the nuclear fraction. Therefore, we investigated the possibility that PGE_2 promotes HIF-1α protein nuclear translocation independent of hypoxia using a nucleo-cytosolic trafficking assay. We generated a pEGFP-HIF-1α vector that expresses GFP-tagged HIF-1α protein. The vector was transiently transfected into PC-3ML cells. The cells were then treated with 1 μM PGE_2 for various times under normoxic conditions, and the intracellular localization of the GFP-HIF-1α fusion protein was visualized using a laser scanning confocal microscope. Consistent with the results obtained by Western blotting, treatment with 1 μM PGE_2 induced a time-dependent nuclear accumulation of the protein (Fig. 3).

The Effect of PGE_2 on HIF-1a Is Mediated by Specific EP Receptor Subtypes—The family of EP receptors consists of four subtypes of G protein-coupled receptors, designated EP_1, EP_2, EP_3, and EP_4. Through these receptors, PGE_2 modulates a variety of physiological and pathological functions (25). We next examined the expression of EP receptor subtypes in PC-3ML cells by RT-PCR using specific oligonucleotide primers reported by Sheng *et al.* (26). As demonstrated in Fig. 4*A*, EP_2, EP_3, and EP_4 are clearly expressed in PC-3ML cells. In contrast, EP_1 mRNA was undetectable in this cell line. To evaluate the functional role of EP receptor subtypes in PC-3ML cells, we tested the effects of butaprost (a selective EP_2 receptor agonist), sulprostone (a selective EP_3 receptor agonist), and PGE_1 alcohol (a selective EP_4 receptor agonist) (25) on HIF-1α protein expression under normoxic conditions. Although the EP_3 receptor agonist sulprostone had no detectable effect (data not shown), both butaprost (an EP_2 receptor agonist) and PGE_1 alcohol (an EP_4 receptor agonist) significantly stimulated the expression level of HIF-1α in both the cytosolic and nuclear fractions (Fig. 4*B*), mimicking the effects of PGE_2 alone. The data demonstrate that PGE_2-induced up-regulation of HIF-1α protein is mediated through the EP_2 and EP_4 receptor signaling pathways in this cell line.

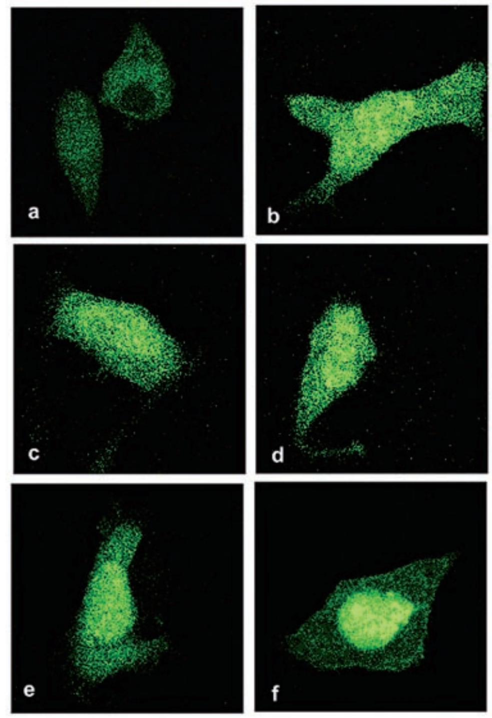

FIG. 3. **PGE_2-induced nuclear translocation of HIF-1α protein.** PC-3ML cells were transiently transfected with the chimeric pEGFP-HIF-1α construct. After transfection, cells were cultured under normoxic conditions and treated with either vehicle as control (*a*) or 1 μM PGE_2 for 4 (*b*), 6 (*c*), 8 (*d*), 12 (*e*), and 16 h (*f*). Cells were then fixed with 4% formaldehyde in PBS and covered by mounting medium. The intensity and subcellular localization of pGFP-HIF-1α were examined using a laser scanning confocal microscope.

The Roles of MAPK and PI3K/AKT in PGE_2-induced HIF-1α Expression—We next determined the possible involvement of several intracellular kinase pathways in PGE_2-induced HIF-1α expression. As shown in Fig. 5*A*, Western blot analysis demonstrated that PD98059, a MAPK inhibitor (50 μM), had no effect on basal HIF-1α expression but did significantly suppress PGE_2-induced up-regulation of HIF-1α protein expression in a dose-dependent fashion (Fig. 5*B*). It was notable that whereas PD98059 inhibited both high and low molecular weight bands of HIF-1α protein in the nucleus, it specifically suppressed the higher molecular weight band of the protein in the cytosolic fraction. These results indicate that inhibition of HIF-1α phosphorylation in the cytosol may prevent its nuclear translocation. LY294002, a PI3K inhibitor, strongly inhibited basal HIF-1α nuclear accumulation but only partially inhibited PGE_2-inducible nuclear and cytosolic HIF-1α protein levels. Staurosporine, a protein kinase C inhibitor, had no effect on either basal and PGE_2-induced HIF-1α expression in both the nuclear and cytosolic fractions (Fig. 5*A*). These

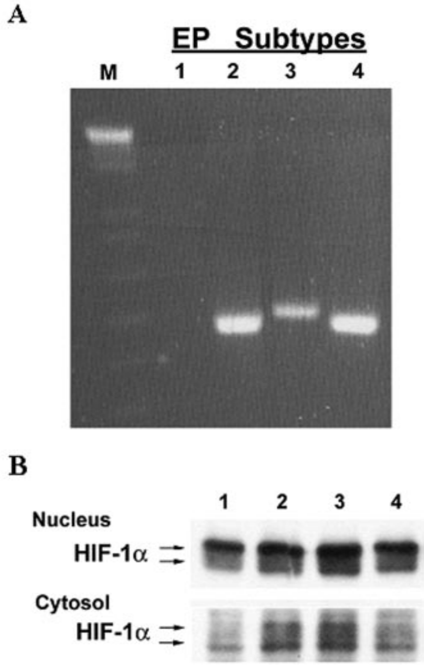

FIG. 4. **Determination of the role of EP receptor subtypes.** *A*, mRNA expression of PGE$_2$ receptor subtypes was examined using RT-PCR. One μg of total RNA extracted from PC-3ML cells was subjected to reverse-transcription and amplified by specific primers for EP$_1$, EP$_2$, EP$_3$, and EP$_4$ for 35 cycles. The amplified products were visualized on 1.5% agarose gels. *M*, 1 kb plus DNA ladder. *B*, the effect of EP receptor agonists on HIF-1α protein expression. PC-3ML cells were incubated in serum-free medium and treated with either vehicle (*lane 1*), 1 μM PGE$_2$ (*lane 2*), 0.1 μM PGE$_1$ alcohol (*lane 3*), or 10 μM butaprost (*lane 4*) for 4 h. Proteins were extracted from the nuclear or cytosolic fractions and subjected to Western blot analysis.

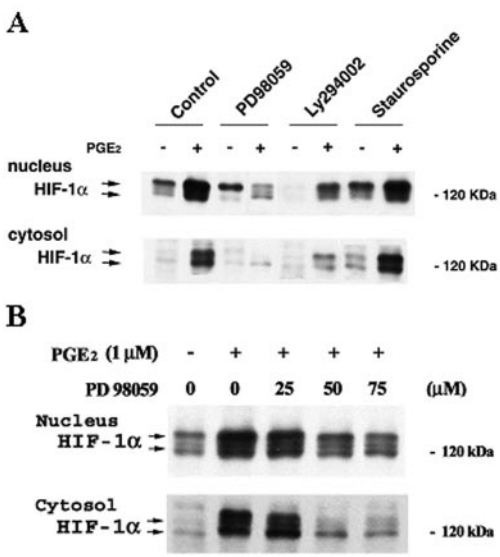

FIG. 5. **The effects of kinase inhibitors on PGE$_2$-induced HIF-1α stabilization.** *A*, PC-3ML cells were pretreated with 50 μM PD98059, 5 μM LY294002, or 20 nM staurosporine for 20 h, and incubations were continued with or without 1 μM PGE$_2$ for an additional 4 h. Proteins in the nuclear and cytosolic fractions were isolated and subjected to Western blot analysis. *B*, cells were pretreated with various concentrations of PD98059 as indicated for 20 h prior to 1 μM PGE$_2$ treatment for 4 h. Proteins in the nuclear and cytosolic fractions were then isolated and subjected to Western blot analysis.

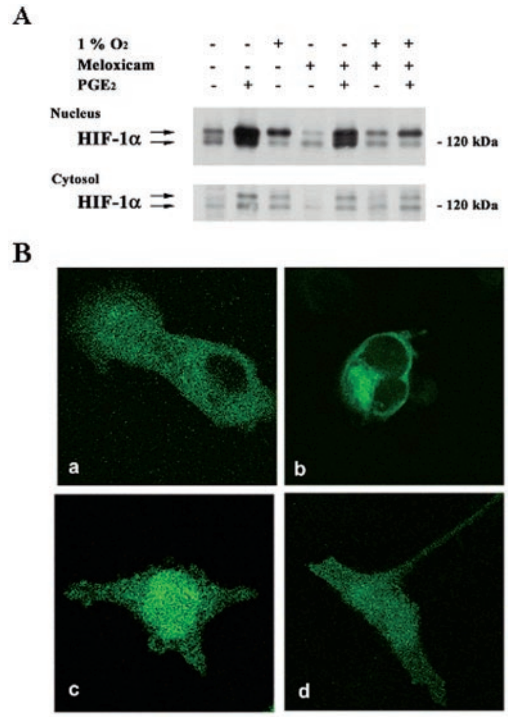

FIG. 6. **The effects of COX-2 inhibitors on hypoxia-induced nuclear accumulation of HIF-1α.** *A*, PC-3ML cells were cultured in serum-free medium for 24 h prior to various treatments as follows: *lane 1*, vehicle only as control; *lane 2*, 1 μM PGE$_2$ for 4 h; *lane 3*, 1% O$_2$ for 12 h; *lane 4*, 10 μM meloxicam for 48 h; *lane 5*, 10 μM meloxicam for 44 h followed by 1 μM PGE$_2$ for an additional 4 h; *lane 6*, 10 μM meloxicam for 36 h followed by 1% O$_2$ for 12 h; *lane 7*, 10 μM meloxicam plus 10 μM PGE$_2$ for 36 h prior to 1% O$_2$ treatment for 12 h. Proteins in the nuclear and cytosolic fractions were then isolated and subjected to Western blot analysis. *B*, NS398-suppressed basal and hypoxia-induced nuclear relocalization of HIF-1α protein. PC-3ML cells were transiently transfected with the chimeric pEGFP-HIF-1α construct. After transfection, cells were treated with vehicle as control (*a*), 50 μM NS398 for 2 days under normoxic conditions (*b*), hypoxia (1% O$_2$) for 12 h (*c*), and pretreated with NS398 (50 μM) for 36 h under normoxic conditions followed by hypoxia (1% O$_2$) treatment for an additional 12 h (*d*). Cells were then fixed with 4% formaldehyde in PBS and covered by mounting medium. The intensity and subcellular localization of pGFP-HIF-1α were examined using a laser scanning confocal microscope.

results demonstrate that the MAP kinase inhibitor (PD98059) specifically inhibits PGE$_2$-induced stabilization and nuclear localization of HIF-1α in this cell line, whereas the PI3K inhibitor (LY294002) acts non-specifically to inhibit both the basal and PGE$_2$-induced effects on the protein.

Inhibition of COX-2 Activity Suppresses Hypoxia-induced HIF-1α Nuclear Accumulation—We reported previously that NS398, a selective COX-2 inhibitor, prevents VEGF up-regulation in response to cobalt chloride-simulated hypoxia (21). Similar results were observed when the same cell line was exposed to true hypoxia (1% O$_2$). We tested whether this effect of the COX-2 inhibitor is due to inhibition of hypoxic effects on HIF-1α protein expression. However, NS398, in addition to its COX-2 inhibitory activity, has also been reported to inhibit MAPK activity directly (27). Therefore, we examined the effect of another selective COX-2 inhibitor, meloxicam, on HIF-1α protein expression. Meloxicam has not been reported to have any direct effect on MAPK activity. As shown in Fig. 6*A*, Western blotting confirmed that both PGE$_2$ and hypoxia significantly up-regulated HIF-1α protein levels. In the nuclear fraction, pretreatment of cells with meloxicam (10 μM) for 2 days inhibited both the basal and hypoxia-induced accumulation of the protein in both the nuclear and cytosolic fractions. These inhibitory effects were predominantly observed in the higher molecular weight bands. Fig. 6*A* also demonstrates that the addition of PGE$_2$ completely reversed the inhibitory effects of meloxicam on hypoxia-induced up-regulation of HIF-1α protein.

We performed a nucleo-cytosolic trafficking assay (laser scanning of confocal microscopy) using a GFP-tagged HIF-1α system to confirm the results noted on immunoblotting. Fig. 6*B* demonstrates that COX-2 inhibition prevents both basal and hypoxia-induced nuclear accumulation of HIF-1α protein. These results demonstrate that PGE_2 production via the COX-2-catalyzed pathway plays a critical role in hypoxia-stimulated effects on HIF-1α protein.

DISCUSSION

There is ample evidence that COX-2 overexpression contributes to carcinogenesis and that COX-2 disruption can both prevent and treat a variety of solid tumors (14, 16, 28–30). COX-2 inhibitors have demonstrated direct effects on tumor cells (induction of apoptosis) (31, 32). In addition to these direct effects, prostaglandins derived from COX-2 exert indirect effects on tumor growth via the promotion of tumor angiogenesis (18–20, 33–35). COX-2 effects on angiogenesis are mediated, at least in part, by modulation of VEGF expression (18, 20). Several studies (20, 36) have shown that inhibition of COX-2 activity (via genetic knockout or pharmacologic inhibitors) results in decreased tumor and stromal VEGF levels with resultant impairment of tumor growth and angiogenesis. PGE_2, one of the major eicosanoid products of the COX-2-catalyzed reaction, has also been specifically implicated in the promotion of VEGF expression and tumor angiogenesis in a variety of cell types (37, 38).

Our previous studies with human prostate cancer cells *in vitro* delineated one cell line, PC-3 ML, in which VEGF levels were dramatically up-regulated by cobalt chloride-simulated hypoxia. Administration of a selective COX-2 inhibitor prevented hypoxia-simulated up-regulation of VEGF, and the co-administration of PGE_2 restored the ability of hypoxia to increase VEGF in the presence of the COX-2 inhibitor (21). In the current report, we studied the effects of true hypoxia and PGE_2 on the expression of the hypoxia-inducible factor-1α (HIF-1α), a master oxygen sensor, in the same prostate cancer cell line. PGE_2 had no effect on HIF-1α mRNA levels but did modulate its protein expression. The lack of effect of PGE_2 on transcriptional activation of HIF-1α was not surprising in view of the fact that hypoxic regulation of HIF-1α is also primarily determined by stabilization of HIF-1α protein, which is otherwise rapidly degraded in oxygenated cells (23).

The mechanisms underlying hypoxic effects on the ubiquitin-ligase complex responsible for HIF-1α protein degradation have been well elucidated recently by the identification of an oxygen- and iron-dependent proline hydroxylase responsible for the post-translational modification of HIF-1α. Proline hydroxylation of the oxygen-dependent domain of HIF-1α results in recognition of the protein by pVHL and subsequent ubiquitination and degradation (39, 41). In addition, hypoxia induces the nuclear translocation of HIF-1α protein (6). The precise mechanisms underlying this nuclear accumulation and its role in HIF-1α stabilization and activation have not yet been established. Nuclear targeting of the protein has been proposed as a regulatory step whereby HIF-1α escapes from proteasomal degradation (11, 24). In addition, nuclear localization of HIF-1α has been shown to be essential, although not sufficient, for activation of the protein (6, 11).

In the current report, we provide evidence that PGE_2 alone, under normoxic conditions, increases HIF-1α protein levels. In addition, PGE_2 potentiates hypoxia-induced HIF-1α expression and nuclear localization. Moreover, inhibition of COX-2 activity significantly suppressed hypoxia-induced nuclear accumulation of HIF-1α protein, as demonstrated by Western blotting and a GFP-tagged HIF-1α system. Finally, PGE_2 addition to meloxicam-treated cells restored the ability of hypoxia to induce HIF-1α nuclear accumulation, establishing a specific role for PGE_2 in this process.

We reported that PC-3ML cells express relatively high constitutive levels of COX-2 (21) but not COX-1 (data not shown), suggesting that PGE_2 production is mainly derived from COX-2 activity in this cell line. The selective COX-2 inhibitors decreased HIF-1α nuclear protein levels in PC-3ML cells under both normoxic and hypoxic conditions. Concomitant addition of PGE_2 to meloxicam-treated hypoxic cells completely reversed the effects of the selective COX-2 inhibitor. In addition, we examined the possible effects of other PGs that are also derived from the COX-2-catalyzed pathway. Our results indicate that neither PGD_2, PGI_2, nor $PGF_{2\alpha}$ significantly modulate HIF-1α protein expression or reverse the effects of the COX-2 inhibitors in PC-3ML cells (data not shown). These data support our conclusion that PGE_2 production via the COX-2-catalyzed pathway specifically mediates hypoxic effects on HIF-1α protein in this cell line.

Several reports demonstrated that hypoxia induces the phosphorylation of HIF-1α by p42/p44 MAPK (also called extracellular signal-regulated kinase, Erk1/2), which increases both HIF-1α nuclear localization and transcriptional activity (10–13, 42). In addition, PGE_2 has been shown to enhance directly MAPK activity in colon cancer cells (26). In the present study, we observed that hypoxia and PGE_2, alone and in combination, promote the nuclear accumulation of HIF-1α protein. Both hypoxia and PGE_2 also induce a higher molecular weight HIF-1α band in the cytosol, which may represent an effect on post-translational modification, presumably phosphorylation, of the protein. This induction was blocked by PD98059 (a MAP kinase inhibitor) and COX-2 inhibitors. These results suggest that PGE_2 induces phosphorylation of HIF-1α protein in the cytosol and imply that this phosphorylation, presumably by the MAP kinase (Erk1/2), is a prerequisite step for nuclear translocation of the protein and necessary for its stabilization. Consistent with these findings, COX inhibitors were recently shown to inhibit cancer cell growth and T cell activation by inhibiting the MAPK pathway (43–45).

The PI3K/AKT signaling pathway was demonstrated previously (46, 47) to be involved in hypoxia-induced effects on VEGF and HIF-1α expression. Moreover, recent reports (26, 31) reveal that PGE_2, acting via the prostaglandin EP_4 receptor, activates both the PI3K/AKT and Erk1/2 pathways, resulting in increased growth, motility, and resistance to apoptosis in cancer cells. Our data, however, demonstrate that LY294002, a potent PI3K inhibitor, completely inhibited basal expression patterns of HIF-1α protein but only partially inhibited the PGE_2 effects on the protein. These results indicate an important role for PI3K/Akt pathway in the maintenance of basal HIF-1α protein expression but fail to demonstrate a specific role for PI3K/Akt in the PGE_2-induced nuclear accumulation of HIF-1α protein.

The effects of PGE_2 are mediated through a specific family of transmembrane G protein-coupled receptors (EP receptors) (25). Three of the four EP receptor subtypes (EP_2, EP_3, and EP_4) are expressed in PC-3ML cells. Our data indicate that both the EP_2 and EP_4 receptor subtypes mediate the observed PGE_2 effects on HIF-1α regulation in this cell line.

Prostate cancer is the most common cancer and second leading cause of cancer deaths in males in the United States. A number of clinical investigations have demonstrated a relationship between the degree of neovascularization and cancer grade (48), metastatic behavior (49), and cancer-specific survival (50). Prostate cancer cells have been reported to overexpress COX-2 and produce elevated levels of PGE_2 (15, 51). We reported previously (21) that the PC-3ML cell line, an androgen-insensitive, highly invasive and metastatic human prostate cancer cell line, expresses high constitutive and hypoxia-inducible levels of COX-2. In addition, this cell line is distinguished from other prostate cancer cell lines by its ability to dramatically up-regulate VEGF (5–6-fold) in response to cobalt chloride-simulated hypoxia, which is suppressed by a selective COX-2 inhibitor (21). In the current report, we demonstrate further that PGE_2 production via the COX-2-catalyzed pathway plays critical roles in the hypoxic regulation of HIF-1α in this cell line.

Intratumoral hypoxia cannot be manipulated. Hypoxia regulates the expression of the α-subunit of HIF, the primary transcription factor involved in the hypoxic response. HIF induces the expression of genes essential for oxygen homeostasis and angiogenesis including VEGF, erythropoietin, tyrosine hydroxylase, inducible nitric-oxide synthase, and glycolytic enzymes. Our data demonstrate that selective COX-2 inhibitors prevents hypoxic up-regulation of HIF-1α in a human prostate cancer cell line. Given the seminal role of HIF-1α in the regulation of genes associated with cancer cell survival and tumor angiogenesis, our data provide a rationale for the use of COX-2 inhibitors as both anti-tumor and anti-angiogenic therapy in the treatment of prostate cancer.

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