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Vascular Endothelial Growth Factor Gene Expression in Colon Cancer Cells Exposed to Prostaglandin E_2 Is Mediated by Hypoxia-inducible Factor 1[1]

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ABSTRACT

Prostaglandin E_2 (PGE_2) has been implicated as an inducer of angiogenesis in human colon cancer. Here, we demonstrate that PGE_2 exposure induces the expression of vascular endothelial growth factor (VEGF) mRNA in HCT116 human colon carcinoma cells that is mediated by the transcriptional activator hypoxia-inducible factor 1 (HIF-1). PGE_2 exposure induces the phosphorylation of extracellular signal-regulated kinase (ERK) and AKT. Pharmacologic inhibition of ERK phosphorylation blocks the induction of VEGF mRNA and HIF-1α protein expression in response to PGE_2 stimulation. Inhibition of C-SRC tyrosine kinase activity also blocks PGE_2-induced HIF-1α protein and VEGF mRNA expression without blocking ERK phosphorylation. In contrast, phosphorylation of AKT is dependent on ERK and C-SRC activity. Thus, the activity of multiple signal transduction pathways is required for the HIF-1-mediated induction of VEGF expression in colon cancer cells exposed to PGE_2.

INTRODUCTION

Colorectal cancer is the second leading cause of mortality from neoplastic disease in the United States. Tumor microvessel density is an important determinant of colon cancer metastasis and patient mortality (1, 2). The level of VEGF[3] expression is a major determinant of microvessel density in colon cancers, and VEGF expression is also correlated with metastasis and mortality. Recent studies of human colon cancers have demonstrated that expression of COX2 is also correlated with VEGF expression, angiogenesis, and patient mortality (3–5). COX2 catalyzes the production of PGE_2, and high levels of PGE_2 have been demonstrated in colorectal adenocarcinomas in comparison with adjacent normal tissue (6, 7). In mice, pharmacologic or genetic inhibition of COX2 activity suppresses colon carcinogenesis (8–10) and can block the growth of established tumors (11). In humans, the use of nonsteroidal anti-inflammatory drugs that nonselectively inhibit COX2 is associated with a reduced risk of colorectal cancer (12–15).

The mechanism by which COX2-generated PGE_2 induces VEGF expression in colon cancer cells has not been determined. VEGF expression is also induced in colon cancer cells by other stimuli, including hypoxia, activation of the IGF-1R, or p53 loss-of-function, and, in these cases, transcriptional activation of the *VEGF* gene is mediated by HIF-1 (16, 17). HIF-1 is a heterodimeric protein consisting of a constitutively expressed HIF-1β subunit and a HIF-1α subunit, the expression of which is regulated by the cellular O_2 concentration and diverse signal transduction pathways leading from cell surface receptors to kinase cascades (18). Under hypoxic conditions, the O_2-dependent hydroxylation of proline residues 402 and 564, which is required for ubiquitination and proteasomal degradation of HIF-1α, is inhibited (19–21). In contrast, activation of the PI3K and ERK pathways after ligand binding to the IGF-1R stimulates increased synthesis of HIF-1α protein (16). HIF-1α overexpression has been demonstrated in human colon cancer biopsies (22, 23), and forced overexpression of HIF-1α in human colon carcinoma HCT116 cells increases tumor growth and angiogenesis in nude mice (17).

In the present study, we investigated the mechanism by which PGE_2 exposure induces *VEGF* gene expression in HCT116 human colon carcinoma cells. We demonstrate that PGE_2 induces expression of HIF-1α protein and VEGF mRNA and that inhibition of HIF-1α expression by RNA interference blocks the induction of VEGF mRNA. We also provide evidence that the activity of multiple signal transduction pathways is required for the HIF-1-mediated induction of VEGF expression in colon cancer cells exposed to PGE_2.

MATERIALS AND METHODS

Tissue Culture and Reagents. HCT116 cells (wild type with respect to p53 expression) were cultured in McCoy's 5A medium with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Corp., Carlsbad, CA). PGE_2, PD98059, Wortmannin, rapamycin, SB203580, cycloheximide, and cobalt chloride ($CoCl_2$) were from Sigma (St. Louis, MO). SC-51322 and 17-pt-PGE_2 were from Biomol Research Laboratories (Butler Pike, PA). PP2 and JNK inhibitor were from Calbiochem. For hypoxic exposures, cells were placed in a modulator incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with 1% O_2/5% CO_2/balance N_2, sealed, and incubated at 37°C.

PGE_2 and Inhibitor Treatments. HCT116 cells were plated at a density of 2.5×10^6/10-cm or 8.6×10^5/6-cm dish. Subconfluent cells were serum starved (0.1% FBS) for 24 h before PGE_2 or 17-pt-PGE_2 was added. Kinase inhibitors PD98059, Wortmannin, rapamycin, and SB203580 were added 1 h before exposure to PGE_2, 1% O_2, or 100 μM $CoCl_2$. JNK inhibitor and PP2 were added 20 min and 2 h, respectively, before the exposure to PGE_2 or 1% O_2. EP_1 receptor antagonist SC-51322 was added 30 min before exposure to PGE_2. Cycloheximide was added to the media of HCT116 cells that had been serum starved and treated with $CoCl_2$ for 4 h or PGE_2 for 24 h, and whole cell extracts were prepared at 0, 20, and 40 min.

Immunoblot Assays. Whole cell extracts were prepared using radioimmunoprecipitation assay buffer, fractionated by SDS-PAGE, and transferred to a nitrocellulose filter. For HIF-1α and HIF-1β, 150-μg aliquots of protein were analyzed using a monoclonal antibody against HIF-1α (H1α67; Ref. 23) or HIF-1β (H1β234; Novus Biologicals, Littleton, CO; Ref. 24) at 1:1000 dilution (16). Aliquots (50 μg) were analyzed using antibodies (1:1000 dilution) specific for phosphorylated (Thr202/Tyr204) or total p44/p42 MAP kinase and phosphorylated (Ser473) or total AKT (Cell Signaling Technology, Beverly, MA, and Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies against mouse and rabbit IgG (1:2500 dilution) and enhanced chemiluminescence reagents were from Amersham Biosciences (Piscataway, NJ).

RT-PCR Assays. Total RNA was extracted from HCT116 cells using TRIzol reagent (Invitrogen Corp.). Aliquots (5 μg) of RNA were reverse transcribed to cDNA using Superscribe First-Strand Synthesis System (Invitrogen Corp.). Aliquots (1, 2, and 4 μl) of cDNA were used as template for PCR of HIF-1α, VEGF, and 18S rRNA sequences. The following oligonucleotides were used as primers: (*a*) 5′-GGGAGAAAATCAAGTCGTGC-3′ and 5′-AGCAAGGAGGGCCTCTGATG-3′ (HIF-1α); (*b*) 5′-TACCTCCACCATGCCAAGTG-3′ and 5′-AAGATGTCCACCAGGGTCTC-3 (VEGF); and (*c*) 5′-ATCCTGCCAGTAGCATATGC-3′ and 5′-ACCGGGTTGGTTTTGAT-

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; ERK, extracellular signal-regulated kinase; COX2, cyclooxygenase 2; HIF-1, hypoxia-inducible factor 1; PI3K, phosphatidylinositol-3-kinase; 17-pt-PGE_2, 17-phenyl-trinor-prostaglandin E_2; IGF-1R, insulin-like growth factor-1 receptor; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; JNK, c-Jun NH_2-terminal kinase; PGE_2, prostaglandin E_2; MAP, mitogen-activated protein; RT-PCR, reverse transcription-PCR.

CTG-3′ (18S rRNA). Thermocycling conditions were 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C for 25 (HIF-1α), 27 (VEGF), or 13 (18S rRNA) cycles.

RNA Interference. To generate $siRNA_{HIF\text{-}1\alpha}$, two oligonucleotides consisting of ribonucleosides, except for the presence of 2′-deoxyribonucleosides at the 3′ end (5′-AGAGGUGGAUAUGUGUGGGdTdT-3′ and 5′-CCCACACAUAUCCACCUCUdTdT-3′), were synthesized and annealed (Dharmacon Research, Inc., Lafayette, CO). HCT116 cells were plated at 2×10^6 cells/10-cm dish and exposed to 100 nM $siRNA_{HIF\text{-}1\alpha}$ in the presence of Oligofectamine (Invitrogen Corp.) for 4 h and then cultured for 24 h in complete media (25). Cells were serum starved for another 24 h and exposed to 100 μM PGE_2 or vehicle for 24 h, and total RNA was isolated for RT-PCR analysis of HIF-1α and VEGF mRNA. As control, cells were exposed to Oligofectamine without $siRNA_{HIF\text{-}1\alpha}$. Neither mock transfection nor transfection with an siRNA targeted to an irrelevant mRNA inhibited HIF-1α mRNA or protein expression (26).

RESULTS

We first performed a time course experiment in which HCT116 cells were serum starved and exposed to PGE_2 for 0–40 h before preparation of whole cell protein lysates or isolation of total RNA. Immunoblot assay revealed that HIF-1β protein was constitutively expressed and HIF-1α protein expression was induced with peak levels observed 18–32 h after the addition of PGE_2 to the culture media (Fig. 1*A*). In contrast, we have demonstrated previously that maximal induction of HIF-1α protein expression is observed 8 h after IGF-1 addition to HCT116 cells (16). Thus, compared with IGF-1, PGE_2 induces HIF-1α protein expression with delayed kinetics. HIF-1α mRNA expression was not induced by PGE_2 stimulation (Fig. 1*A*), indicating that the increased HIF-1α protein levels resulted from either increased protein synthesis or decreased protein degradation. VEGF mRNA expression was induced by PGE_2 with kinetics similar to those observed for HIF-1α protein expression (Fig. 1*A*). Exposure of HCT116 cells to various concentrations of PGE_2 for 24 h induced HIF-1α protein and VEGF mRNA expression in a dose-dependent manner, although the responses were modest in comparison with the responses induced by hypoxia (Fig. 1*B*).

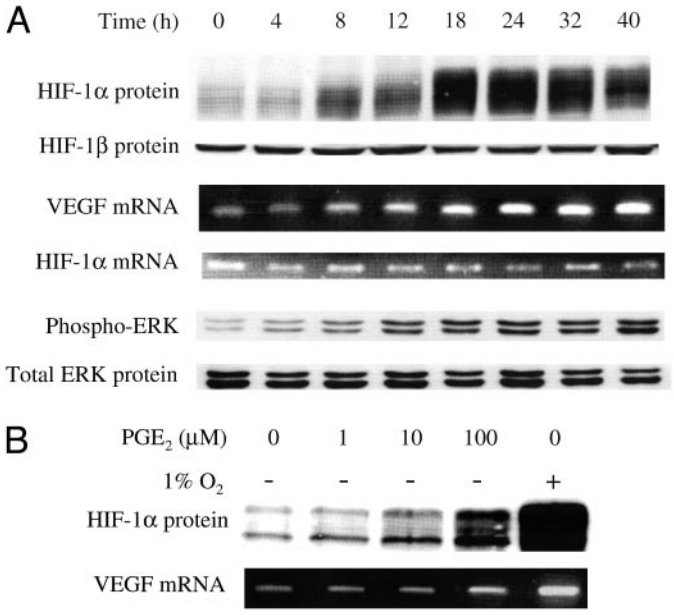

Fig. 1. PGE_2-induced responses in HCT116 cells. *A*, time course. Cells were serum starved for 24 h; 100 μM PGE_2 was added to the media for the indicated time, and cells were harvested for immunoblot (HIF-1α, HIF-1β, phospho-ERK [Thr202/Tyr204], and total ERK protein) and RT-PCR (VEGF and HIF-1α mRNA) assays. *B*, dose response. Cells were untreated (*Lane 1*) or exposed to 1–100 μM PGE_2 (*Lanes 2–4*) or 1% O_2 (*Lane 5*) for 24 h and harvested for analysis of HIF-1α protein or VEGF mRNA.

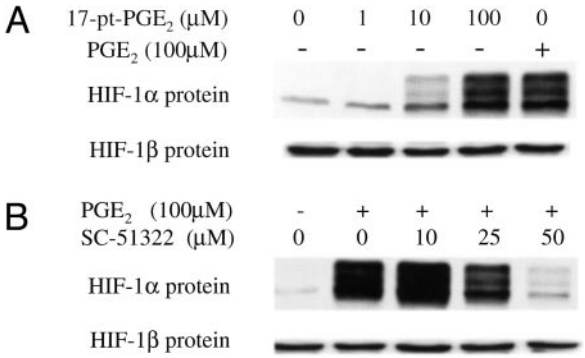

Fig. 2. Effect of EP_1 receptor agonist and antagonist. In *A*, HCT116 cells were untreated (*Lane 1*) or exposed to the EP_1 receptor agonist 17-pt-PGE_2 (*Lanes 2–4*) or PGE_2 (*Lane 5*) for 24 h. In *B*, cells were untreated (*Lane 1*) or exposed to PGE_2 for 24 h in the presence of 0–50 μM EP_1 receptor antagonist SC-51322 (*Lanes 2–5*).

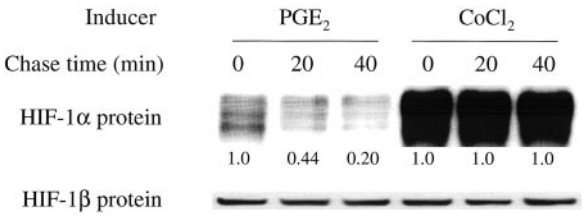

Fig. 3. Analysis of HIF-1α protein stability. HCT116 cells were exposed to 100 μM PGE_2 for 24 h (*Lanes 1–3*) or 100 μM $CoCl_2$ for 4 h (*Lanes 4–6*). Cycloheximide was added to a final concentration of 100 μM, and cells were harvested 0, 20, or 40 min later. The fraction of HIF-1α remaining is indicated.

PGE_2 binds to the G protein-coupled receptors EP_1, EP_2, EP_3, and EP_4. The EP_1 receptor-selective agonist 17-pt-PGE_2 was as effective as PGE_2 in stimulating HIF-1α protein expression in HCT116 cells (Fig. 2*A*). Furthermore, the EP_1 receptor-selective antagonist SC51322 inhibited PGE_2-induced HIF-1α expression in a dose-dependent manner (Fig. 2*B*). Neither 17-pt-PGE_2 nor SC51322 had any effect on HIF-1β expression. Thus, EP_1 receptor activation appears to be necessary and sufficient to induce HIF-1α expression in HCT116 cells.

In HCT116 cells subjected to hypoxia or IGF-1 treatment, increased HIF-1α protein levels result from a decreased rate of degradation and an increased rate of synthesis, respectively (16). To determine which of these mechanisms is involved in PGE_2-induced HIF-1α expression, cells were cultured for 24 h in the presence of PGE_2 or $CoCl_2$, which inhibits HIF-1α degradation, similar to the effect of hypoxia. The half-life of HIF-1α protein was $>$40 min in $CoCl_2$-treated cells but $<$20 min in PGE_2-treated cells (Fig. 3). These results indicate that, unlike $CoCl_2$, PGE_2 does not increase the half-life of HIF-1α protein and therefore must stimulate HIF-1α protein synthesis, similar to the effect of IGF-1.

To investigate the signal transduction pathways activated by PGE_2, we first analyzed the phosphorylation status of ERK. Phosphorylation of ERK was induced with delayed kinetics in response to PGE_2 stimulation (Fig. 1*A*). PD98059 inhibits the activity of MEK, which is responsible for the activating phosphorylation of ERK. PD98059 blocked the induction of HIF-1α protein and VEGF mRNA that was induced by PGE_2 (Fig. 4*A*). Wortmannin and rapamycin are inhibitors of PI3K and the downstream serine-threonine kinase FKBP rapamycin-associated protein (also known as mammalian target of rapamycin), respectively. Both Wortmannin and rapamycin partially inhibited PGE_2-induced HIF-1α protein and VEGF mRNA expression. Neither the inducers or inhibitors had any consistent effect on the expression of HIF-1β protein or HIF-1α mRNA (Fig. 4*A*). In the presence of a

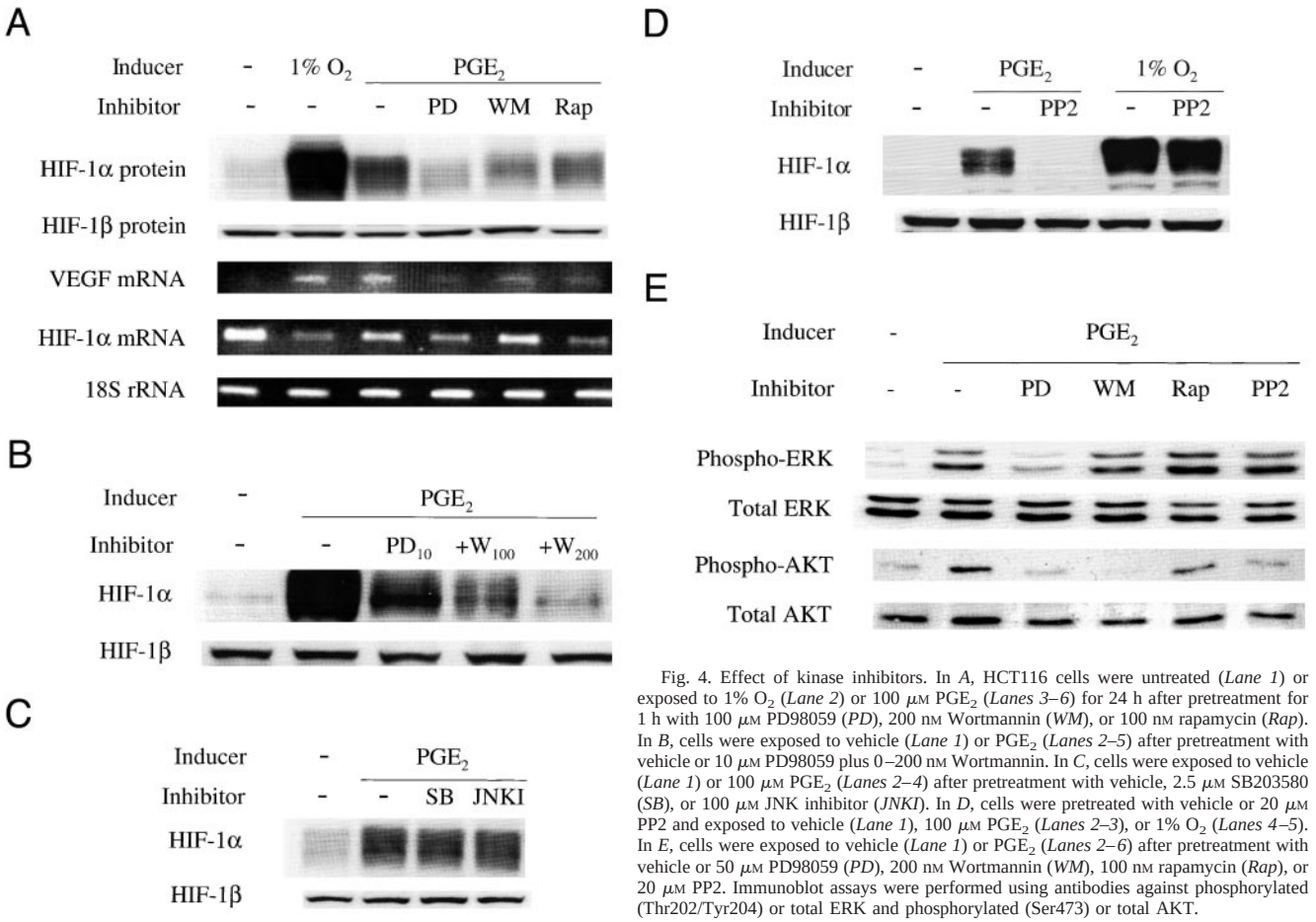

Fig. 4. Effect of kinase inhibitors. In *A*, HCT116 cells were untreated (*Lane 1*) or exposed to 1% O_2 (*Lane 2*) or 100 μM PGE_2 (*Lanes 3–6*) for 24 h after pretreatment for 1 h with 100 μM PD98059 (*PD*), 200 nM Wortmannin (*WM*), or 100 nM rapamycin (*Rap*). In *B*, cells were exposed to vehicle (*Lane 1*) or PGE_2 (*Lanes 2–5*) after pretreatment with vehicle or 10 μM PD98059 plus 0–200 nM Wortmannin. In *C*, cells were exposed to vehicle (*Lane 1*) or 100 μM PGE_2 (*Lanes 2–4*) after pretreatment with vehicle, 2.5 μM SB203580 (*SB*), or 100 μM JNK inhibitor (*JNKI*). In *D*, cells were pretreated with vehicle or 20 μM PP2 and exposed to vehicle (*Lane 1*), 100 μM PGE_2 (*Lanes 2–3*), or 1% O_2 (*Lanes 4–5*). In *E*, cells were exposed to vehicle (*Lane 1*) or PGE_2 (*Lanes 2–6*) after pretreatment with vehicle or 50 μM PD98059 (*PD*), 200 nM Wortmannin (*WM*), 100 nM rapamycin (*Rap*), or 20 μM PP2. Immunoblot assays were performed using antibodies against phosphorylated (Thr202/Tyr204) or total ERK and phosphorylated (Ser473) or total AKT.

submaximal concentration of PD98059, Wortmannin further decreased HIF-1α protein expression in a dose-dependent manner (Fig. 4*B*). Inhibitors of two other MAP kinases, p38 and JNK, had no effect on PGE_2-induced HIF-1α protein expression (Fig. 4*C*).

PP2, an inhibitor of C-SRC tyrosine kinase activity, completely blocked HIF-1α protein expression induced by PGE_2 but not by hypoxia (Fig. 4*D*). Although PD98059, Wortmannin, rapamycin, and PP2 all inhibited PGE_2-induced HIF-1α protein expression (Fig. 4*A–D*), only PD98059 blocked PGE_2-induced ERK phosphorylation (Fig. 4*E*). These results indicate that neither C-SRC nor PI3K pathway activation was required for PGE_2-induced ERK activation. PGE_2 induced phosphorylation of AKT that was blocked by pretreatment with Wortmannin (Fig. 4*E*). Pretreatment with PD98059 or PP2 partially inhibited PGE_2-induced AKT phosphorylation, suggesting that PI3K-AKT signaling was dependent on both ERK and C-SRC activity.

The data presented above (Figs. 1, *A* and *B* and 4*A*) demonstrate a remarkably consistent correlation between HIF-1α protein and VEGF mRNA expression in cells treated with various kinase inhibitors and/or PGE_2. To further demonstrate that HIF-1α is required for the induction of VEGF mRNA expression in response to PGE_2, HCT116 cells were mock transfected or transfected with a small interfering RNA ($siRNA_{HIF\text{-}1\alpha}$) that targets HIF-1$\alpha$ mRNA for degradation. After transfection, the cells were serum starved and exposed to vehicle or PGE_2. As expected, HIF-1α mRNA levels were reduced in cells transfected with $siRNA_{HIF\text{-}1\alpha}$ (Fig. 5). VEGF mRNA levels were slightly reduced in $siRNA_{HIF\text{-}1\alpha}$-transfected cells treated with vehicle and dramatically reduced in PGE_2-treated cells. Thus, inhibition of HIF-1α expression is sufficient to block PGE_2-induced VEGF mRNA expression.

DISCUSSION

The present studies have delineated molecular mechanisms by which PGE_2 stimulates *VEGF* mRNA expression in human colon carcinoma cells, thus providing a link between COX2 activity and tumor angiogenesis. Although most experiments involved exposing cells to relatively high concentrations of PGE_2, concentration-dependent effects were observed over the range of 1–100 μM. Induction of VEGF mRNA expression is mediated by binding of PGE_2 to the EP_1 G protein-coupled receptor and activation of the MEK-ERK and PI3K-AKT pathways. We have demonstrated previously that transfection of HCT116 cells with an expression vector encoding constitutively active MEK results in HIF-1α protein and VEGF mRNA expression (16). We demonstrate that, unlike hypoxia, PGE_2 treatment is not associated with an increase in the half-life of HIF-1α protein. Our data suggest that, as in the case of IGF-1, PGE_2 increases the rate of HIF-1α protein synthesis. The induction of HIF-1α protein and

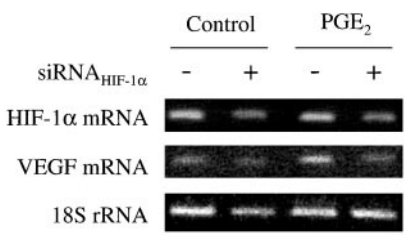

Fig. 5. Effect of HIF-1α RNA interference on VEGF mRNA expression. HCT116 cells were exposed to Oligofectamine in the absence (−) or presence (+) of a small interfering RNA directed against HIF-1α (*siRNA$_{HIF\text{-}1\alpha}$*). A day after transfection, the cells were serum starved for 24 h and then exposed to vehicle (*Control*) or 100 μM PGE_2 for 24 h.

VEGF mRNA expression in PGE_2-treated cells is completely or partially blocked by inhibitors of MEK and PI3K, respectively, as was also observed in IGF-1-treated HCT116 cells (16). Inhibition of C-SRC tyrosine kinase activity also blocks the induction of HIF-1α protein and VEGF mRNA expression in PGE_2-treated cells without blocking ERK phosphorylation. Our data indicate that the signal transduction from PGE_2 receptor binding to HIF-1α expression is complex, and additional studies are required to determine the mechanisms and consequences of ERK, PI3K, and C-SRC activation in PGE_2-treated HCT116 cells.

Although HIF-1α protein and VEGF mRNA expression are induced in HCT116 cells exposed to either IGF-1 or PGE_2, there are several notable differences in the respective signal-transduction pathways: (*a*) in contrast to IGF-1, PGE_2 induces ERK phosphorylation and HIF-1α expression with delayed kinetics, which suggests a requirement for gene expression; and (*b*) AKT phosphorylation is dependent on ERK activation in PGE_2-treated HCT116 cells but independent of ERK activation in IGF-1-treated cells. PGE_2-induced AKT phosphorylation is also dependent on C-SRC tyrosine kinase activity. Induction of HIF-1α protein and VEGF mRNA expression in V-SRC-transfected rodent cells has been demonstrated previously (27).

While this study was in preparation, the induction of HIF-1α expression by PGE_2 treatment of PC-3ML human prostate cancer cells was reported (28). HIF-1α expression was induced by EP_2 and EP_4 but not EP_3 receptor-selective agonists, whereas the EP_1 receptor was not expressed. PD98059 inhibited PGE_2-induced HIF-1α expression. However, HIF-1α expression was induced within 4 h in PGE_2-treated PC-3ML cells (28). These results suggest that different signal transduction pathways are activated by PGE_2 in prostate and colon cancer cells, although MEK-ERK activation appears to play an essential role in both cases. The effect of PGE_2 in PC-3ML cells was attributed to stabilization of HIF-1α protein, but no data were presented to support this conclusion. Additional studies are required to address this issue.

VEGF expression is induced in colon and other cancer cells as a result of hypoxia and multiple genetic alterations, including p53 and PTEN loss-of-function, RAS and SRC gain-of-function, and autocrine tyrosine kinase signaling pathways involving epidermal growth factor receptor, $HER2^{neu}$, and IGF-1R (29–35). In each case, *VEGF* gene expression is activated by HIF-1 (16, 17, 27, 36–39). Based on the analysis of prostate cancer cells recently reported (28) and colon cancer cells presented in this study, this list can now be extended to include the increased *VEGF* expression resulting from COX2-generated PGE_2. COX2 inhibitors, either alone or in combination with traditional cancer therapies such as radiation, have antiangiogenic effects (5, 40). As in the case of the tyrosine kinase-signaling pathways described above, the antiangiogenic effects of COX2 inhibitors appear to be attributable in part to their inhibition of HIF-1α expression (28, 41). Efforts to identify small molecules that directly inhibit HIF-1 activity are under way (42), and such compounds may represent useful additions to the armamentarium of anticancer agents that target signal transduction pathways and angiogenesis.

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