Vascular Endothelial Growth Factor Gene Expression in Colon Cancer Cells Exposed to Prostaglandin E2 Is Mediated by Hypoxia-inducible Factor 1

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ABSTRACT

Prostaglandin E2 (PGE2) has been implicated as an inducer of angiogenesis in human colon cancer. Here, we demonstrate that PGE2 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). PGE2 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 PGE2 stimulation. Inhibition of C-Src tyrosine kinase activity also blocks PGE2-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 PGE2.

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 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 metastasis and mortality (1, 2). The level of VEGF expression is a major determinant of colon cancer metastasis and patient mortality (1, 2). The level of VEGF expression is a major determinant of microvessel density in colon cancers, and VEGF expression is also correlated with metastasis and mortality (1, 2).

In the present study, we investigated the mechanism by which PGE2 exposure induces VEGF gene expression in HCT116 human colon carcinoma cells. We demonstrate that PGE2 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 PGE2.

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). PGE2, PD98059, Wortmannin, rapamycin, SB203580, cycloheximide, and cobalt chloride (CoCl2) were from Sigma (St. Louis, MO). SC-51322 and 17-PT-PGE2, 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% O2/5% CO2 balance N2, sealed, and incubated at 37°C.

PGE2 and Inhibitor Treatments. HCT116 cells were plated at a density of 2.5 × 104/10-cm or 8.6 × 106/cm dish. Subconfluent cells were serum starved (0.1% FBS) for 24 h before PGE2 or 17-PT-PGE2 was added. Kinase inhibitors PD98059, Wortmannin, and rapamycin, and SB203580 were added 1 h before exposure to PGE2, 1% O2, or 100 μM CoCl2. JNK inhibitor and PP2 were added 20 min and 2 h, respectively, before the exposure to PGE2 or 1% O2. EP1 receptor antagonist SC-51322 was added 30 min before exposure to PGE2. Cycloheximide was added to the media of HCT116 cells that had been serum starved and treated with CoCl2 for 4 h or PGE2 for 24 h, and whole cell extracts were prepared at 0, 20, and 40 min.

Immunoblot Assays. Whole cell extracts were prepared using radiomunoprecipitation 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 for HIF-1α, VEGF, and 18S rRNA sequences. The following oligonucleotides were used as primers: (a) 5′-GGGAGAAATCAAGTTGTCG-3′ and 5′-AGCAAGGGGCGTCAGTG-3′ (HIF-1α); (b) 5′-TACCTCCACCATGGCAAGTG-3′ and 5′-AAGATGTCACCAAGGTTTC-3′ (VEGF); and (c) 5′-ATCTGCGCAGTACGATGTCG-3′ and 5′-ACCGGGATTGTTTGTG-
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-1α, two oligonucleotides consisting of ribonucleosides, except for the presence of 2′-deoxyribonucleosides at the 3′ end (5′-AGAGGGUGGUAUGUGGGGdTdT-3′ and 5′-CCCAACAUCCACCUCCUdTdT-3′), were synthesized and annealed (Dharmacon Research, Inc., Lafayette, CO). HCT116 cells were plated at 2 × 10⁶ cells/10-cm dish and exposed to 100 nM siRNA HIF-1α 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 PGE₂ 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-1α. 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 PGE₂ for 0–40 h before preparation of whole cell protein lysates or isolation of total RNA. Immunoblot assay revealed that HIF-1α protein was constitutively expressed and HIF-1α protein expression was induced with peak levels observed 18–32 h after the addition of PGE₂ to the culture media (Fig. 1A). 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, PGE₂ induces HIF-1α protein expression with delayed kinetics. HIF-1α mRNA expression was not induced by PGE₂ stimulation (Fig. 1A), indicating that the increased HIF-1α protein levels resulted from either increased protein synthesis or decreased protein degradation. VEGF mRNA expression was induced by PGE₂ with kinetics similar to those observed for HIF-1α protein expression (Fig. 1A). Exposure of HCT116 cells to various concentrations of PGE₂ 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. 1B).

**Fig. 1.** PGE₂-induced responses in HCT116 cells. A, time course. Cells were serum starved for 24 h, 100 μM PGE₂ 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 PGE₂ (Lanes 2–4) or 1% O₂ (Lane 5) for 24 h and harvested for analysis of HIF-1α protein or VEGF mRNA.

**Fig. 2.** Effect of EP₁ receptor agonist and antagonist. In A, HCT116 cells were untreated (Lane 1) or exposed to the EP₁ receptor agonist 17-pt-PGE₂ (Lanes 2–4) or PGE₂ (Lane 5) for 24 h. In B, cells were untreated (Lane 1) or exposed to PGE₂ for 24 h in the presence of 0–50 μM EP₁ receptor antagonist SC51322 (Lanes 2–5).

<table>
<thead>
<tr>
<th>Inducer</th>
<th>PGE₂ (μM)</th>
<th>CoCl₂ (μM)</th>
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<tbody>
<tr>
<td>Chase time (min)</td>
<td>0</td>
<td>20</td>
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<tr>
<td>HIF-1α protein</td>
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<td>0.44</td>
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<tr>
<td>HIF-1β protein</td>
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**Fig. 3.** Analysis of HIF-1α protein stability. HCT116 cells were exposed to 100 μM PGE₂ for 24 h (Lanes 1–5) or 100 μM CoCl₂ 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.

PGE₂ binds to the G protein-coupled receptors EP₁, EP₂, EP₃, and EP₄. The EP₁ receptor-selective agonist 17-pt-PGE₂ was as effective as PGE₂ in stimulating HIF-1α protein expression in HCT116 cells (Fig. 2A). Furthermore, the EP₁ receptor-selective antagonist SC51322 inhibited PGE₂-induced HIF-1α expression in a dose-dependent manner (Fig. 2B). Neither 17-pt-PGE₂ nor SC51322 had any effect on HIF-1β expression. Thus, EP₁ 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 PGE₂-induced HIF-1α expression, cells were cultured for 24 h in the presence of PGE₂ or CoCl₂, which inhibits HIF-1α degradation, similar to the effect of hypoxia. The half-life of HIF-1α protein was >40 min in CoCl₂-treated cells but <20 min in PGE₂-treated cells (Fig. 3). These results indicate that, unlike CoCl₂, PGE₂ 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 PGE₂, we first analyzed the phosphorylation status of ERK. Phosphorylation of ERK was induced with delayed kinetics in response to PGE₂ (Fig. 1A). Exposure of HCT116 cells to 17-pt-PGE₂ resulted in the phosphorylation of ERK (Thr202/Tyr204) and total ERK protein. This phosphorylation was inhibited by the EP₁ receptor-selective antagonist SC51322 (Fig. 2B). PGE₂ induced the phosphorylation of ERK in a dose-dependent manner, similar to the effect of IGF-1.

To determine the effect of PI3K and the downstream serine-threonine kinase FKBP rapamycin, we treated cells with the PI3K inhibitor PI3K and the downstream serine-threonine kinase FKBP rapamycin. This treatment inhibited the phosphorylation of ERK, similar to the effect of IGF-1.
remarkably consistent correlation between HIF-1α activation was required for PGE2-induced ERK activation. PGE2 increased HIF-1α protein expression in a dose-dependent manner (Fig. 4B). Inhibitors of two other MAP kinases, p38 and JNK, had no effect on PGE2-induced HIF-1α protein expression (Fig. 4C).

PP2, an inhibitor of C-SRC tyrosine kinase activity, completely blocked HIF-1α protein expression induced by PGE2 but not by hypoxia (Fig. 4D). Although PD98059, Wortmannin, rapamycin, and PP2 all inhibited PGE2-induced HIF-1α protein expression (Fig. 4A–D), only PD98059 blocked PGE2-induced ERK phosphorylation (Fig. 4E). These results indicate that neither C-SRC nor PI3K pathway activation was required for PGE2-induced ERK activation. PGE2-induced phosphorylation of AKT that was blocked by pretreatment with Wortmannin (Fig. 4E). Pretreatment with PD98059 or PP2 partially inhibited PGE2-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 4A) demonstrate a remarkably consistent correlation between HIF-1α protein and VEGF mRNA expression in cells treated with various kinase inhibitors and/or PGE2. To further demonstrate that HIF-1α is required for the induction of VEGF mRNA expression in response to PGE2, HCT116 cells were mock transfected or transfected with a small interfering RNA (siRNAHIF-1α) that targets HIF-1α mRNA for degradation. After transfection, the cells were serum starved and exposed to vehicle or PGE2. As expected, HIF-1α mRNA levels were reduced in cells transfected with siRNAHIF-1α (Fig. 5). VEGF mRNA levels were slightly reduced in siRNAHIF-1α-transfected cells treated with vehicle and dramatically reduced in PGE2-treated cells. Thus, inhibition of HIF-1α expression is sufficient to block PGE2-induced VEGF mRNA expression.

**DISCUSSION**

The present studies have delineated molecular mechanisms by which PGE2 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 PGE2, concentration-dependent effects were observed over the range of 1–100 μM. Induction of VEGF mRNA expression is mediated by binding of PGE2 to the EP1 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, PGE2 treatment is not associated with an increase in the half-life of HIF-1α protein. Our data suggest that, in the case of IGF-1, PGE2 increases the rate of HIF-1α protein synthesis. The induction of HIF-1α protein and

![Image](cancerres.aacrjournals.org)
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$_3$ and EP$_4$ but not EP$_2$ 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, HER2neo, 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). Attempts 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.

REFERENCES


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