Direct Transcriptional Up-regulation of Cyclooxygenase-2 by Hypoxia-Inducible Factor (HIF)-1 Promotes Colorectal Tumor Cell Survival and Enhances HIF-1 Transcriptional Activity during Hypoxia

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Abstract

Cyclooxygenase (COX)-2, the inducible key enzyme for prostanoid biosynthesis, is overexpressed in most colorectal carcinomas and a subset of colorectal adenomas. Genetic, biochemical, and clinical evidence indicates an important role for COX-2 in colorectal tumorigenesis. Although COX-2 can be induced by aberrant growth factor signaling and oncogene activation during colorectal tumorigenesis, the role of micro-environmental factors such as hypoxia in COX-2 regulation remains to be elucidated. For the first time, we report that under hypoxic conditions COX-2 protein levels increase in colorectal adenoma and carcinoma cells. Rigorous analyses reveal that COX-2 up-regulation is transcriptional and is associated with hypoxia-inducible factor (HIF)-1α induction. Oligonucleotide pull-down and chromatin immunoprecipitation assays reveal that HIF-1α binds a hypoxia-responsive element on the COX-2 promoter. COX-2 up-regulation during hypoxia is accompanied by increased levels of prostaglandin E2 (PGE2), which promote tumor cell survival under hypoxic conditions. In addition, elevated levels of PGE2 in hypoxic colorectal tumor cells enhance vascular endothelial growth factor expression and HIF-1 transcriptional activity by activating the mitogen-activated protein kinase pathway, showing a potential positive feedback loop that contributes to COX-2 up-regulation during hypoxia. This study identifies COX-2 as a direct target for HIF-1 in colorectal tumor cells. In addition, COX-2 up-regulation represents a pivotal cellular adaptive response to hypoxia with implication for colorectal tumor cell survival and angiogenesis. We propose that using modified COX-2-selective inhibitors, which are only activated under hypoxic conditions, could potentially be a novel more selective strategy for colorectal cancer prevention and treatment. (Cancer Res 2006; 66(13): 6683-91)

Introduction

Colorectal cancer results from the accumulation of intrinsic genetic and epigenetic changes that alter signaling pathways, which regulate cell proliferation, differentiation, and cell death (1) as well as the selection process imposed by environmental and microenvironmental factors, which ultimately leads to clonal evolution and tumor progression (2). Cyclooxygenase (COX)-2 is overexpressed in most colorectal carcinomas and a subset of adenomas (3, 4), and accumulating evidence supports an important role for COX-2 in colorectal tumorigenesis (5). COX-2-selective inhibitors have a potent antitumor effect in vitro (6, 7) and in vivo (8), and genetic depletion of the COX-2 gene results in a marked decrease in adenoma burden in the Apc1776 mouse model of intestinal tumorigenesis (9). Enhanced COX-2 expression in colon cancer cells results in increased levels of proangiogenic factors, such as vascular endothelial growth factor (VEGF), thus the promotion of angiogenesis (10). COX-2, like its isoenzyme COX-1, catalyses the conversion of arachidonic acid to endoperoxide intermediates, which are ultimately converted to prostanoids. Recent evidence points to an important role for the COX-2 downstream metabolite prostaglandin E2 (PGE2) in colorectal tumorigenesis (11). Increased levels of PGE2 were reported in human colorectal cancers and adenomas in familial adenomatous polyposis (FAP) patients (12). Additionally, PGE2 can stimulate cell proliferation and motility (13) while inhibiting apoptosis in colorectal cancer cells (14). More recently, PGE2 was shown to enhance intestinal adenoma growth via activation of the Ras-mitogen-activated protein kinase (MAPK) cascade in Apcmin mice (15). Given the critical role for COX-2 up-regulation in colorectal cancer and other cancers, it is important to achieve a greater understanding of the regulatory networks that control COX-2 expression.

Numerous factors, including growth factors, cytokines, oncogenes, and tumor promoters, stimulate COX-2 transcription via transcription factors, such as activator protein, NF-IL6, NF-κB, NFAT, and PEA3 (5). In the context of colorectal tumorigenesis, although mutations resulting in aberrant Wnt and Ras signaling have been implicated in COX-2 up-regulation (16), the role of microenvironmental factors, such as hypoxia in COX-2 regulation during colorectal tumorigenesis, has not been investigated.

Hypoxia, which refers to oxygen deficiency in tissues, is a universal hallmark of solid tumors and it represents a key regulatory factor in tumor growth and survival (17). Although, the hypoxic microenvironment is thought to be associated with tumor progression (18), persistent hypoxia can also result in cell death (19). Therefore, hypoxia represents an important selection pressure that drives clonal progression of tumors (20). Adaptation to hypoxia is critical for tumor cell survival and is mediated largely by activation of genes that facilitate short-term adaptation (e.g., increased vascular permeability) as well as long-term adaptive mechanisms (e.g., angiogenesis; refs. 17, 21, 22).

The coordinated homeostatic response to hypoxia is largely transcriptional and is mediated primarily through the activation of...
the heterodimeric transcription factor hypoxia-inducible factor (HIF)-1α (23). HIF-1α is composed of two subunits: the oxygen-sensitive HIF-1α and the constitutively expressed HIF-1β subunit (23). In normoxia, HIF-1α is hydroxylated at key proline residues facilitating von Hippel-Lindau protein binding, which in turn allows ubiquitination and subsequent proteasome-targeted degradation (24). Under hypoxic conditions, proline hydroxylation is inhibited, thereby stabilizing HIF-1α, which can then translocate into the nucleus and bind to constitutively expressed HIF-1β, forming the active HIF-1 complex (25). The HIF-1 complex recruits the transactivator p300/CREM, resulting in enhanced transcriptional activity (26). HIF-1 binds a conserved DNA consensus on promoters of its target genes known as the hypoxia-responsive element (HRE; ref. 23). The core sequence of the HRE is 5'-CGTG-3', and the optimum mammalian HRE was defined to be 5'-BAα(G)CGTGVBBB-3' (where B refers to all bases except A and V refers to all bases except T; ref. 27).

HIF-1α is overexpressed in various types of cancer, including colorectal cancer (28), and compelling evidence supports a role for HIF-1 in tumorigenesis (29, 30). The HIF-1 transcriptional response largely allows cellular adaptation to the hypoxic microenvironment (31). Therefore, identification and characterization of the mechanisms underlying the adaptive responses to hypoxia are vital for an increased understanding of the tumorigenic process and, most importantly, for the development of novel therapeutic approaches.

Because COX-2 expression increases during colorectal tumor progression (3, 4) and given the key role of COX-2 in colorectal tumorigenesis (9), we hypothesized that COX-2 may be a hypoxia-responsive gene whose up-regulation may facilitate adaptation to cellular stress imposed by hypoxia. Indeed, we describe a novel mechanism for COX-2 up-regulation in colorectal tumor cells during hypoxia through HIF-1α. During hypoxia, COX-2 up-regulation results in higher levels of PGE2, which then promotes cellular stress imposed by hypoxia. Furthermore, we describe a novel mechanism for COX-2 up-regulation in colorectal tumor cells during hypoxia through HIF-1α. During hypoxia, COX-2 up-regulation results in higher levels of PGE2, which then promotes cellular stress imposed by hypoxia. Collectively, our results identify COX-2 up-regulation as a critical adaptive response to hypoxia with implications for colorectal tumor cell survival and angiogenesis.

Materials and Methods

Cell culture and treatments. The carcinoma cell lines HT29 and HCT116 were from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in DMEM or McCoy’s 5A media supplemented with 10% fetal bovine serum, respectively. The adenoma cell line AA/C1 was derived in this laboratory and maintained as described previously (2). AA/C1 is derived from a single adenoma from a patient with FAP, and AA/C1/SB10C represents an in vitro–transformed variant of AA/C1 (2). For hypoxic exposure, cells were placed in a modulator-incubator in an atmosphere exposed, cells were placed in a modulator-incubator in an atmosphere

Determination of cell yield and cell death. After treatment, the cell yield was determined by counting the adherent cells. The level of cell death was assessed by measuring the proportion of floating apoptotic cells, which had detached from the tissue culture flask, and cell death was represented as a percentage of total cell number as described previously (33). The induction of cell death was characterized as apoptotic both morphologically (following acridine orange staining) and biochemically as shown by poly(ADP-ribose) polymerase cleavage as described in detail previously (33).

Cell cycle analysis. Adherent HT29 cells were collected and fixed in 70% ethanol. Cells were resuspended in PBS containing 10 μg/ml propidium iodide (Sigma) and 5 μg/ml RNase A (Sigma) and incubated at room temperature for 30 minutes before fluorescence-activated cell sorting analysis was done using a FACScan (BD Biosciences, Oxford, United Kingdom). Data analysis was done using Cell Quest software (BD Biosciences).

Transient transfection and reporter assays. The expression vector pcDNA3/HIF-1α/P402A-P564G (generous gift from Peter Ratcliffe, University of Oxford, Oxford, United Kingdom) encodes a mutant version of HIF-1α, which is resistant to degradation (34). HT29 cells were transiently transfected with either HIF-1α expression plasmid or empty vector (pcDNA3) plasmid using Tfx (Promega, Southampton, United Kingdom) following the manufacturer's protocol.

The HRE reporter p11w was from the ATCC, and the COX-2 promoter reporter was a generous gift from Stephen Prescott (University of Utah, Salt Lake City, UT). COX-2 promoter deletions were carried out starting from the full-length COX-2 promoter using unique restriction sites and subcloned into a pGL3-basic vector. For luciferase reporter assays, HT29 cells were transfected with the firefly luciferase reporter construct and the control renilla luciferase reporter pRL-SV40 using Tfx reagent. After treatment, the luciferase activity was measured using the dual-luciferase reporter assay system (Promega) following the manufacturer's protocol.

Western blot analysis. Whole-cell extracts were prepared by lysing cells with Cell Signalling Technology lysis buffer supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was quantified using Bio-Rad Dc protein assay kit (Bio-Rad, Hemel Hempstead, United Kingdom). Protein lysates were resolved on SDS-PAGE, and proteins were detected by Western blotting with mouse monoclonal antibodies against HIF-1α, HIF-1β (BD Biosciences), COX-2 (Cayman Europe), and α-tubulin (Sigma) and rabbit antibodies against ERK and pERK using enhanced chemiluminescence detection system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

RNA extraction and Northern analysis. Total RNA was isolated using the RNeasy kit (Qiagen, Crawley, United Kingdom). RNA (20 μg) samples were resolved on a 1% formaldehyde agarose gel and analyzed by Northern blotting. COX-2 mRNAs were detected using a biotinylated human COX-2 probe. The COX-2 probe was prepared by amplifying a fragment from COX-2 cDNA using the biotinylated primers: forward primer (5'-GGTGCCGATG-GATGGCCCGACTCC-3') and reverse primer (5'-TTGTGTGTTCTCCTCCGAC-CAGATTG-3'). Visualization was carried out using the nonradioactive NorthSouth hybridization and detection kit (Pierce Biotechnology, Rockford) following the manufacturer's protocol.

Extraction of nuclear DNA-binding proteins. Extraction of DNA-binding proteins from HT29 cells was done following a standard procedure. Briefly, HT29 cells were washed with ice-cold PBS and resuspended in 400 μL ice-cold low-salt buffer [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L aminoethyl-benzenesulfonyl fluoride]. The nuclei were collected by centrifugation and then resuspended in 40 μL ice-cold high-salt buffer [20 mmol/L HEPES-KOH (pH 7.9), 25% v/v glycerol, 420 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride] and incubated on ice for 20 minutes, and then the lysates were cleared by centrifugation.

Oligonucleotide pull-down assay. Nuclear DNA-binding protein extracts (100 μg) were incubated at 30°C for 10 minutes with either 0.5 mmol 5'-biotinylated double-stranded wild-type (WT) oligonucleotide (5'-ATTTCCTTATGCTTGGTGTAAACACCCC-3') or mutant oligonucleotide (5'-ATTTCCTTACCTATGCTTGGTGTAAACACCCC-3') (Sigma-Genosys, Haverhill, United Kingdom) coupled previously to streptavidin agarose beads (Sigma). After incubation, the biotinylated oligonucleotide-coupled streptavidin beads were washed six times. Samples were denatured in SDS sample buffer and subjected to SDS-PAGE. HIF-1α was detected by Western blotting. For the competition assay, excess nonbiotinylated oligonucleotides were used.

Chromatin immunoprecipitation assay. The procedure was done using chromatin immunoprecipitation (ChIP) kit (Upstate, Lake Placid, NY). Briefly, HT29 cells growing in T25 flasks under normoxia or hypoxia were

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cross-linked using 1% formaldehyde at 37°C for 10 minutes. After washing with PBS, cells were resuspended in 300 μL lysis buffer [50 mmol/L Tris-HCl (pH 8.1), 10 mmol/L EDTA, 1% SDS, protease inhibitor cocktail]. DNA was sheared to small fragments of 200 to 900 bp by sonication. The supernatant was recovered, diluted, and preclued using herring sperm DNA/protein G-Sepharose slurry (Sigma). The recovered supernatant was incubated with either anti-HIF-1α antibody or an isotype control IgG for 2 hours in the presence of herring sperm DNA and protein G-Sepharose beads. The beads were washed with low-salt, high-salt, and LiCl buffers. The immunoprecipitated DNA was retrieved from the beads with 1% SDS and 1.1 mol/L NaHCO₃ solution at 65°C for 6 hours. DNA was then purified using a PCR purification kit (Qiagen), and PCR was done on the extracted DNA using COX-2 promoter-specific primers forward (5’-GAATTTACCTTTCCCGCCTCTC-3’) and reverse (5’-AAGCCCGGTGGG-GGCAGGGTTT-3’).

VEGF quantification. VEGF level was determined in growth medium using a VEGF ELISA (R&D Systems, Arbington, United Kingdom) following the manufacturer’s instructions. The growth medium was removed and cleared by centrifugation, and adherent cells were counted. The level of VEGF was determined in samples at two different dilutions (1:1 and 1:10) in triplicate and standardized to cell number.

Determination of PGE₂ levels. PGE₂ levels were measured using competitive enzyme immunoassay for PGE₂ (Cayman Europe) following the manufacturer’s protocol.

Statistical analysis. Statistical tests were carried out using t test done on Microsoft Excel. Statistical significance was determined and expressed as P ≤ 0.01.

Results

COX-2 protein expression is up-regulated in colorectal tumor cells in response to hypoxia. To achieve a further understanding of the regulatory networks controlling COX-2 expression, we sought to investigate the role of an important microenvironmental factor, hypoxia, in COX-2 regulation. For this, colorectal carcinoma (HT29 and HCT116), colorectal adenoma (AA/C1), and in vitro–transformed adenoma (AA/C1/SB10C) cells were subjected to growth under hypoxic conditions (1% O₂), and protein extracts were prepared over time (Fig. 1A). Immunoblot analysis confirmed that cells were exposed to hypoxia, as HIF-1α was rapidly induced in both colorectal adenoma and carcinoma cells (Fig. 1A). Interestingly, COX-2 protein levels increased in a time-dependent manner on exposure to hypoxia in all the cell lines used (Fig. 1A) irrespective of the basal levels of COX-2 expressed by these cells. It is important to note that, in response to hypoxia, HIF-1α induction preceded COX-2 up-regulation (Fig. 1A).

Interestingly, COX-1 protein levels did not change in response to hypoxia in any of the cell lines used (data not shown).

COX-2 is transcriptionally up-regulated during hypoxia. Having shown COX-2 up-regulation by hypoxia in colorectal tumor cells, we aimed to gain some insights into the mechanism underlying this up-regulation. Initially, we used a luciferase reporter assay using a COX-2 promoter to assess COX-2

Figure 1. COX-2 up-regulation in colorectal tumor cells during hypoxia. A, Western blot analyses for HIF-1α and COX-2 protein levels during hypoxia. Colorectal tumor cell lines (carcinoma cells, HT29 and HCT116; adenoma cells, AA/C1; and in vitro–transformed adenoma, AA/C1/SB10C), which express different basal levels of COX-2 (6, 50), were exposed to hypoxia (1% O₂), and HIF-1α and COX-2 protein levels were analyzed by Western blotting over time as indicated. α-Tubulin was used as a protein loading control. B, HT29 cells were transiently transfected with the COX-2 promoter luciferase reporter and pRL-SV40 plasmids and grown in either normoxia (N) or hypoxia (H) for 16 hours. The luciferase activity was determined. Columns, mean of three independent experiments; bars, SD. C, Northern blot analysis for COX-2 mRNA levels in HT29 cells exposed to hypoxia for the indicated time using a biotinylated COX-2 probe. The 18S rRNA was used as a control for equal loading. D, examination of COX-2 mRNA decay in HT29 cells after growth in hypoxia/normoxia. HT29 cells were exposed to hypoxia/normoxia for 16 hours and transferred to normoxia after treatment with 10 μmol/L actinomycin-D. Cells were harvested every hour over a 3-hour period, and COX-2 mRNA levels were analyzed by Northern blotting.

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Figure 2. Direct involvement of HIF-1 in COX-2 up-regulation during hypoxia in colorectal cancer cells. A, association between HIF-1α induction and COX-2 up-regulation. A, left, Western blot analysis for HIF-1α and COX-2 protein levels in HT29 cells treated with increasing concentrations of the HIF-1α-specific inducer deferoxamine (DFO). A, middle, HT29 cells were transiently transfected with either the pcDNA3 (V), vector control, or the pcDNA3-HIF-1α (HIF-1α) expression vector, and the levels of HIF-1α and COX-2 were analyzed by Western blotting. A, right, effect of HIF-1α inhibition on COX-2 up-regulation during hypoxia in HT29 cells. HT29 cells were exposed to hypoxia in the presence of increasing concentrations of the guanylyl cyclase inhibitor YC-1 for 16 hours and then followed by Western blotting for HIF-1α and COX-2. 

B, nucleotide sequence of the COX-2 promoter. Bold uppercase letters, core sequences of the four HREs, CGTG; bold lowercase letters, transcription start point; underlined, restriction sites used in the subcloning of shorter fragments of the COX-2 promoter. Diagrammatic representation of the progressively shorter fragments of the COX-2 promoter [full length (P1), deleted HRE1 (P2), deleted HRE1 and HRE2 (P3), and deleted HRE3 (P4)] and their relative location. Relative luciferase reporter activity of the different promoter constructs after transfection in HT29 cells and exposure to hypoxia. Columns, mean of three independent experiments done in triplicates; bars, SD. C, top, oligonucleotides used in the pull-down assay. The WT oligonucleotide corresponds to fragment –519 to –490 from the COX-2 promoter [highlighted in (B)] and contains a HRE core sequence (underlined, position –506); this sequence was mutated in the mutant (Mut) oligonucleotide (underlined). C, bottom, oligonucleotide pull-down assay. HT29 cells were grown in normoxia or hypoxia for 16 hours, and nuclear protein extracts were prepared. The pull-down assay was done as described in Materials and Methods. Samples were denatured and resolved on SDS-PAGE, and then gels were probed with HIF-1α monoclonal antibody. D, ChIP assay. HT29 cells were grown in normoxia/hypoxia for 16 hours, cells were then fixed and lysed, and DNA was sheared by sonication to 200- to 900-bp fragments (DNA shearing efficacy was assessed by gel electrophoresis). Immunoprecipitation was carried out using an HIF-1α monoclonal antibody or an irrelevant mouse IgG. The protein DNA complexes were denatured, and DNA was recovered and purified using a PCR purification kit. A PCR using COX-2 promoter primer was done on the input (genomic DNA) from normoxia- and hypoxia-treated HT29 cells as well as the immunoprecipitated DNA (using HIF-1α and mouse IgG). PCR products were analyzed by gel electrophoresis, and the size of the COX-2 promoter fragment was determined using the DNA marker (M).
Hypoxia-Induced COX-2 Promotes Cell Survival

Figure 3. The effect of COX-2 up-regulation on colorectal cancer cell growth and survival during hypoxia. A, COX-2 up-regulation during hypoxia results in increased PGE2 levels. HT29 cells were exposed to normoxia or hypoxia in the presence or absence of 10 μmol/L COX-2-selective inhibitor NS-398 for 24 hours, and then PGE2 levels were determined in the growth medium using a commercial ELISA and standardized to cell number. Columns, mean of three experiments done in triplicate; bars, SD. B, effect of hypoxia on cell growth and survival during hypoxia. HT29 cells were exposed to normoxia or hypoxia for 24 hours, and the number of adherent and floating apoptotic cells were determined as described in Materials and Methods. Columns, mean of three independent experiments done in triplicate; bars, SD. C, PGE2 promotes cancer cell growth and survival under hypoxic conditions. HT29 cells were treated in hypoxia with NS-398 (10 μmol/L), PGE2 (1 μmol/L), or a combination of both for 24 hours to examine the effect of PGE2 on cell growth and survival under hypoxic conditions. The number of attached and floating apoptotic cells were determined as described in Materials and Methods. Columns, mean of three different experiments done in triplicate; bars, SD.

COX-2 up-regulation is associated with HIF-1α induction. Because COX-2 was transcriptionally up-regulated in hypoxic HT29 cells and given the critical role that HIF-1 plays in the cellular transcriptional response to hypoxia, we investigated whether HIF-1 is involved in hypoxia-mediated COX-2 up-regulation. To examine the association between HIF-1α induction and COX-2 up-regulation, HT29 cells were treated with increasing concentrations of the hypoxia surrogate deferoxamine (a specific HIF-1α inducer) for 16 hours. Western blot analysis revealed that hypoxia-mediated COX-2 protein levels in HT29 cells in a dose-dependent manner (Fig. 2A, left). Additionally, transient ectopic expression of HIF-1α in HT29 cells, achieved by transfection with a mutant HIF-1α that is resistant to degradation (34), resulted in a 2.5-fold increase in COX-2 protein levels in normoxia compared with HT29 cells transfected with the vector-only control plasmid (Fig. 2A, middle). This increase in COX-2 protein levels was consistent in several experiments, ranging from 2- to 3-fold. Furthermore, inhibiting HIF-1α induction during hypoxia using 200 μmol/L YC-1 (32) abolished hypoxia-mediated COX-2 up-regulation (Fig. 2A, right). Altogether, these observations suggest that HIF-1 is involved in hypoxia-induced COX-2 up-regulation.

HIF-1 directly binds to the COX-2 promoter. Having established an association between HIF-1α induction and COX-2 up-regulation, a direct involvement for HIF-1 in COX-2 transcription was investigated. HIF-1 has been shown to bind HRE on promoters of target genes and activate their transcription (23). Screening of the COX-2 promoter region for HIF-1-binding sites revealed the presence of four putative HRE consensuses...
(HRE1, HRE2, HRE3, and HRE4) in the sense orientation (Fig. 2B). From these four HRE sites, only the HRE3 (5'-GACGTAACAT-3') located at position 506 upstream the transcription initiation point (~506) fulfilled the criteria for mammalian HRE. Indeed, COX-2 promoter deletion analysis suggested the importance of HRE3 in the responsiveness of COX-2 promoter to hypoxia, as deletion of HRE3 abolished hypoxia-mediated activation of the COX-2 promoter reporter (Fig. 2B). To test the physical interaction of HIF-1 with the HRE3 of the human COX-2 promoter, we used biotinylated double-stranded oligonucleotides (Fig. 2C) coupled to streptavidin agarose beads to pull-down proteins interacting with the HRE3 in HT29 cells growing in normoxia or hypoxia. The bound protein complexes were then analyzed by Western blotting.

The HRE3 in HT29 cells growing in normoxia or hypoxia. The streptavidin agarose beads to pull-down proteins interacting with a HRE3 mutated. The binding capacity of HIF-1 oligonucleotide but not to a similar oligonucleotide with the HIF-1 in a competition assay (Fig. 2C). These results indicate that HIF-1α directly binds the HRE3 derived from the COX-2 promoter. We next did ChIP to determine whether HIF-1 binds the COX-2 promoter in HT29 cells. As shown in Fig. 2D, the anti-HIF-1α antibody, but not the control IgG antibody, precipitated the COX-2 promoter fragment spanning HRE3 in hypoxic HT29 cells. These data show that HIF-1 binds the COX-2 promoter at HRE3 site located at ~506.

COX-2 up-regulation in hypoxia results in increased PGE2 levels. Previous reports have shown a critical role for the COX-2 metabolite PGE2 in colorectal tumorigenesis (15). Having shown COX-2 up-regulation in hypoxia, we examined whether PGE2 levels also increase in hypoxia. HT29 cells were subjected to growth under hypoxic conditions in the presence or absence of the COX-2-selective inhibitor NS-398 for 16 hours, and the levels of PGE2 in the growth medium were determined. There was a 4-fold increase in PGE2 levels in hypoxia compared with normoxia (Fig. 3A). Furthermore, the increase in PGE2 during hypoxia was inhibited by treatment with 10 μmol/L COX-2-selective inhibitor NS-398 (this dose was reported previously to selectively inhibit COX-2 activity with no effect on cell growth; ref. 35). Therefore, we conclude that, in response to hypoxia, there is a COX-2-dependent increase in PGE2 levels.

PGE2 contributes to tumor cell growth and survival under hypoxic conditions. PGE2 has been shown previously to promote colorectal tumor cell growth and survival in normoxia (13, 14). This is believed to be one of the mechanisms underlying the oncogenic role of COX-2 in colorectal tumorigenesis. Here, we hypothesized that the increased levels of PGE2 during hypoxia may also promote cell growth and survival under hypoxic conditions. Initially, exposure of HT29 cells to hypoxia for 24 hours resulted in a reduction in cell yield (Fig. 3B, top, left), increased levels of cell death (Fig. 3B, top, right), and a decreased rate of cell proliferation with more cells accumulating in G1 phase of the cell cycle (Fig. 3B, bottom). To assess the direct consequence of COX-2 on cell survival under hypoxia, COX-2 was selectively inhibited with 10 μmol/L COX-2-selective inhibitor NS-398, and cell yield was determined. In normoxia, NS-398 (10 μmol/L) treatment had no significant effect on cell yield (data not shown; reported in ref. 35), and as predicted, PGE2 treatment on its own significantly enhanced cell growth (data not shown; reported in ref. 36). In hypoxia, NS-398 (10 μmol/L) treatment potentiated the inhibitory effect of hypoxia on cell yield (Fig. 3C, top) with a significant increase in cell death (Fig. 3C, bottom).

**Figure 4.** PGE2 enhances HIF-1 transcriptional activity and VEGF production. A, HT29 cells were grown in normoxia/hypoxia and treated with NS-398 (10 μmol/L), PGE2 (1 μmol/L), or a combination of both for 16 hours. Western blot analyses were used to assess HIF-1α/HIF-1β protein levels. An HRE luciferase reporter assay was used as described in Materials and Methods to assess HIF-1 transcriptional activity after treatment with PGE2. B, increased PGE2 levels during hypoxia result in enhanced VEGF production. HT29 cells were treated as described previously, and VEGF level was determined in the growth medium using a commercial ELISA. Columns, mean of three independent experiments standardized to cell counts; bars, SD.
The inhibitory effect of NS-398 on cell survival under hypoxia was overcome by the addition of exogenous PGE2 (Fig. 3C). Treatment with PGE2 on its own promoted cancer cell growth and survival in hypoxia (Fig. 3C). Taken together, these results indicated that PGE2 as a result of COX-2 activity plays an important role in promoting colorectal cancer cell growth and survival during hypoxia.

**PGE2 enhances HIF-1 transcriptional activation during hypoxia.** To examine a possible cross-talk between COX-2/PGE2 and HIF-1 pathways, we decided to investigate whether PGE2 affected HIF-1 expression or activity. PGE2, at relatively high concentrations, has been reported previously to increase the expression of HIF-1α in HCT116 cells (37). In normoxia, PGE2 alone at a relatively low dose (1 μmol/L) that we have shown previously to stimulate growth and survival of colorectal cancer cells (36) failed to induce HIF-1α (Fig. 4A) and had no effect on HIF-1β levels. Similarly, under hypoxic conditions, exogenous PGE2 (1 μmol/L) and NS-398 (10 μmol/L) treatment had no effect on HIF-1α/HIF-1β expression levels (Fig. 4A). Interestingly, however, hypoxia-induced HIF-1 transcriptional activity was partly inhibited by NS-398 treatment, suggesting that PGE2 may enhance HIF-1 transcriptional activity in hypoxia (Fig. 4A). Indeed, subsequent addition of PGE2 overcame the inhibitory effect of NS-398 on HIF-1 transcriptional activity. These observations suggest that PGE2 enhances HIF-1 transcriptional activity in hypoxia.

VEGF, the critical proangiogenesis factor, is an important target of HIF-1. As PGE2 enhances HIF-1 transcriptional activity in hypoxia, we examined the role of PGE2 in VEGF regulation. PGE2 treatment resulted in the induction of VEGF in HT29 cells growing under normoxia (Fig. 4B). Exposing HT29 cells to hypoxia resulted in a further increase in VEGF production, which was partly inhibited by NS-398. The inhibitory effect of NS-398 on VEGF expression under hypoxia was recovered by PGE2 treatment (Fig. 4B). These observations correlate with HIF-1 transcriptional activity shown in Fig. 4A and indicated that PGE2 potentiates hypoxia-induced VEGF by enhancing HIF-1 transcriptional activity in the colon cancer cell line HT29.

**PGE2 enhances HIF-1 transcriptional activity in hypoxia through the MAPK pathway.** PGE2 has been shown to promote cell proliferation through the activation of MAPK pathway (15); we therefore examined whether increased levels of PGE2 during hypoxia activate the MAPK pathway. Consistent with previous reports, in normoxia, PGE2 activated the MAPK pathways by inducing ERK phosphorylation in HT29 cells (Fig. 5A). In hypoxia, ERK was also activated, and its activation was partly inhibited with NS-398 treatment (Fig. 5A). However, the inhibitory effect of NS-398 on ERK activation in hypoxia was overcome by PGE2 treatment (Fig. 5A). These results indicate that increased levels of PGE2 during hypoxia activate the MAPK pathway.

Because the MAPK pathway has been reported to enhance HIF-1 transcriptional activity (38), it was hypothesized that increased levels of PGE2 by hypoxia may increase HIF-1 activity through activating the MAPK pathway. To test this hypothesis, HIF-1 activity was assessed in hypoxic HT29 cells treated with the MEK inhibitor U0126. Inhibition of MEK resulted in reduced transcriptional activity of HIF-1 similar to that observed with NS-398 treatment (Fig. 5B). However, whereas the addition of PGE2 overcame the effect of NS-398 on HIF-1 transcriptional activity, PGE2 did not reverse the effect of the MEK inhibitor. This suggests that PGE2 enhances HIF-1 transcriptional activity through ERK activation.

**Figure 5.** PGE2 activates the MAPK pathway during hypoxia and enhances HIF-1 transcriptional activity through ERK activation. A, Western blot analysis was carried out to examine ERK activation in HT29 cells grown in normoxia/hypoxia in the presence or absence of NS-398, PGE2, or both. B, PGE2 enhances HIF-1 transcriptional activity through ERK activation. HT29 cells were exposed to growth under hypoxic conditions and treated as indicated, and, subsequently, ERK activation and HIF-1 transcriptional activity were examined.

**Discussion**

Adaptation to hypoxia is critical for tumor cell growth and survival and is achieved largely by transcriptional activation of genes that facilitate short- and long-term adaptive responses (17, 31). In the current study, we report for the first time that hypoxia induces COX-2 in colorectal tumor cells and show that this up-regulation is mediated directly by HIF-1. In addition, COX-2 up-regulation by hypoxia represents a critical adaptive mechanism that promotes colorectal tumor cell survival and angiogenesis under hypoxic conditions.

COX-2 overexpression has been described in tumors originating from different tissues (39), including colorectal tumors (3, 4), and compelling evidence supports an important role for COX-2 in tumorigenesis (8, 40, 41). Therefore, identifying the regulatory mechanisms that underlie COX-2 up-regulation is crucial for further understanding of the tumorigenic process and the development of novel approaches for cancer prevention and therapy. For colorectal tumors, deregulation of the Wnt and Ras signaling pathways have been reported to contribute to COX-2 up-regulation (16). Our findings identify hypoxia as a novel tumor microenvironmental factor that contributes to COX-2 up-regulation in colorectal tumor cells. It is of interest to note that, in response to hypoxia, COX-2 expression...
is enhanced in both colorectal adenoma and carcinoma cells, suggesting that hypoxia may contribute to COX-2 overexpression at early stages of colorectal tumorigenesis. Additionally, hypoxia up-regulates COX-2 expression in colorectal tumor cells with different basal levels of COX-2, suggesting that hypoxia may act synergistically with other pathways implicated in COX-2 up-regulation.

COX-2 up-regulation by hypoxia has been described previously in human umbilical vascular endothelial (42) and corneal epithelial cells (43) to be mediated by NF-κB and peroxisome proliferator-activated receptors, respectively. In addition, while this article was in preparation, Csiki et al. reported that COX-2 is up-regulated in hypoxic lung cancer cells in an HIF-1-dependent manner (44). Although the study of Csiki et al. was the first report to describe COX-2 as a HIF-1 target gene, we provide the first evidence to show that HIF-1 directly binds a specific HRE located at −506 on the COX-2 promoter and highlight the biological significance of COX-2 up-regulation during hypoxia. The oncogenic role of COX-2 in colorectal and other tumors is largely attributed to its role in prostaglandin biosynthesis (11). For colorectal tumorigenesis, PGE2 has been particularly and extensively studied (11). Here, we report that COX-2 up-regulation during hypoxia, which could have important implications for colorectal adaptation (angiogenesis). Because tumor hypoxia could be a target for selective cancer therapy (49), our findings suggest that pharmacologic targeting of COX-2 using modified COX-2-selective inhibitors that can only be activated under hypoxic conditions could increase the selectivity of the current COX-2-selective inhibitors.

Figure 6. Model for COX-2 regulation and its role in colorectal tumorigenesis. Mutations in Wnt and Ras signaling pathways can induce COX-2 in normoxic conditions, resulting in increased levels of PGE2, which promotes tumor cell growth/survival and stimulates angiogenesis. As reported by Wang, PGE2 can activate the Ras-MAPK pathway, thereby amplifying the expression of COX-2 and showing a positive feedback loop that contribute to COX-2 up-regulation (15). This positive feedback loop can be extended to our study under hypoxic conditions, where we show that HIF-1 can directly up-regulate COX-2 and PGE2 levels. PGE2 then activates the MAPK pathway and enhances HIF-1 transcriptional activity, resulting in a potential positive feedback loop that may act to maintain high COX-2 levels under hypoxic conditions.

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Hypoxia-Induced COX-2 Promotes Cell Survival

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Direct Transcriptional Up-regulation of Cyclooxygenase-2 by Hypoxia-Inducible Factor (HIF)-1 Promotes Colorectal Tumor Cell Survival and Enhances HIF-1 Transcriptional Activity during Hypoxia

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