Thioredoxin-1 Modulates Transcription of Cyclooxygenase-2 via Hypoxia-Inducible Factor-1α in Non–Small Cell Lung Cancer

Ildiko Csiki,[1] Kiyoshi Yanagisawa,[2] Nobuhiro Haruki,[2] Sorena Nadaf,[2] Jason D. Morrow,[3] David H. Johnson,[2] and David P. Carbone[1,2]

Departments of [1]Cancer Biology, [2]Medicine (Hematology-Oncology), and [3]Pharmacology, Vanderbilt-Ingram Cancer Center, Nashville, Tennessee

Abstract

Hypoxic induction of gene expression occurs mainly via the hypoxia-inducible factor-1 (HIF-1) transcription factor and is a critical step in tumor growth. Cyclooxygenase-2 (COX-2) is commonly overexpressed in non–small cell lung cancer (NSCLC). In this study, we sought to determine the role of HIF-1 in the induction of COX-2 expression during hypoxia. Through sequence comparison of hypoxia-responsive genes, COX-2 promoter deletion analysis, and site-directed mutagenesis, we identified a hypoxia-responsive element within the COX-2 promoter that interacts with HIF-1α and underlies the mechanism of hypoxic activation of COX-2 in lung cancer cells. Proteomic analysis of NSCLC identified thioredoxin-1 as a redox protein overexpressed in NSCLC correlated with poor prognosis. We also show that thioredoxin-1 stabilizes HIF-1α to induce hypoxia-responsive genes under normoxic conditions. Our results identify two new mechanisms for regulation of COX-2 expression in NSCLC. (Cancer Res 2006; 66(1): 143-50)

Introduction

Hypoxia is observed in nearly all solid tumors and has been associated with poor prognosis (1). Decreased oxygen tension directly induces the expression of many genes causing accumulation of hypoxia-inducible factor (HIF-1), a heterodimer of the hypoxia-induced HIF-1α and the constitutively expressed HIF-1β (ARNT). This dimer binds specifically to a 5′-RCGTG-3′ hypoxia-responsive element (HRE) in the promoter or enhancer region of hypoxia-inducible genes (2), leading to hypoxic gene activation (3). HIF-1 has been implicated in numerous cellular events, such as angiogenesis, cell survival, apoptosis, cell motility, and others (4, 5). Cyclooxygenase (COX), and especially the COX-2 isoform (6), has been associated with all of these properties of tumors as well and is frequently overexpressed in non–small cell lung cancer (NSCLC) and associated with poor outcome (7–12). It is known that COX-2 is induced in hypoxic human umbilical vascular endothelial cells (HUVEC; ref. 13) via the nuclear factor-κB (NF-κB) transcription factor (14). The mechanism of COX-2 overexpression and induction by hypoxia in tumors was unknown. In this study, we show that HIF-1 directly induces the transcriptional activation of COX-2 in lung cancer cells under hypoxic conditions.

However, even well vascularized tumors can overproduce COX-2. A recent study proposes other mechanisms besides hypoxia for stabilization of the HIF-1α protein (15). Thioredoxin-1, a member of the redox family proteins (16), has been shown to enhance HIF-1 DNA binding and stimulate HIF-1 transactivation function (15, 17). For example, thioredoxin-1 overexpression has been shown to activate HIF-1 binding to the HRE, leading to increased vascular endothelial growth factor (VEGF) production (18). Increased thioredoxin-1 expression is found in NSCLC and is associated with lymph node status and negative prognosis (19). Here, we also show that in human lung cancer cells, overexpression of thioredoxin directly induces COX-2 expression via HIF-1 under normoxic conditions.

Materials and Methods

Cell culture and hypoxic treatment. The human adenocarcinoma cell lines (A549 and H358) and the normal human bronchial epithelial cell line (BEAS-2B) were obtained from the American Type Culture Collection (Rockville, MD). The A549 and H358 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, whereas BEAS-2B cells were grown in DMEM (Invitrogen) with 10% FCS. A549 stably overexpressing redox-active and redox-inactive thioredoxin were cultured in RPMI 1640 with 10% FCS supplemented with 200 μg/mL of G418. Cells were grown under humidified 95% air, 5% CO_2 at 37°C. Cells in experimental plates were exposed to hypoxia for various times in a humidified chamber at 37°C with a gas mixture containing 1% O_2, 94% N_2, and 5% CO_2 maintained using an oxygen sensor (Pro/Ox 110; Reming Bioinstruments Co., Redfield, NY).

Plasmids. A full-length COX-2 promoter luciferase (Luc) construct (~2.0 kb) and several smaller deletion constructs were generous gifts from D. Dixon (Vanderbilt University Medical Center, Nashville, TN). The empty pGL3 control plasmid and the pRL-CMV *Renilla* Luc containing plasmid used to control for transfection efficiency were obtained from Promega (Madison, WI). Mutant transcription factor binding sites were introduced into the full-length COX-2 promoter Luc construct by using a site-specific mutagenesis kit from Stratagene (La Jolla, CA). Successful mutagenesis was confirmed using an automated sequencer. HIF-1α expression plasmid was generously supplied by O. Iliopoulos (Massachusetts General Hospital, Boston, MA), and HIF-1β expression plasmid was a generous gift from F. Bunn (Harvard Medical School, Boston, MA). The dominant-negative HIF-1α expression plasmid was a kind gift from E. Berra (Institut de Recherche/Centre National de la Recherche Scientifique/Institut National de la Sante et de la Recherche Medicale/Universite de Nice-Sophia Antipolis, Nice, France) The sense and antisense thioredoxin-1 expression plasmids were constructed using pIRESpuro3 (BD Biosciences Clontech, Palo Alto, CA). Thioredoxin-1 cDNA was amplified using 5′-GCTTTGGATCCATTTC-CATCGGTC-3′ (forward) and 5′-ATCGGATCCAATAGCC AATGGCTG-3′ (reverse) primers by PCR. Thioredoxin-1 constructs had their sequences confirmed through the open reading frame. All expression plasmids produced appropriately sized peptides detected in Western blot analysis by specific anti-thioredoxin-1 and anti-HIF-1 antibodies after transfection.

Western blot analysis. Nuclear extracts from cells grown in hypoxic or normoxic conditions were prepared using the NE-PER nuclear/cytoplasmic

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: David P. Carbone, Vanderbilt-Ingram Cancer Center, 685 Preston Research Building, Nashville, TN 37232-6838. Phone: 615-936-3524; Fax: 615-936-3222; E-mail: d.carbone@vanderbilt.edu.

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extraction reagent (Pierce, Rockford, IL) following the manufacturer's instructions. Protein (15-100 μg) was separated by 10% SDS-PAGE and subsequently blotted onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Proteins were immunoblotted with monoclonal anti-HIF-1α, monoclonal anti-HIF-1β (BD Biosciences, Lexington, KY), or polyclonal anti-COX-2 antibody (Oxford Biomedical Research, Oxford, MI). Super Signal substrate (Pierce) was used for chemiluminescence-based detection of appropriate secondary antibodies.

Real-time reverse transcription-PCR. Total RNA was isolated from A549 cells exposed to hypoxia or normoxia for 12 and 24 hours using the Versagene RNA cell kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. For real-time PCR analysis, oligonucleotide reverse transcription-PCR (RT-PCR) primers for COX-2 (5′-ATAAGC-GAGGGCCAGCTTTCA-3′ and 5′-GTGGGAGGA TACATCTCTCCA-3′) and β-actin (5′-TCCTTCCTGGGCATGGAGTC-3′ and 5′-TTCT GCATCCT-GTCGGCAATG-3′) were generated. Quantitative fluorogenic reverse transcription of mRNA and amplification of cDNA was done in the iCycler (Bio-Rad, Hercules, CA) using the Quantitect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The following conditions were used: reverse transcription for 30 minutes at 50°C; initial activation for 15 minutes at 95°C; 42 cycles of 15 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C followed by 10 minutes at 72°C, and finally 1 minute at 95°C. The relative abundance of mRNA in samples was determined from standard curves generated from the amplification from serially diluted standard pools of A549 RNA and normalized to β-actin mRNA.

COX-2 transcriptional activity. Cells are seeded at 7.5×10^4 per well in 12-well plates and serum starved for 24 hours before transfection. Fugene 6 reagent (0.02 μg; Roche Applied Sciences, Indianapolis, IN) is used for cotransfection into cell lines with each of the reporter constructs (empty PGL3 control plasmid, full-length COX-2 promoter Luc constructs, deletion or specific site-directed mutant constructs) +/− HIF-1α and HIF-1β and/or +/− thioredoxin sense and thioredoxin antisense, and 1 ng/well of pRL-CMV-*Renilla* Luc plasmid was used as an internal control. Five hours after transfection, cells are placed either at 21% O_2 or at 1% O_2, 5% CO_2 controlled by a hypoxia chamber and allowed to proliferate for 24 hours. Firefly and *Renilla* Luc activities were determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Plasmid Luc activities are determined by dividing firefly Luc activity with *Renilla* Luc activity for each sample to normalize for variable transfection efficiency between the samples. COX-2-plasmid Luc activity during hypoxia and normoxia is normalized by setting the activity of the empty PGL3 control vector during hypoxia and normoxia at 1% and obtaining a relative fold induction of COX-2/Luc constructs over the empty vector during hypoxia and normoxia, respectively.

HIF-1α binding and competition assay. HIF-1 binding and competition assay was done with a TransAM kit (Active Motif, Carlsbad, CA), which measures binding to a 26-bp HRE oligonucleotide from the *EPO* gene attached to a 96-well plate. Nuclear extracts were prepared from A549 cells as described above following exposure to normoxia or hypoxia for 24 hours. To examine HIF-1 activation during hypoxia, 2.5, 5, and 10 μg of nuclear extract was used per well. To measure HIF-1 binding and for the competition assay, 10 μg of nuclear extract were used per well. Synthetic oligonucleotide probes for the COX-2 promoter containing the wild-type and mutated HREs were synthesized and annealed. The following 26-bp double-stranded probes from the COX-2 promoter were used for the HIF-1 binding and competition experiment (the sequence of the sense strand is shown): COX2HRE-WT, 5′-GTCTGTCCCGACGTGACTTCCTC GAC-3′; COX2HRE-MT, 5′-GTCTGTCCCGA**AAA**GACTTCCCTCGAC-3′ (the HRE is underlined and the mutated bases are in bold). For binding and competition experiments, 20 pmol (1×) or 100 pmol (5×) of either the wild-type or the mutant oligonucleotide of the *EPO* gene promoter or the *COX-2* gene promoter was first added to the appropriate well before addition of the nuclear extract and incubation for 1 hour. After washing three times, HIF-1α binding was assessed by incubation for 1 hour each with an anti-HIF-1α antibody and then with a horseradish peroxidase–conjugated secondary antibody. A_{450} was then determined.

Results

COX-2 mRNA and protein is induced by hypoxia. A549 cells were serum starved for 24 hours to minimize the effect of growth factors on COX-2 expression and then incubated in the presence of 21% O_2 or 1% O_2 for variable hours. HIF-1α accumulation during hypoxia leading to HIF-1 expression has been shown in different cell lines (20, 21). Similarly, after exposure of A549 cells to hypoxia, HIF-1α began to accumulate in the cells and was easily detected by Western blot analysis; however, it was not seen in normoxic cells (Supplementary Fig. 1). Western blot analysis of A549 cells showed induction of COX-2 after ~12 hours of continuous exposure to 1% O_2, and the extent of COX-2 induction during hypoxia had further increased by 24 hours ($P < 0.005$). Intracellular COX-2 protein in hypoxic A549 cells increased 3-fold after 24 hours (Fig. 1*A* and *B*).

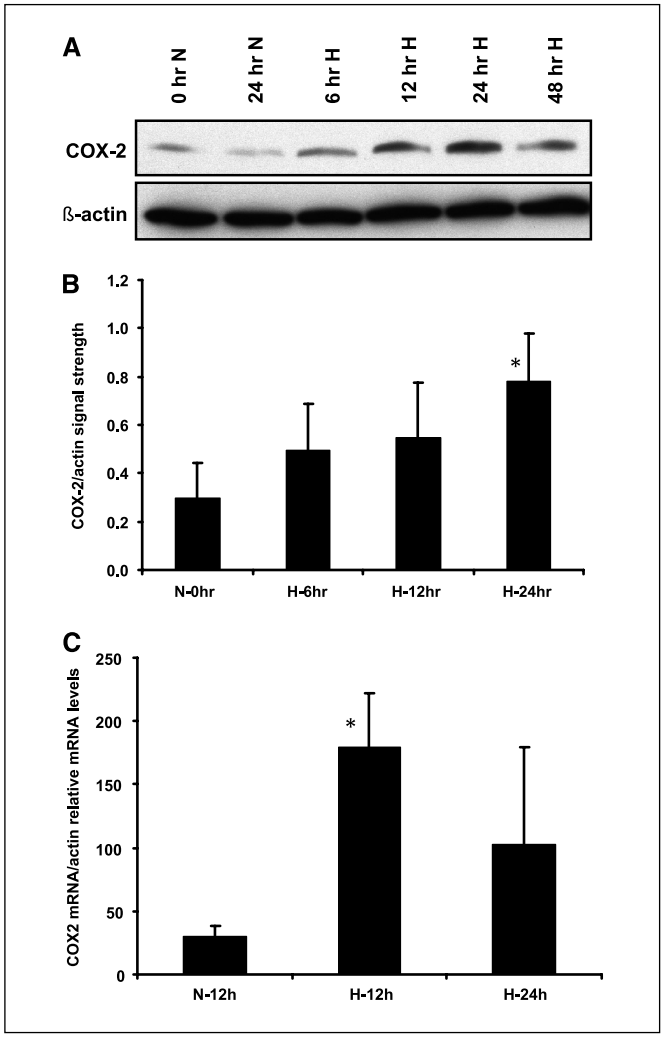

Figure 1. Hypoxic induction of COX-2 protein and mRNA in A549 cells. *A,* time course analysis of COX-2 expression by Western blot. Cells were incubated in normoxia (*N*) or hypoxia (*H*) for various time points, and total protein extracts were analyzed for COX-2 protein expression using anti-COX-2 antibodies. Anti-actin antibodies were used as control of protein loading. *B,* densitometry of COX-2 expression under hypoxia at various time points and normalized to loading controls using NIH Image 3.2. *Columns,* mean of three independent experimental determinations; *bars,* SD. *C,* real-time PCR. Cells were subjected to 24 hours of normoxia and 12 and 24 hours of hypoxia before COX-2 mRNA levels normalized to β-actin levels were determined by real-time PCR. *Columns,* mean of three to six experimental determinations; *bars,* SD.

Quantitative real-time PCR also showed induction of COX-2 mRNA consistent with results from the Western blot analysis (Fig. 1*C*). COX-2 mRNA was increased 6-fold ($P < 0.005$) by 12 hours and about 3-fold at 24 hours after hypoxic treatment.

Functional analysis of COX-2 5′-flanking sequences. To determine whether the 5′-flanking region of the *COX-2* gene could mediate transcriptional responses to cellular hypoxia, we used reporter plasmids in which COX-2 5′-flanking sequences were fused to Luc coding sequences (Fig. 2*A*). This series of 5′-deletion mutants was transfected into the A549 human adenocarcinoma cell line and maintained in 10% serum overnight. Following 24 hours of serum starvation at 21% O_2, transfectants were incubated at 1%, 21% O_2 or 21% O_2 + 150 μmol/L $CoCl_2$ for 48 hours. To correct for variable transfection efficiency, cells were cotransfected with pRL-CMV-*Renilla*. Cell extracts were assayed for Luc and *Renilla* activity, and the Luc/*Renilla* ratio was determined. The Luc/*Renilla* ratio from cells at 1% O_2 or cells treated with $CoCl_2$ was normalized to the results obtained with the same reporter plasmids in cells at 21% O_2 to generate the relative Luc activity. We calculated the fold reporter-promoter construct induction at normoxic and hypoxic condition over the empty PGL3 control vector to obtain the relative COX-2/Luc induction. Hypoxia had no effect on background Luc activity measured using the empty PGL3 control vector (P0). The full-length COX-2 reporter containing ~2 kb of 5′-flanking DNA (P1), mediated a 2.5-fold greater level of Luc expression in cells exposed to 1% O_2 (Fig. 2*B*) and in cells treated with $CoCl_2$ compared with cells exposed to 21% O_2 (data not shown). In contrast, the ability of the COX-2 reporter to respond to hypoxia decreased markedly as the 5′-flanking sequences were deleted. Marked loss of induction to hypoxia occurred when sequences beyond −466 bp upstream of the start site were deleted. These results suggest that (*a*) COX-2 5′-flanking sequences mediate transcriptional responses to hypoxia in A549 cells, (*b*) one or more important *cis*-acting regulatory elements are located in the promoter region between ~2 kb and 466 bp, and (*c*) these regulatory elements are specifically involved in mediating hypoxia-inducible transcription.

Stimulation of COX-2 reporter activity by exogeneous HIF-1α. To determine whether the COX-2 5′-flanking sequences that mediated hypoxia-inducible transcription could functionally interact with HIF-1, we cotransfected BEAS-2B and A549 cells with COX-2 reporter and pRL-CMV-*Renilla* plasmids in the presence or absence of HIF-1α and HIF-1β (ARNT) expression vectors (rHIF-1). Cells were incubated at 1% or 21% O_2 for 24 hours, and the Luc/*Renilla* values were normalized to the results from cells transfected with P1 incubated at 21% O_2. In cells cotransfected with rHIF-1, there was an activation of P1 expression both at 1% and 21% O_2. In BEAS-2B cells at 21% O_2, Luc expression was 4-fold greater in the presence than in the absence of HIF-1 expression vectors and reached the level of endogenous response to hypoxia (Fig. 3*B*). Furthermore, at 1% O_2 reporter gene expression was 9.6-fold greater in the presence than in the absence of rHIF-1 expression vectors and 35.3-fold greater in hypoxic cells cotransfected with rHIF-1 than in nonhypoxic cells that were not cotransfected with rHIF-1 (Fig. 3*B*). In A549 cells, P1 expression was 6-fold higher in cells at 1% O_2 than in cells at 21% O_2 in the absence of rHIF-1. Compared with cells transfected with COX-2 reporter only, cotransfection with rHIF-1 increased P1 expression 1.8-fold in cells at 21% (Fig. 3*C*). In hypoxic cells transfected with rHIF-1, there was a 2-fold increase in the full-length COX-2 reporter expression compared with that in hypoxic cells transfected with reporter only and an 11.7-fold increase compared with levels in nonhypoxic cells without rHIF-1 (Fig. 3*C*). In both BEAS-2B and A549 cells, similar results were obtained when the cells were treated with $CoCl_2$

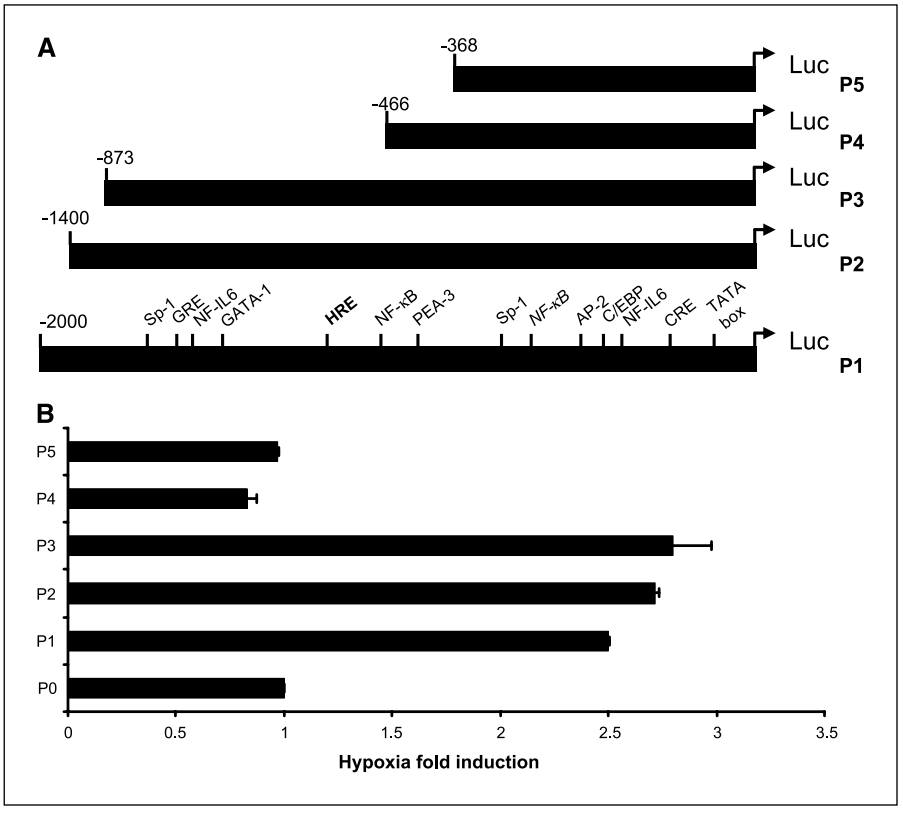

Figure 2. Hypoxia induces COX-2 transcription. *A,* COX-2/Luc reporter constructs. Each deletion construct is given a name (P1-P5) along with the terminal nucleotide at the 5′-end relative to the transcription initiation start site. The 3′-end of each construct (+38) was fused to Luc coding sequences. *B,* functional analysis of the human COX-2 5′-flanking sequences in transient expression assays in A549 cells. Cells were transfected with the various promoter constructs (P1-P5) and then incubated in normoxic or hypoxic conditions. Luc activity was normalized for transfection efficiency using a *Renilla* reporter plasmid.

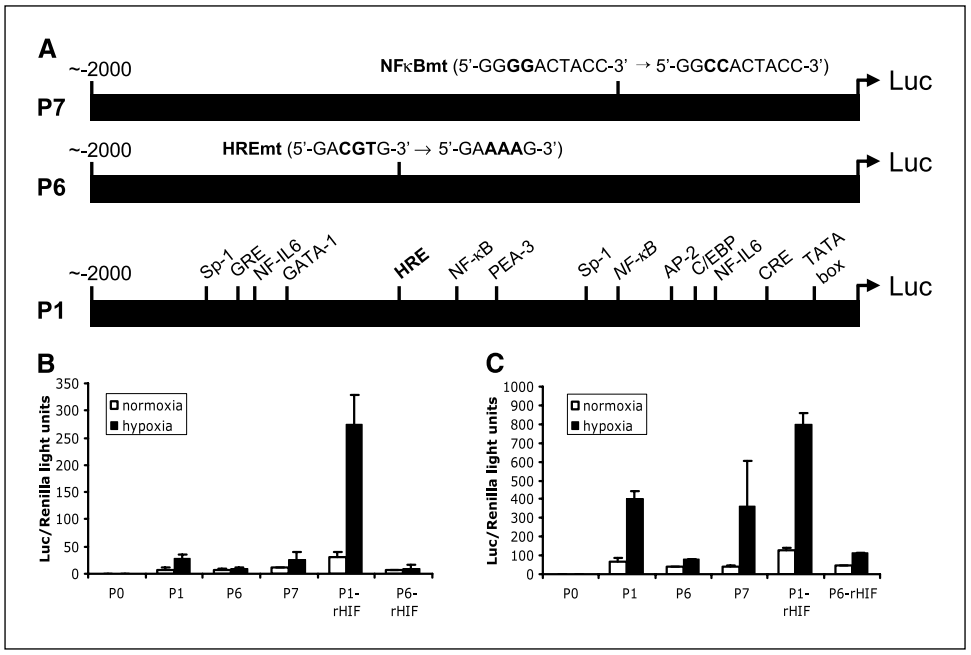

Figure 3. Modulation of wild-type and mutant COX-2 reporter expression by HIF-1α. *A,* COX-2/Luc mutant reporter constructs. P6 designates an ~2-kb COX-2 reporter construct (P1, with major transcription factor binding sites of the COX-2 promoter shown) with the putative HRE mutated (HIF-1 binding site shown above with mutated bases in bold), and P7 refers to the P1 with NF-κB binding site mutated (NF-κB binding site shown above and mutated bases are in bold). *B-C,* hypoxic induction of wild-type COX-2 promoter (P1) and the specific site-directed mutants in the presence or absence of exogenous HIF-1α and HIF-1β expression plasmids (rHIF-1) in BEAS-2B (*B*) and A549 cells (*C*). Cells were analyzed as in B and the activity of P1 was compared with the activity of mutagenized constructs (P6 and P7) in which putative transcriptional elements had been destroyed by site-directed mutagenesis.

instead of 1% O_2 (data not shown). These results suggest that HIF-1 was capable of activating transcription selectively through DNA sequences required for hypoxia-inducible transcription.

COX-2 promoter contains a functional HRE. Analysis of COX-2 5′-flanking sequence revealed a potential HIF-1 binding site that matched with the consensus binding site sequence 5′-BACGTGCK-3′ that was derived from known HIF-1 binding sites. The P1 vector contained this putative HIF-1 binding site. The results based on reporter deletion constructs shown in Fig. 2*B* were consistent with the localization of the potential HIF-1 binding site present in P1, P2, and P3. To further analyze this putative HRE, we constructed reporter P6 (Fig. 3*A*), which contained the sequences of the full-length wild-type COX-2 reporter, but the putative HIF-1 binding site was mutated by introducing a 3-bp substitution. This mutation altered the putative HIF-1 binding site from 5′-GACGTG-3′ to 5′-GAAAAG-3′. An analogous mutation in the VEGF and EPO promoters completely eliminated hypoxia-inducible reporter gene expression (22, 23).

In addition to the putative HIF-1 site, P1 also contains a 3′-NF-κB binding site that was shown to be involved in hypoxic up-regulation of COX-2 in HUVECs (14). To analyze the potential involvement of this site in the hypoxic regulation of COX-2 in lung cancer cells, we constructed reporter P7 (Fig. 3*A*). This construct contained the sequences of the full-length wild-type COX-2 reporter (P1) with the exception of the 3′-NF-κB site, which was mutated by site-directed mutagenesis from GGGGACTACC to GGCCACTACC, previously shown to abrogate hypoxia-induced up-regulation of COX-2 in HUVECs (14).

P6 and P7 reporter constructs were transfected into A549 and BEAS-2B cells in the presence or absence of rHIF-1 and incubated at 1% or 21% O_2 for 24 hours. The expression pattern of P7 was essentially identical to that of P1 (Luc expression about 25% less than that of the P7 reporter), indicating that the 3′-NF-κB site plays only a minor role in the transcriptional response of COX-2 to hypoxia in A549 and BEAS-2B cells. P6 showed a pattern of expression significantly different from that of P1 and P7 in hypoxic conditions, whereas during normoxic conditions the pattern of expression did not differ from that of P1 and P7. Expression of P6 was not significantly induced by hypoxia and/or rHIF-1 (Fig. 3*B* and *C*). Taken together, these data show that in A549 and BEAS-2B cells hypoxia induces transcription from the COX-2 promoter, and that this transcriptional activation is mediated primarily by a HRE and to a much smaller extent by the 3′-NF-κB site.

Inhibition of COX-2 reporter gene and protein expression in hypoxic cells by a dominant-negative form of HIF-1α. The pcDNA3-HA-DN-HIF-1α construct encodes a form of HIF-1α lacking the COOH-terminal transactivation domains. Dominant-negative HIF-1α can heterodimerize with HIF-1β and can inhibit HRE-driven reporter genes (24). A549 cells were cotransfected with P1 and wild-type and dominant-negative HIF-1α expression vector and incubated at normoxic and hypoxic conditions. The expression of P1 transfected with the dominant-negative HIF-1α under hypoxia was similar to the baseline expression of P1 under normoxia (Fig. 4*A*). Overall, cells transfected with P1 and wild-type HIF-1α had 7.6-fold increased P1 reporter expression under hypoxia compared with baseline P1 levels under normoxia, whereas cells transfected with P1 and the dominant-negative HIF-1α had only a 1.6-fold increase in P1 reporter expression under hypoxia (Fig. 4*A*), suggesting that in hypoxic cells reporter gene activation is mediated by HIF-1. We also determined the effect of pcDNA3-HA-DN-HIF-1α cotransfection on the expression of reporter plasmids containing COX-2 promoter sequences that lacked the hypoxia response element (P6). As expected, there was no activation of P6 expression observed under hypoxia (data not shown). In addition to the effect of dominant-negative HIF-1α on transcriptional activation of the COX-2 reporter, we examined the effect of dominant-negative HIF-1α on endogenous COX-2 activation during hypoxia. Cells transfected with the dominant-negative HIF-1α did not show up-regulated COX-2 expression following hypoxic exposure compared with vector controls (Fig. 4*B*). These data complement the results obtained by cotransfection of wild-type HIF-1α and HIF-1β expression vectors, and together, these experiments provide evidence that the hypoxia-inducible expression of COX-2 is mediated by HIF-1.

HRE in the COX-2 promoter competes with the EPO HRE for HIF-1 binding. We carried out a HIF-1 binding and competition ELISA in which the binding of HIF-1α to the HRE of the *EPO* gene was assessed in the presence or absence of a competing HRE. Nuclear extracts from hypoxia-treated A549 cells showed strong binding to the EPO HRE (increased binding was observed with the addition of increasing amounts of nuclear extract), indicating the presence of activated HIF-1α (data not shown). Minimal HIF-1α activation was observed in the nuclear extracts from cells exposed to normoxia, even with the addition of increasing amounts of nuclear extract (2.5, 5, and 10 μg). We then used the hypoxic nuclear extracts for the competition assay. To correlate the transcriptional assay and DNA binding assay, we synthesized a wild-type and a mutant oligonucleotide that contained the same 3-bp substitution that resulted in the loss of function of P6 reporter. Pretreatment of the nuclear extracts with 20 pmol of a 26-bp wild-type DNA duplex spanning the HRE region of the COX-2 promoter was able to abolish HIF-1α binding to the EPO HRE (Fig. 4*C*). However, the mutant COX-2 HRE could not compete with the EPO HRE, even at a 5-fold higher concentration. As expected, pretreatment of the extract with the 26-bp wild-type EPO DNA duplex competed for binding, whereas a mutant EPO DNA duplex was ineffective at both the 20 and 100 pmol concentration (Fig. 4*C*).

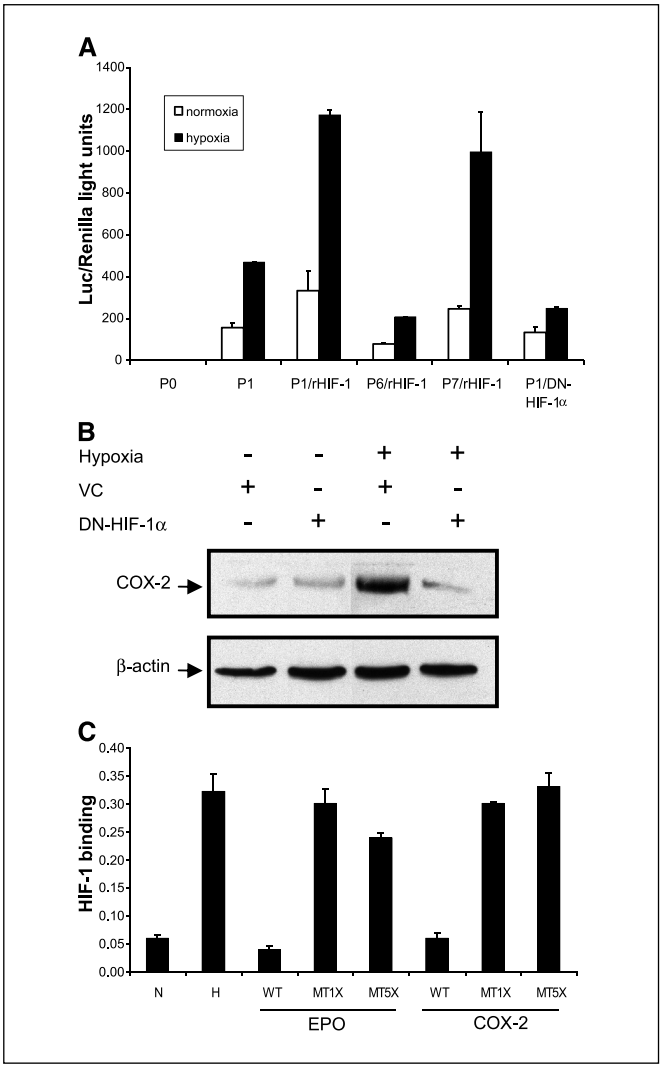

Figure 4. Dominant-negative HIF-1α inhibits hypoxic activation of COX-2. *A,* effect of a dominant negative form of HIF-1α on the transcriptional response to hypoxia. Cells were transfected with wild-type or dominant-negative HIF-1α and the transcriptional activation of P1 under hypoxia was examined. One representative experiment of three independent experiments carried out in triplicate. *B,* Western blot analysis of COX-2 and actin expression in cells transfected with an expression vector containing no insert (*VC*) or dominant-negative form of HIF-1α. Cells were selected with puromycin for 2 days and then exposed to 24 hours of hypoxia or normoxia before Western blot analysis. *C,* HIF-1 binding and competition assay. Nuclear extracts were prepared from A549 cells exposed to normoxia (*N*) or hypoxia (*H*). Ten micrograms of nuclear extract was incubated with 20 pmol (1×) or 100 pmol (5×) of wild-type (*WT*) or mutant (*MT*) probe from the 26-bp *EPO* gene promoter HRE oligo or the 26-bp COX-2 promoter HRE oligo. Binding to the 26-bp HRE oligo from the *EPO* gene was assessed as described in Materials and Methods. For competition experiments, the promoter probe was first added to the appropriate well before addition of the nuclear extracts. *Left,* results of a control experiment with nuclear extracts with an EPO probe but no competing oligo. *Columns,* means of duplicate determinations in one representative experiment of three; *bars,* SD.

Thioredoxin transactivates COX-2 via HIF-1. Proteomic analysis identified thioredoxin-1 as a marker of poor prognosis in NSCLC (25).[4] We confirmed the high expression of thioredoxin-1 in human NSCLC and various cancer cell lines using Western blot analysis in agreement with previous reports (Supplementary Fig. 2*A*; ref. 25). Thioredoxin expression level is unaltered during hypoxia (Supplementary Fig. 2*B*). Thioredoxin-1 overexpression in MCF7, HT29, and mouse WEH17.2 cells has been previously shown to increase HIF-1α protein levels (18). We also confirmed that overexpression of thioredoxin-1 resulted in stabilization of HIF-1α protein in BEAS-2B cells (Supplementary Fig. 3*A*). Next, we transfected the thioredoxin-1 expression plasmids into A549, BEAS-2B, and H358 cells in combination with the HIF-1 expression vectors and a COX-2/Luc reporter plasmid containing HIF-1 binding site of COX-2 promoter region (P1). As shown in Fig. 5*A-D*, overexpression of sense thioredoxin-1 markedly activated COX-2 promoter activity together with rHIF-1 under normoxic conditions. P1 expression increased 9-fold in the BEAS-2B cells, 29-fold in the H358 cells, and 4-fold in the A549 cells when rHIF-1 was cotransfected with sense thioredoxin-1. The same effect was observed using A549 cells subjected to hypoxia (Fig. 5*B*). When A549 cells were cotransfected with rHIF-1 in addition to sense thioredoxin-1, there was an additive effect on reporter expression observed both under normoxia and hypoxia. Under normoxic conditions, reporter expression of P1 increased 1.8-fold when rHIF-1 was cotransfected into the cells and increased 4.2-fold when sense thioredoxin-1 was cotransfected along with rHIF-1. Similarly, under hypoxic conditions, P1 expression increased 2-fold when rHIF-1 was cotransfected into the cells and increased 2.6-fold when sense thioredoxin-1 was cotransfected along with rHIF-1. Cotransfection of rHIF-1 and sense thioredoxin-1 under hypoxic conditions increased P1 expression 15.3-fold over P1 expression during normoxia (Fig. 5*B*). We also observed that antisense thioredoxin-1 suppressed COX-2 promoter activity in all of cell lines analyzed (Fig. 5*A-D*). Under hypoxic conditions, the suppression of P1 when rHIF-1 was cotransfected with antisense thioredoxin-1 was not maximal presumably due to hypoxic activation of HIF-1α and the presence of rHIF-1 within the cells, which offset the lack of HIF-1α stabilization by antisense thioredoxin-1 (Fig. 5*A*). As expected, P6 expression was not modulated with cotransfection of sense thioredoxin-1 and rHIF-1 due to lack of functional HIF-1 binding site, whereas expression of P7 under both hypoxia and normoxia

[4] Manuscript in preparation.

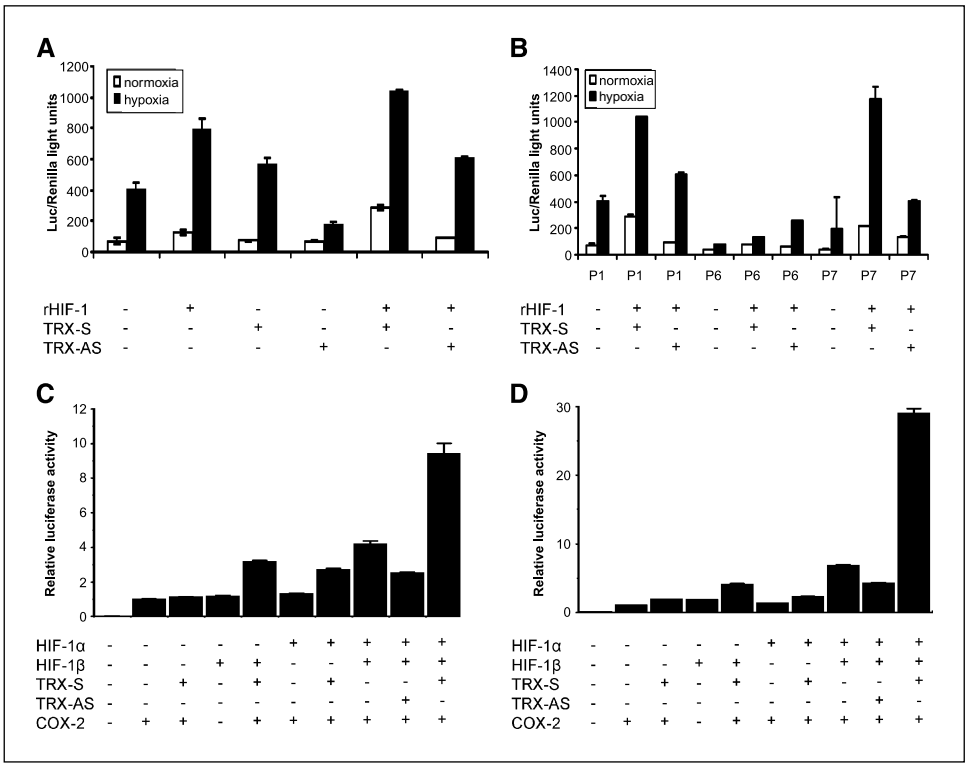

Figure 5. Effect of thioredoxin-1 on trans-activation of COX-2 through HIF-1. *A,* A549 cells were cotransfected with various combination of thioredoxin-1 and HIF-1 expression plasmid and P1 and the Luc expression of P1 was measured under normoxia and hypoxia. Transfection efficiency was normalized using transfection with *Renilla* Luc, and activation of the COX-2 promoter was determined by calculating the increase in firefly/*Renilla* Luc activity compared with the empty vector control activity. One representative experiment of three experiments carried out in triplicate. *Columns,* mean; *bars,* SD. *B,* P1, P6 (containing the mutated HIF-1 binding site), or P7 (containing the mutated NFκB site) was cotransfected into A549 cells with an expression vector containing no insert, sense thioredoxin (*TRX-S*) along with rHIF-1 or antisense thioredoxin (*TRX-AS*) along with rHIF-1, and the cells were exposed to 24 hours of normoxia or hypoxia. The relative Luc activity was calculated as in (*A*). One representative experiment of three experiments carried out in triplicate. *Columns,* mean; *bars,* SD. BEAS-2B (*C*) and NCI-H358 (*D*) cells were transfected with various combination of thioredoxin-1 and HIF-1 expression plasmids, and the Luc expression of P1 was measured under normoxia. The relative luciferase activity was calculated as in (*A*). One representative experiment of three experiments carried out in triplicate. *Columns,* mean; *bars,* SD.

was comparable to that of P1 due to the presence of a functional HIF-1 binding site (Fig. 5*B*). These results suggest that thioredoxin overexpression under both normoxic and hypoxic conditions can result in increased COX-2 expression via HIF-1 stabilization, which can activate the COX-2 promoter.

Thioredoxin modulates endogenous COX-2 expression. We transfected the thioredoxin-1 expression plasmid into BEAS-2B in various combinations with the HIF-1 expression vectors. After a 2-day selection with puromycin, induction of COX-2 protein was observed when sense thioredoxin was overexpressed together with HIF-1α and HIF-1β under normoxic conditions (Fig. 6*A*). Neither thioredoxin-1 nor HIF-1 alone was sufficient to induce COX-2 in human lung epithelial cells. We then generated A549 stably expressing redox-active and redox-inactive thioredoxin. The redox-inactive thioredoxin (C32S/C35S thioredoxin-1) has previously been shown to inhibit the effects of wild-type thioredoxin-1 by acting in a dominant-negative manner through competitive inhibition of thioredoxin reductase (18). When we analyzed these cells for endogenous COX-2 expression, we found that COX-2 expression was induced in the cells overexpressing redox-active thioredoxin, whereas COX-2 expression was decreased in the cells expressing redox-inactive thioredoxin (Fig. 6*B*). Similarly, thioredoxin-1 inhibitors have been shown to decrease endogenous levels of other HIF-target genes, such as the iNOS protein in MCF-7 cells and VEGF protein in MCF7-xenografts (26).

Discussion

Clinical and experimental data indicate that increased COX-2 activity is a feature of NSCLC associated with poor patient survival. In this study, we show that the *COX-2* gene in lung cancer cells is directly transcriptionally activated in response to hypoxia by HIF-1 binding to a functional HRE within the proximal COX-2 promoter and that this promoter can be activated in the absence of hypoxia by overexpression of thioredoxin-1.

HIF-1α activity in human tumors is associated with expression of many genes associated with increased angiogenesis and poor prognosis and thus is a potential therapeutic target (27). In normal cells HIF-1α levels are tightly regulated but are consistently elevated in cancer cells through several proposed mechanisms

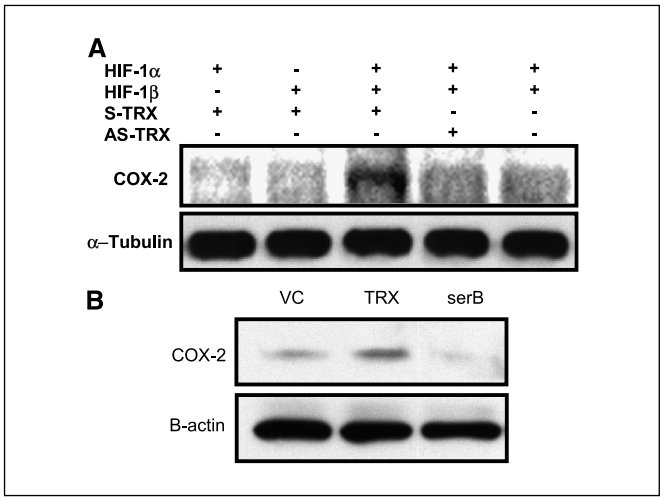

Figure 6. Modulation of endogenous COX-2 expression by redox-active or redox-inactive thioredoxin-1. *A,* Western blot analysis of (*top*, 70-kDa band) COX-2 and (*bottom*, loading control) α-tubulin in human bronchial epithelial cell line, BEAS-2B, which was transfected with various combination of thioredoxin-1 and HIF-1 expression plasmids. Transfected cells were selected with puromycin for 2 days. *B,* Western blot analysis of COX-2 and β-actin in A549 cells stably transfected with a redox-active and redox-inactive thioredoxin. Transfected cells were maintained in 200 μg/mL G418. *VC,* vector containing no insert.

(28, 29). Previous studies have shown that thioredoxin-1 increases HIF-1α protein in both normoxic and hypoxic cancer cells, leading to increased angiogenesis (18), and thioredoxin-1 inhibitors have been shown to inhibit HIF-1 transactivation both *in vivo* and *in vitro*, resulting in decreased expression of downstream angiogenesis-associated genes, such as *VEGF* and *iNOS* (26). We show here that COX-2 induction by HIF-1 occurs during both hypoxic and normoxic conditions: during hypoxia, HIF-1α is physiologically stabilized and activates COX-2 transcription, whereas in normoxic conditions overexpression of thioredoxin stabilizes HIF-1 with the same effect.

COX-2 up-regulation is an early event in the development of NSCLC and COX-2 is overexpressed in about one third of atypical adenomatous hyperplasias and carcinoma *in situ* specimens and in up to 70% to 90% of invasive lung adenocarcinomas (8–10). Our study suggests that inhibition of the thioredoxin-1 redox system may represent an effective approach for these early stages of disease because it may affect multiple downstream target genes involved in tumor growth. Recent data show that prostaglandin E_2 (PGE_2) can directly induce expression of HIF-1α protein (30, 31). These findings suggest the possibility of autocrine stimulation in which elevated PGE_2 levels due to increased COX-2 overexpression stimulate expression of HIF-1 that induces continued COX-2 expression. Thus, once COX-2 is activated in preneoplasia, the changes may tend to persist and promote the development of invasive cancer.

Hypoxic induction of COX-2 has only been studied before in endothelial cells, where it seems to involve the cooperation of the transcription factors NF-κB (14), Sp1, and the high-mobility group protein I(Y) (32, 33) binding to the COX-2 promoter. We report here that deletion analysis of the human COX-2 promoter indicated the presence of a *cis*-acting element in the region between −873 and −466 that affected the transcriptional response to hypoxia in A549 cells. We found that this transcriptional activation of COX-2 during hypoxia is associated with overexpression of HIF-1 and that a dominant-negative HIF-1α construct in lung cancer cells prevented this hypoxic up-regulation of COX-2. Thus, it seems that HIF-1 is the major hypoxic regulator of COX-2 in these lung cancer cells.

However, it is clear that regulation of COX-2 expression is complex and differs depending on stimuli and cell type. For example, in pulmonary artery smooth muscle cells (PASMC), hypoxia failed to induce expression of *iNOS* and *HO-1*, two of the main HIF target genes (34). However, in rat aortic vascular smooth muscle cells, *HO-1* is induced by hypoxia (35). Importantly, COX-2 expression in cancers is induced by a variety of other stimuli including oncogenes, growth factors, tumor promoters, chemotherapy agents, etc. (36) such that hypoxic induction is only one of the numerous mechanisms of COX-2 induction in the cancer cell. Sequences that mediate increased RNA stability have also been identified in the 3′-untranslated region of the COX-2 mRNA (37, 38). These findings suggest that post-transcriptional events also participate in the regulation of COX-2 expression. The effectiveness of manipulating HIF-1 in lung cancer cells suggests, however, that this may be the predominant regulatory mechanism in these cells.

The effect of COX-2 induction during hypoxia on the production of specific eicosanoids in lung cancer cells is not yet understood. Other studies have found that endothelial and PASMCs have increased production of a variety of prostaglandins during hypoxia (34), whereas rat microvascular and corneal epithelial cells show decreased prostaglandin production during hypoxia (39). The specific biochemical consequences of hypoxic induction of COX-2 or normoxic induction of COX-2 due to HIF-1α stabilization *in vivo* remain to be determined. Interestingly, a recent study identified prostacyclin synthase (*PTGIS*) and prostaglandin-endoperoxide synthase 1 (*PTGS1*; enzymes in the prostaglandin production pathway) as genes whose expression is modulated by HIF-1 during hypoxia as well (40).

Our studies thus show that the *COX-2* gene promoter can be activated by HIF-1α in A549 lung cancer cells exposed to hypoxia and that this mechanism may be more important than NF-κB in these cells. This represents the first identification and characterization of a functional HRE in the *COX-2* gene promoter. We also found that thioredoxin, frequently overexpressed in NSCLC, induces COX-2 expression through HIF-1α stabilization. Our results establish a novel regulatory mechanism underlying COX-2 overexpression in lung cancer and suggest a possible relationship between thioredoxin overexpression and induction of a set of genes, including *COX-2*, that confer poor prognosis in lung cancer.

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Ildiko Csiki, Kiyoshi Yanagisawa, Nobuhiro Haruki, et al.

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