Hypoxia Induces Cyclooxygenase-2 via the NF-κB p65 Transcription Factor in Human Vascular Endothelial Cells*

(Received for publication, May 17, 1996, and in revised form, October 4, 1996)

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The inducible cyclooxygenase, COX-2, has been associated with vascular inflammation and cellular proliferation. We have discovered that hypoxia increases expression of the COX-2 gene in human vascular endothelial cells in culture independent of other stimuli. Western analysis of human umbilical vein endothelial cells (HUVEC) revealed a greater than 4-fold induction of protein by hypoxia (1% O2). The steady-state level of COX-2 mRNA was correspondingly elevated by both Northern blot and reverse transcriptase-polymerase chain reaction analysis. Using electrophoretic mobility shift assays with antibody supershifting, we also found that hypoxia causes increased binding of NF-κB p65 (Rel A) to the one out of the two NF-κB consensus elements in the COX-2 promoter which is closest to the transcription start site of the COX-2 gene. Transfection of an immortalized human microvascular endothelial cell line (HMEC-1) with mutation reporter gene constructs and HUVEC with both mutation and deletion reporter gene constructs suggested that transcription of the COX-2 gene was enhanced by hypoxia. In transcription factor decoy experiments, hypoxic HUVEC were exposed in culture to 20 μM of the same NF-κB element found to bind NF-κB protein. The wild type transcription factor decoy prevented hypoxic induction of COX-2, presumably by binding with cytoplasmic p65; however, mutated or scrambled oligonucleotides did not prevent the increase in COX-2 protein expression by hypoxia. Thus, the intracellular signaling mechanism that leads to induction of COX-2 by hypoxia includes binding of p65 to the relatively 3' NF-κB consensus element in the COX-2 upstream promoter region in human vascular endothelial cells.

The cyclooxygenases, also referred to as prostaglandin synthases, catalyze the rate-limiting step in prostaglandin synthesis. A constitutive cyclooxygenase (COX-1) and an inducible cyclooxygenase (COX-2) have been identified. COX-2 is the inducible cyclooxygenase in most tissues (1, 2). Both COX-1 and COX-2 perform two enzymatic functions; as cyclooxygenases, they convert arachidonic acid to PGG2, and as peroxidases, they convert PGG2 to PGH2. Prostaglandin synthesis can be stimulated by various physical and chemical agents in human vascular endothelial cells, including shear stress, hypoxia, esterified fatty acids from the cell membrane, calcium ionophores, bradykinin, thrombin, angiostatin II, and growth factors (3). Glucocorticoids and non-steroidal anti-inflammatory agents decrease prostacyclin production (3). However, the level of prostaglandin synthesis in the vascular endothelium is not simply regulated by posttranslational modification of cyclooxygenases. Although aspirin acetylates and deactivates cyclooxygenases, indomethacin induces expression of both COX-2 protein and mRNA (4). Mitogens such as tumor necrosis factor, phorbol ester, lipopolysaccharide, or interleukin-1α also increase the steady-state levels of COX-2 mRNA in human umbilical vein endothelial cells (HUVEC) (5).

Given the previously reported finding that hypoxic HUVEC in culture produce increased prostacyclin (6), we hypothesized that the enzyme which catalyzes the rate-limiting step in prostacyclin synthesis, COX-2, might also be induced by hypoxia in this setting. Herein we present the first demonstration that the expression of the COX-2 gene is induced by hypoxia, along with an investigation of the mechanism of this induction by NF-κB p65.

NF-κB p65 (Rel A) is one of the NF-κB family of transcriptional activator proteins. NF-κB p65 enters the nucleus and binds to DNA in a dimerized state, typically with NF-κB p50. The p65 subunit is known to be responsible for initiating transcription by DNA binding (7), but it is also likely that a number of other proteins bind with dimerized p65-p50 to initiate NF-κB-mediated transcription so as to allow a gene-specific response to this ubiquitous transcription factor (8). Phosphorylation by protein kinase C appears to be an essential part of this pathway, both through the removal of NF-κB p65 from the cytoplasmic inhibitory binding protein 1κB-α and through augmentation of the transactivation potential of NF-κB p65 itself (9).

There are two NF-κB consensus sites in the promoter region of the human COX-2 gene (10): the NF-κB-5’ site (5′-GGCGGG-GAGAGGATTCTTCGGCCCC-3′, element underlined, within −455 to −428 bases from the transcriptional start site in the sequence HSU04636; accession no. U04636 (1994)) and the NF-κB-3’ site (5′-CAGGAGAGTTGCCGCCTCCCTCTGCT-3′, element underlined, within −232 to −205 bases from the transcriptional start site). NF-κB-5′ has been shown to have a role in the mechanism of COX-2 induction by tumor necrosis factor α in a murine osteoblast cell line (11). NF-κB-3’ may play a role in facilitating the induction of COX-2 by lipopo-
nuclear protein interaction. Furthermore, we then synthesized mutant oligonucleotides containing a restriction endonuclease site within the NF-κB consensus regions. These products were used directly for electrophoretic mobility shift assay and were also applied to PCR-based synthesis of mutant promoter constructs for luciferase reporter gene analysis using PGL2-Basic (Promega). In addition, we used the wild-type and mutant oligonucleotides for selective analysis of DNA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemical reagents were obtained from Sigma, except as noted. HMEC-1 medium (Lonza, lots 072041 and 072045) and the basic fibroblast growth factor from Promocea, Madison, WI as cryopreserved primary cultures, which demonstrated factor VIII-related antigen and low density lipoprotein uptake. Human microvascular endothelial cells (HUVEC) (a generous gift of Ann McEwen, The Woodlands, TX), a human immortal endothelial cell (EaeI, UT, Houston, TX) or IgG monoclonal antibody to chicken IgG (Cappel, West Chester, PA) were obtained from HUVEC grown in HMEC-1 medium (Life Technologies, Inc.) with 10% FBS (HyClone Laboratories, Logan, UT), 50 μg/ml heparin, and 1.0 μg/ml trypsin inhibitor, and 10.0 μg/ml soybean trypsin inhibitor, and 0.03% bromphenol blue. The sample was boiled for 5 min. After electrophoresis in an SDS-PAGE gel, the proteins were electrotransferred to nitrocellulose. Non-specific binding was blocked by soaking the membrane in a blocking buffer containing 5% non-fat milk and 2% fish gelatin. The membrane was prepared with ambient oxygen concentrations of 1% (using a controlled incubator with CO2/O2 monitoring and CO2/N2 gas sources). Reoxygenation was prevented through immediate replacement of hypoxic medium with lysis buffers while the cells were on ice. The medium was blocked by soaking the membrane in a blocking buffer containing 5% non-fat milk, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.25 mM MgCl2, 0.2 mM dNTPs, and 2.5 units of Taq DNA polymerase in a total volume of 100 μl at 94°C for 2 min, followed by 28 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s.

**Western Blotting**—Cell culture dishes were briefly washed with phosphate-buffered saline before adding lysis buffer (50 mM Hepes (pH 7.4), 0.5% Triton X-100, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml pepstatin A, 1.0 μg/ml leupeptin, 10.0 μg/ml soybean trypsin inhibitor, and 10.0 μg/ml aprotinin). Protein was measured using a modified Bradford method (14). The lysate (40 μg of protein) was mixed with 2× electrophoresis sample buffer: 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.003% bromphenol blue. The sample was boiled for 5 min. After electrophoresis in an SDS-PAGE gel, the proteins were electrotransferred to nitrocellulose. Non-specific binding was blocked by soaking the membrane in a blocking buffer containing 5% milk, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 for 30 min at 37°C. The membrane was then incubated with IgG monoclonal antibody to COX-2 at 1:1500 dilution (0.17 μg/ml) (Transduction Laboratories) or IgG monoclonal antibody to chicken α-tubulin at 1:2500 dilution (0.1 mg/ml) (Sigma) was also performed for comparative purposes. The membrane was next incubated in Luminal ECL detection reagents (Amersham, Buckinghamshire, United Kingdom) and exposed to film.

**Transcription Factor Decay Experiments**—NF-κB-3′ double-stranded DNA (5′-CAGGAGATGGGGACATCCCCCTTCTGCT-3′, −232 to −205) was synthesized by annealing of sense and antisense oligonucleotides with and without a mutation of the two italicized guanine residues to cytosome (5′-CAGGAGATGGGGACATCCCCCTTCTGCT-3′, −232 to −205). This wild type and mutant DNA was placed directly into cell culture medium at a concentration of 20 μl at the beginning of exposure of the cells to 24 h of hypoxia for Western analysis. Electrophoretic mobility shift assays were first performed with a consensus NF-κB oligonucleotide: 5′-AGTGGGAGGACTTTCCAGGC-3′ (Promega) and later with the NF-κB-5′-WT (5′-CGCGGCGGGAGAGGATTCCTCGGCCC-3′) and NF-κB-3′-WT (5′-AGACAGGAGAATGGGGACATCCCCCTTCTGCT-3′) oligonucleotides for selective analysis of DNA.

**Northern Blotting**—The poly(A)+ RNA sample, 3.0 μg in 2 μl of aqueous solution, was mixed with 1.0 μl of 10× MOPS running buffer (0.4 M MOPS, pH 7.0, 0.1 mM sodium acetate, 0.01 mM EDTA), 1.7 μl of 37% formaldehyde, and 5.0 μl of formamide, with water as needed to provide a total volume of 10 μl. The sample was incubated 15 min at 55°C.
Then 2.0 μl of formaldehyde loading buffer (1 mM EDTA (pH 8.0), 0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 50% glycerol) was added to each sample. A formaldehyde gel (1.2% agarose, 1 × MOPS running buffer, 1% formaldehyde) was loaded with 12 μl of sample in each well. Ethidium bromide (0.5 μg/ml) was added to the gel to allow us to visualize the position of the gel after staining. After electrophoresis at 250 V, it was placed in a tray and washed several times with diethyl pyrocarbonate-treated water. The RNA was transferred to a nylon membrane by blotting and then rinsed in 5 × SSPE, diluted from previously prepared 20 × SSPE (3.0 mM NaCl, 200 mM Na₂HPO₄·H₂O, 25 mM EDTA, pH 7.7). The RNA was subsequently fixed by UV cross-linking. Hybridization occurred in 5 ml of 50% formamide, 5 × SSPE, 5 × Denhardt’s solution, 1% SDS, and 200 μg/ml salmon testes DNA over 4 h at 42°C using a rotating bottle hybridizer. The cDNA reverse-transcription, which was performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM each dNTP, 1 unit/ml RNase inhibitor, 2.5 units/μl MMLV-RT (Perkin Elmer), and 20 pmol each of HSCOX2-S (5′-CCGGACAGGAGGTACTGAGA-3′, sense bases 531–512) and HSCOX2-AS (5′-ATCCATGCGAGCAGGAGG-3′, antisense bases 554–535) (17), which also flanked intron DNA. A total of 4 μl of RNA from cellular extracts was placed in each assay tube for subsequent reverse transcription, which was performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM each dNTP, 1 unit/ml RNase inhibitor, 2.5 units/μl MMLV-RT (Perkin Elmer), and 20 pmol each of HSCOX2-AS and HSTUB1-AS in a total volume of 20 μl, incubated first at 42°C for 60 min and then at 99°C for 5 min. The reaction product was then subjected to PCR with the addition of 20 pmol of either HSCOX2-AS or HSTUB1-AS in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM MgCl₂, 0.2 mM dNTPs, and 2.5 units of Taq DNA polymerase (Perkin Elmer) in a total volume of 100 μl at 95°C for 2 min, followed by 28 cycles of 95°C for 30 s, 55.0°C for 30 s, and 72°C for 60 s. The COX-2 (300 bp) and α-tubulin (200 bp) mRNA amplification products were separated by agarose gel electrophoresis and analyzed using ethidium bromide staining. The product of constitutively expressed α-tubulin mRNA served as the control in this assay. We assert that these data are quantitative (relative to α-tubulin based on the following sets of tests performed prior to data analysis. First, both products were assayed in the linear response range of the RT-PCR amplification process; the cycle number used in this assay (28) was determined by finding the midpoint of linear amplification on a sigmoidal curve for both amplification products with cycle numbers 21–33 plotted against band density. Second, after ethidium bromide staining of sample amplification products, the densitometric “area under the curve” was found to increase by 1 linear unit for each log₂ increase in RNA from HUVEC.

**Electrophoretic Mobility Shift Assay**—Nuclear protein was extracted from cells that had been washed on ice with ice-cold phosphate-buffered saline and scraped off the flask. The duration of hypoxia was 2 h. The cells were isolated by centrifugation at 1000 × g for 4 min at 4°C and resuspended in nuclear extraction cell lysis buffer (10.0 mM Hepes (pH 7.8), 1.5 mM MgCl₂, 10.0 mM KCl, 1.0 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 10.0 μg/ml leupeptin, and 10.0 μg/ml aprotinin). The cells were again isolated by centrifugation at 1000 × g for 4 min at 4°C for 4 min and then 100 μl of 1% Trition X-100 was added per 107 cells. The cells were then isolated by centrifugation at 2000 × g for 4 min at 4°C. Subsequently 50 μl of final nuclear protein isolation buffer (20.0 mM Hepes (pH 7.8), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1.0 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 10.0 μg/ml soybean trypsin inhibitor, 10.0 μg/ml leupeptin, and 10.0 μg/ml aprotinin) was added and allowed to sit on ice for 30 min. Following centrifugation at 15,800 × g for 4 min at 4°C, the supernatant containing the protein was removed. We examined the 5′-flanking region of the COX-2 gene with an electroelution and mobility shift at 25–30°C in the presence of hypoxia-inducible protein-DNA binding sites. There are two regions for NF-κB binding in the COX-2 promoter region: the NF-κB-5′ site (5′-GCCGGAGAAGAGATGCGGCCC-3′), within −455 to −428 from the transcriptional start site at +832 in the sequence HU046360 (10) and the NF-κB-3′ site (5′-CAGGAGATTGGG-GACTACCCCTGCTGTC-3′, −232 to −205). Double-stranded DNA probes were synthesized by annealing of sense and antisense oligonucleotides and each was end-labeled with 50 μCi of [γ-32P]ATP/20 pmol using T4 polynucleotide kinase (Pharmacia, Upplands, Sweden) and then isolated using a Centri-Sep column (Princeton Separations, Adelphia, NJ). DNA probe (20,000 cpm) and 1.0 μg of bulk carrier poly(dC)-DNA DNA was reconstituted with distilled water. After centrifugation at 55°C for 30 min to 1 h depending on the individual experiment. Antibody supershift was performed with polyclonal rabbit antibody to human amino-terminal NF-κB p50 at 5.0 μg/ml or NF-κB p65 at 10.0 μg/ml final concentration (both from Santa Cruz Biotechnology, Santa Cruz, CA). Some supershift experiments were performed with delayed introduction of antibodies. Dideoxynucleotides were added with a 400-fold excess of cold DNA or substitution of 5.0 μg of bovine serum albumin for nuclear protein was performed as a control. The protein-DNA product was then run in a 5% nondenaturing polyacrylamide gel and autoradiographed or subjected to phosphor imaging.

**Transfection of Promoter Constructs**—Transfection analysis using luciferase reporter gene constructs with the promoter region of COX-2 were performed in HMEC-1, which we have shown to be a workable model for analysis of hypoxia-induced transcription (18), and in HUVEC. A 565-bp region of the COX-2 5′ region was amplified by PCR with and without mutation of the NF-κB-3′ site as above. We were originally concerned about the possibility that the induction of COX-2 in hypoxia was dependent on some cofactor in the medium employed. Therefore, the Western analysis was repeated under variant cell culture conditions: (a) in the standard HUVEC medium as noted under “Experimental Procedures” (2A), (b) in the same M199 medium formulation but with 5% instead of 10% FBS, and (c) 2% FBS using the HMEC-1...
MCDB 131 formulation plus 12 μg/ml bovine brain extract and 10 ng/ml human recombinant epidermal growth factor (Fig. 2B). Hypoxia induced COX-2 similarly under all three conditions. The response of the HUVEC to hypoxia is compared with that of the endothelial constitutive nitric-oxide synthase in Fig. 2B. The ecNOS is predictably down-regulated in these cells by hypoxia (19), while COX-2 expression is induced. Fig. 2C displays a statistical summary of repeated analyses using the medium employed in Fig. 2A.

The COX-2 mRNA response of HUVEC to hypoxia is documented in Fig. 3 (A and B). Both Northern blotting and RT-PCR revealed that there was a peak in the accumulation of mRNA for COX-2 between 30 min and 2 h after the induction of hypoxia. A positive control was obtained through a 4-h incubation with PMA/CHX (20 ng/ml phorbol 12-myristate 13-acetate and 10 μg/ml cycloheximide) in the Northern blot. The large response of COX-2 to phorbol ester/cycloheximide has been observed previously (20). The results in Fig. 2A suggest that hypoxia can induce endothelial COX-2 mRNA to a steady-state level comparable to that of the constitutive COX-1 mRNA. Using hybridization to the 2.7-kb band to control for lane loading, and the hybridization to COX-2 mRNA in normoxia for comparison, the steady-state level of COX-2 mRNA was increased 12.7-fold after 30 min of hypoxia and 8.9-fold after 4 h of hypoxia. Clearly there is a lag time between induction of mRNA and protein; this may be attributable to intermediary steps in translational processing that were not the focus of this particular investigation.

Although both cyclooxygenases encode peptides of approximately 600 amino acids, the size of the mRNA differs. COX-1 typically hybridizes at 2.7 kb; COX-2 at 4.2 kb. This difference is mainly due to the length of the 3′ untranslated region that is present in COX-2 (see Fig. 1). We encountered some technical concerns as to whether there might be some hybridization of COX-2 cDNA to a variably observed 2.7-kb band, which can migrate very close to the COX-1 band. For this reason, we confirmed the results of Northern blotting with comparative RT-PCR, illustrated in Fig. 3B. Comparison of the COX-2 (300 bp) and α-tubulin (200 bp) mRNA amplification products was performed in each lane. Using α-tubulin as a control, densitometry revealed a 2.7-fold induction of COX-2 by hypoxia. Given the log-linear relationship between densitometric analysis and template concentration described under “Experimental Procedures” (the densitometric “area under the curve” was found to increase by 1 linear unit for each log10 increase in RNA from HUVEC), we estimated that the steady-state level of COX-2 mRNA was greatly increased after 2 h of hypoxia. After three successful repeats of this assay, we concluded that the rapid accumulation of transcript was mirrored by its rapid disappearance by 4 h after onset of hypoxia. This reflects the instability of the COX-2 mRNA, attributed to the long 3′-untranslated region found in COX-2 mRNA, which is probably destabilized through the presence of the Shaw-Kamen AU repeats in
Hypoxia Induces COX-2 via NF-κB p65 in Vascular Endothelium

A Northern blot of HUVEC mRNA (3 μg of poly(A)+ RNA/lane) extracted after incubating cells in either normoxia (21% O2, 5% CO2) or hypoxia (1% O2, 5% CO2) for either 30 min or 4 h, or normoxic conditions with a 4-h incubation with 20 ng/ml hydrocortisone 12-myristate 13-acetate and 10 μg/ml cycloheximide as a positive, nonphysiologic control. B, reverse transcriptase-polymerase chain reaction assay of HUVEC total RNA (4 μg/lane). HSCOX2-S (5'-CCGAGCGAGACCTATGAGA-3', sense bases 232–252) and HSCOX2-AS (5'-CAATCATGCGACAGAGGG-3', antisense bases 531–512) (1) were used to amplify COX-2. RT-PCR amplification

\[ \text{a-tubulin} \]

and 65 bases downstream, differing only in the addition of an

\[ \text{COX-2} \]

upstream of the NF-κB element (at the same site studied in HMEC-1 transfection) prevented reporter gene induction by hypoxia. The wild type element was necessary for regulation of reporter gene expression by oxygen tension. Furthermore, inclusion of the region just upstream of the NF-κB-3' element yielded progressively more luciferase expression (Fig. 6).

A proof of a direct cause-effect relationship between protein binding to NF-κB-3' and the transcription of COX-2 was necessary, since others have shown that the peroxidase activity of COX-1 itself can lead to NF-κB activation (23). We succeeded in introducing naked dsDNA into HUVEC during standard culture conditions as transcription factor decoys to address this question. This is demonstrated as part of Fig. 2. The peak in COX-2 protein prevalence in response to hypoxia occurs at 24 h; this effect is not altered by incubation of these hypoxic cells with 20 μM mutant NF-κB-3' DNA, but is nearly entirely blocked by wild type NF-κB-3' DNA. Although the relevance of the NF-κB-3' site to the synthesis of COX-2 is suggested by the gel shift and reporter gene construct data, it is proven by the direct inhibitory effect that the wild type NF-κB-3' DNA has on the synthesis of COX-2, presumably by blocking the translocation of NF-κB p65 from cytoplasm to nucleus.

DISCUSSION

Our experiments demonstrate for the first time that COX-2 is induced by hypoxia in vascular endothelial cells, and that this induction is mediated by NF-κB p65 (also known as Rel A). Given the findings of electrophoretic mobility shift assays, mutation/deletion construct reporter gene transfection assays, and the transcription factor decoy experiments presented herein, it appears that binding of the NF-κB-3' site is a necessary (if not sufficient) step, perhaps a final common pathway for hypoxic induction of COX-2. This could be related to modification of stimulatory cytokine-related signal transduction pathways in response to hypoxia. It is also likely that COX-2 levels are regulated (at least in part) through degradation of its mRNA, since the 3' region contains the AU motif initially described by Shaw and Kamen as a mediator of mRNA degradation (21) and reported by other investigators to be associated with instability of the COX-2 transcript (10, 24).

Previous reports indicate that NF-κB transcription factors can be activated by reactive oxygen intermediates. Hydrogen peroxide can induce NF-κB-mediated expression and replication of human immunodeficiency virus-1 in human T cell lines, and N-acetyl-L-cysteine, an antioxidant, can block the activation of NF-κB in this model (25). Although oxygen is required for the formation of reactive oxygen species, hypoxia may decrease the ability of cells to supply NADPH and, hence, reduced glutathione in the presence of chemical oxidants (26). This could lead to an overall increase in net cellular oxidative stress in hypoxia in certain settings. Hypoxia followed by reoxygenuation has been shown to activate NF-κB p50-p65 heterodimer activity in HeLa cells (27). However, in the experiments reported herein, NF-κB activity was facilitated without reperfusion or reoxygenuation. We propose that either incomplete oxidative metabolism in hypoxia or the presence of chemical oxidants in the cellular milieu are possible mechanisms for the hypoxia-mediated effects on NF-κB.

Recently it was shown that hypoxia can increase the tyrosine phosphorylation of IκB-α in Jurkat T-cells, and that this facilitated NF-κB binding to DNA (28). Interestingly, the tyrosine phosphorylation of IκB-α could not be duplicated in their model with phorbol myristate acetate or tumor necrosis factor, suggesting that this phosphorylation is a relatively specific response of these cells to hypoxia. Ubiquitination of IκB-α and subsequent degradation is another of the steps that lead to release of NF-κB subunits from cytoplasmic binding proteins (8). We do not exclude any of these possible mechanisms for
signal transduction in hypoxia.

Cells of varying species and site of origin have different prostacyclin responses to hypoxia (29, 30) and ischemia (31). In HUVEC, prostacyclin synthesis is stimulated by hypoxia (6), but cultured aortic and pulmonary arterial endothelial cells used in some experiments have not demonstrated increased production of prostacyclin in hypoxia (32). COX-2 expression has been shown to be highly regulated in systems in which phenotypic changes in smooth muscle or endothelial cells occur (33). Our experiments demonstrate that hypoxia increases steady-state levels of COX-2 mRNA and protein in hypoxic human umbilical vein endothelial cells. The relevance of this...
cyclooxygenase induction to eicosanoid synthesis in particular vascular tissues remains a subject for future investigation. Hempel et al. have demonstrated that human alveolar macrophages have an opposite, tissue-specific decrease in COX-2 expression in hypoxia (34).

COX-2 gene regulation by hypoxia may be analogous to the regulation of vascular endothelial growth factor. Using RNase protection assays and transfection analysis of promoter fragments (35, 36), a 5'-flanking hypoxia-induced enhancer for this gene was recently described. Regions of homology of the vascular endothelial growth factor promoter region with the previously described hypoxia-inducible enhancer have also been noted (37). However, the binding sequence published for the hypoxia-inducible factor associated with this enhancer (38) is not found adjacent to the COX-2 gene (10, 39).

The up-regulation of COX-2 in response to hypoxic stress is of pathophysiologic significance and might be a target for gene therapy, as demonstrated using the transcription factor decoy method. Increased COX-2 gene expression correlates with the development of gastrointestinal malignancies (16). Blood in hypoxic vessels downstream from an arterial stenosis is known to be more prone to thrombosis (40). This could be related to an increased COX-2 production by hypoxic vascular endothelium and subsequent release of procoagulant thromboxanes. Mitogens increase COX-2 activity in macrophages (41), cells that play a key role in atherosclerosis and may play a role in the proliferative response to vascular injury. COX-2 or its eicosanoid products have been recently found to stimulate monocytes, the precursors of macrophages, to produce 8-epi prostaglandin F$_2$α, a mitogen and vasoconstrictor (42). Platelet-activating factor, a lipid mediator formed in response to cell injury, acts in the presence of retinoic acid, a modulator of cell growth, to induce COX-2 transcription (43). The inflammatory, procoagulant, and proliferative aspects of endothelial dysfunction in atherosclerosis may be linked by the fact that many of the genes for leukocyte adhesion molecules associated with initiation of the atherosclerotic lesion have functional NF-κB binding sites (44). Aspirin is a non-selective inhibitor of the function of both cyclooxygenases (45) that can also inhibit NF-κB-mediated transcription (46). We speculate that the mechanism of the beneficial effect of aspirin in patients with coronary artery disease (47) could be at least partially attributable to inhibition of NFκB-mediated responses of the vascular endothelium associated with atherosclerosis and hypoxia.

Acknowledgments—We gratefully acknowledge the technical assistance of Qing Xu, Yazu Wu, and Brigitte Grochan in the conduct of these experiments.

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J. Biol. Chem. 1997, 272:601-608.
doi: 10.1074/jbc.272.1.601

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