Sp1 Increases Expression of Cyclooxygenase-2 in Hypoxic Vascular Endothelium

IMPLICATIONS FOR THE MECHANISMS OF AORTIC ANEURYSM AND HEART FAILURE*

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Cyclooxygenase-2 (COX-2) catalyzes prostaglandin synthesis from arachidonic acid and is expressed locally in aortic aneurysm and heart failure. Cellular hypoxia is also found in these conditions. We have previously shown that cox-2 is transcriptionally regulated by hypoxia in human umbilical vein endothelial cells (HUVEC) in culture via the transactivation factor NF-κB p65, leading to increased production of prostaglandin E2, an inhibitor of vascular smooth muscle cell proliferation. Sp1 is a transactivation factor known to be important in the regulation of cytokine expression in association with NF-κB. We hypothesized that Sp1 is involved in the induction of cox-2 in hypoxic HUVEC. Electrophoretic mobility shift assays with hypoxic HUVEC nuclear protein showed that both Sp1 and the related protein Sp3 specifically bound to the cox-2 promoter. Immunoblotting demonstrated that hypoxia increased the nuclear localization of Sp1 but did not change the Sp3 content in HUVEC. Overexpression of Sp1 through transfection of HUVEC enhanced cox-2 promoter activity as measured by reporter gene expression and by the production of COX-2. The specificity of the results was confirmed by mutation of the Sp1-binding site in the cox-2 promoter construct and by reproducibility in an Sp-deficient Drosophila SL2 cell line. The regulatory role of Sp1 discovered in this work supports the concept that a mechanistic link exists between vascular cellular hypoxia and mediators of inflammation associated with aortic aneurysm and heart failure.

Although the pharmacologic inhibition of cyclooxygenase activity with aspirin is a cornerstone of modern cardiovascular therapy, we do not yet understand how endothelial cyclooxygenases function in health or in disease. The cyclooxygenases, also referred to as prostaglandin endoperoxide synthases or PGH synthases, catalyze the rate-limiting step in prostaglandin synthesis. A constitutive cyclooxygenase (COX-1) and an inducible cyclooxygenase (COX-2) have been identified. Both COX-1 and COX-2 perform the same enzymatic function, converting arachidonic acid to PGG2 and then PGH2. PGH2 is the progenitor of the thromboxanes, prostacyclin, and PGE2, among other prostaglandins (1, 2).

Myocardial hypoxia has been found in animal models of heart failure (3). Both COX-2 expression and NF-κB activation appear in heart failure (4). The wall of an aneurysmal human aorta is also hypoxic (5) and has increased cellular COX-2 content and PGE2 production (6). These findings are not surprising in light of our earlier work, within which we demonstrated that cox-2 is transcriptionally regulated by hypoxia via the transactivation factor NF-κB p65 in human vascular endothelial cells (7), leading to increased production of PGE2 (8).

Nuclear factor-κB p65 (RelA) is one of the NF-κB family of transcriptional activator proteins. The p65 subunit is known to be responsible for initiating transcription by DNA binding (9), 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 (10). The intracellular signaling mechanism that leads to induction of cox-2 by hypoxia in human vascular endothelium includes binding of p65 to the NF-κB consensus element closest to the transcription start site. However, we learned in earlier deletion experiments that there is a relationship between the length of the region upstream of this NF-κB element and the degree of induced transcription. It appeared to us that binding of the NF-κB p65 is a necessary but not sufficient step in hypoxic induction of COX-2 (7).

After finding that cytoplasmic NF-κB p65 and IκBα (an inhibitory protein that binds NF-κB p65 precursors) levels are not changed by hypoxia, we hypothesized that other factors might play a role in regulating the cox-2 promoter. The HMG I(Y) family of proteins features multiple A/T hooks and is associated with NF-κB-mediated transactivation. We recently discovered that hypoxia increases expression of HMG I(Y) proteins while facilitating transactivation of the cox-2 promoter (8).

Sp1 is typically a positive-acting transcription factor that is ubiquitously expressed and required for the expression of a variety of genes (11). It is known to be important in the regulation of cytokine and human immunodeficiency virus gene activation in association with NF-κB (12). Sp3 is a bi-functional regulator of transcription (repressor/activator, depending on the context) that competes with Sp1 for the same binding site. Sp1 mediates expression of the constitutive cyclooxygenase (COX-1) (13). Transcription of the hypoxia-inducible factor-1α (HIF-1α) gene is initiated just downstream of two Sp1 sites (14). Extension of our previous search for hypoxia-related regulatory elements in the cox-2 promoter (15) revealed an Sp1-binding site just upstream of the NF-κB-3' element that we...
already know enhances cox-2 transcription in hypoxia. We therefore hypothesized that Sp1 is involved in the induction of cox-2 in hypoxic human vascular endothelial cells, and this hypothesis is supported by the results of experiments detailed herein.

EXPERIMENTAL PROCEDURES

Cell Culture—HUVEC were obtained as cryopreserved primary cultures that demonstrated factor VIII-related antigen and low density lipoprotein uptake (Clonetics). HUVEC were grown in Medium 199 with 2.2 g of NaHCO3/Liter, 50 units/ml penicillin, and 50 µg/ml streptomycin (Life Technologies, Inc.) with 5% fetal bovine serum (HyClone Labs), 50 µg/ml endothelial cell growth supplement (Collaborative Biomedical Products), 50 µg/ml heparin, and 1.0 µg/ml hydrocortisone. The medium was prepared with a pH of 7.3. Incubator conditions were either normoxic (21% O2, 5% CO2) or hypoxic (1% O2, 5% CO2, balance N2) in a humidified incubator with an interior temperature of 37°C. The medium was equilibrated to the environmental gas conditions overnight before cellular exposure. HUVEC cells were studied at the third to fifth passages. Hypoxic stimulation was produced with ambient oxygen concentrations of 1% (using a controlled incubator with CO2/O2, balance N2) in a humidified incubator with an interior temperature of 37°C. The medium was equilibrated to the environmental gas conditions overnight before cellular exposure. HUVEC cells were studied at the third to fifth passages. Hypoxic stimulation was produced with ambient oxygen concentrations of 1% (using a controlled incubator with CO2/O2, balance N2) in a humidified incubator with an interior temperature of 37°C. The medium was equilibrated to the environmental gas conditions overnight before cellular exposure. HUVEC cells were studied at the third to fifth passages. Hypoxic stimulation was produced with ambient oxygen concentrations of 1% (using a controlled incubator with CO2/O2, balance N2) in a humidified incubator with an interior temperature of 37°C. The medium was equilibrated to the environmental gas conditions overnight before cellular exposure. 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**FIG. 1.** An overview of the proposed regulation of COX-2 by hypoxia regulatory factors. The sequence of the COX-2 promoter region of interest (−250 to −205) is GGGAGGAGGGATCGAGAGGAAGGGATCGACA-3′. The Sp1 element is the 1st underlined segment, and NF-κB-3′ is the 2nd underlined segment.

**FIG. 2.** Electrophoretic mobility shift assay of Sp1 reveals specific DNA-protein interaction in hypoxia. Nuclear protein of HUVECs treated with continued normoxia or 2 h of hypoxia is presented. An oligonucleotide CX9 corresponding to the Sp1-binding site in the COX-2 promoter (5′-GGAGAGGGATCGAGAGGAAGGGATCGACA-3′) and a consensus Sp1 oligonucleotide c-Sp1 (5′-ATTCGATCGGGGCGGGCGGGCGAGC-3′) were used as probes and competitors. The CX9 mutant CX9M (5′-GGAGAGGAAGGGATCGACA-3′, mutated bases are italicized and bold) was used to confirm specificity of Sp1 binding. Oligonucleotides were end-labeled, and 2 × 10^5 cpm was used as probe to incubate with the nuclear protein in each lane. Bovine serum albumin (5 μg) was used in lane 1 instead of 3 μg of nuclear protein (other lanes). Lanes 2 and 3 and lanes 13 and 14 demonstrate that hypoxia enhances the binding of both CX9 and c-Sp1 to the nuclear protein. Hypoxic nuclear protein was incubated with antibodies to Sp1 (lane 4, duplicated in lane 12), Sp3 (lane 5), NF-κB (lane 6), or α-tubulin (lane 7) for 30 min at room temperature prior to incubation with labeled DNA probe. Sp1 antibody supershifted primarily the upper band of the DNA-protein complex. Sp3 antibody supershifted both the upper and lower bands. Nonspecific NF-κB and α-tubulin antibodies had no effect. The specific DNA-protein binding was blocked by 100-fold excess of cold CX9 (lane 8) or c-Sp1 (lane 9). Lanes 10 and 11 show no shift when mutant CX9M DNA was used as a probe, indicating the specificity of Sp1 binding.

EMSA. The binding profile was identical to that observed with CX9. The DNA-protein complexes were competed away with 100-fold excess of unlabeled CX9 or c-Sp1. Antibodies specific for Sp1 and Sp3 supershifted the DNA-protein complexes. Sp1 antibody partially eliminated the upper complex. Sp3 antibody almost completely eliminated the lower complex and diminished the upper complex. As controls, the nonspecific NF-κB and α-tubulin antibodies were used to perform supershift. None of them had an effect on the DNA-protein complexes, indicating the binding is Sp1/Sp3-specific. The binding specificity was further confirmed by a mutation experiment. We mutated the Sp1 site of CX9 by only two bases and created CX9M. No DNA-protein complex was observed when CX9M was used as a probe, suggesting that the intact Sp1 site is required for interaction of Sp1/Sp3 with the cox-2 promoter.

**Sp1/Sp3 Regulates cox-2 Promoter Activity in Drosophila SL2 Cells and HUVEC—**To determine directly the role of Sp1 and Sp3 on regulation of cox-2 promoter activity, a Drosophila Schneider SL2 cell line that does not express endogenous Sp1 and Sp3 was used to perform co-transfection experiments. The wild type cox-2 promoter luciferase reporter construct pWT was transfected into SL2 cells with the expression vectors pPacSp1 or pPacSp3. As shown in Fig. 4, overexpression of pPacSp1 (over the range 0–100 ng/well) caused a dose-dependent increase of cox-2 promoter activity as measured by luciferase reporter gene expression, with a maximal 16-fold increase. Overexpression of pPacSp3 also increased the cox-2 promoter activity, with a maximal 3-fold increase.

Similar dose-response co-transfection experiments were also performed in HUVECs except that mammalian expression vectors pCMVSp1 and pCMVSp3 were used instead of pPacSp1 and pPacSp3. As illustrated in Fig. 5, the promoter activity of pWT was enhanced by increasing the amount of pCMVSp1 (over the range 0–1000 ng/well) with a maximal 5-fold increase but not by pCMVSp3. Increasing amount of pCMVSp3 (over the range 0–1000 ng/well) actually caused a decrease of the pro-
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**DISCUSSION**

We previously demonstrated that cox-2 is induced by hypoxia via the NF-κB p65 transcription factor in human vascular endothelium, leading to increased production of PGE2, and that the expression of the high mobility group protein HMG I(Y), known to be associated with NF-κB-mediated transactivation, treated with 24-h hypoxia. Compared with normoxia control, hypoxia significantly increased the cox-2 promoter activity of pWT by 2.2-fold but not that of pSPM (Fig. 6). This suggests that the Sp1-binding site in the cox-2 promoter region is critical to the regulation of cox-2 expression by hypoxia.

**Sp1 Enhances cox-2 Promoter Activity in Hypoxic HUVECs**—To determine the effect of Sp1 and Sp3 on cox-2 promoter activity in hypoxic HUVECs, 300 ng of pCMVSp1 or pCMVSp3 along with 3 μg of pWT were co-transfected into HUVECs. After 24 h normoxia or hypoxia treatment, cells were harvested and assayed for promoter activity. A blank vector pCMV5 (19) was added as a control and to keep the total amount of transfected DNA constant. Compared with base line (co-transfection with blank vector under normoxia), overexpression of sp1 synergistically increased the cox-2 promoter activity with hypoxia. In contrast, overexpression of sp3 not only caused a lower cox-2 promoter activity than that of base line but also decreased the transactivation of the cox-2 promoter by hypoxia (Fig. 7). It appears that Sp3 represses cox-2 gene expression under both normoxia and hypoxia in HUVECs.

**Sp1 Increases COX-2 Protein in Hypoxic HUVECs**—To determine the effect of overexpression of sp1 on COX-2 protein expression in hypoxic HUVECs, 300 ng of expression plasmid pCMVSp1 was transfected into HUVECs. In the control experiment, 300 ng of blank vector pCMV5 was transfected into HUVECs as we found DNA transfection itself resulted in slightly less COX-2 production. After 24 h normoxia or hypoxia treatment, cell lysates were collected and employed for Western blot analysis using COX-2 antibody. As demonstrated in Fig. 8, Sp1 progressively increased COX-2 immunoreactive protein in hypoxic HUVECs. We did not demonstrate a clear reduction of COX-2 protein by Sp3 (data not presented) as there was very little COX-2 protein production at base-line normoxia.

**FIG. 5.** Dose response of COX-2 promoter activity to the over-expression of Sp1 and Sp3 in HUVECs. The indicated amounts of pCMVSp1 or pCMVSp3 were co-transfected with 2.5 μg of wild type (pWT) or Sp1 mutant (pSPM) COX-2 promoter constructs. The total amount of transfected DNA was maintained constant with control plasmid (pCMV5). The pWT promoter activity was enhanced by increasing amounts of pCMVSp1 (solid circle) but not by pCMVSp3 (open circle). Increasing the amount of pCMVSp1 transfected let to minimal change in pSPM promoter activity as reflected by reporter gene expression (triangle). Results were expressed as mean ± S.E. of LUC/PAP ratio.

**FIG. 4.** Effect of Sp1 and Sp3 on the COX-2 promoter in Drosophila SL2 cells. The indicated amounts of pPacSp1 or pPac Sp3 were co-transfected with 2.5 μg of pWT into the Drosophila SL2 cells. Transfection efficiency was controlled with the inclusion of 200 ng of pPac-gal in each transfection. The total amounts of transfected DNA were kept constant at 2.8 μg with control plasmid (PUC19). Co-transfection with pPacSp1 (solid circle) caused a dose-dependent increase of the COX-2 promoter activity. Co-transfection with pPacSp3 (open circle) also increased the COX-2 promoter activity but to a less extent than that with pPacSp1. Results were expressed as mean ± S.E. of LUC/gal ratio from three independent experiments.

**FIG. 3.** Effects of hypoxia on Sp1 and Sp3 prevalence in nuclear protein. HUVECs were treated with normoxia and a time course of hypoxia. Nuclear protein (15 μg) was loaded in each lane and separated on 10% SDS-polyacrylamide gel. The gel was blotted with nitrocellulose membrane and hybridized with Sp1 antibody or Sp3 antibody. The loading consistency of each lane was confirmed by Coomassie Blue staining of the membrane. Sp1 blot density content was increased, but nuclear prevalence of Sp3 was not changed by hypoxia. A bar graph of relative band density (mean ± S.E.) from three separate experiments reveals that Sp1 content was increased approximately 2-fold by hypoxia (*p < 0.05 versus normoxia).

**FIG. 2.** Sp1 at 100 kD (nuclear protein) and Sp3 at 110/80 kD (nuclear protein).

**The Sp1 Site Is Required for cox-2 Induction by Hypoxia**—To determine if Sp1-site mutation affects hypoxia-mediated cox-2 promoter induction, either the wild type construct pWT or Sp1 mutant construct pSPM was transfected into HUVECs and
HUVECs were co-transfected with 0.3 μg of wild-type COX-2 promoter constructs containing -531 to +65 of the human COX-2 site with hypoxic HUVEC. The two vectors differ in two bases (underlined) within the Sp1-binding site (bold). After 24 h of hypoxia, the reporter gene expression with pWT increased compared with normoxia. However, hypoxia did not change the reporter gene expression with pSPM. The results are presented as LUC/PAP ratios. Statistics (mean ± S.E.) were based on three independent experiments.

FIG. 6. Transfection analysis with wild type and Sp1 mutant constructs. Wild type (pWT) and Sp1 mutant (pSPM) COX-2 promoter constructs containing -531 to +65 of the human COX-2 site were introduced into HUVECs. The two vectors differ in two bases (underlined) within the Sp1-binding site (bold). After 24 h of hypoxia, the reporter gene expression with pWT increased compared with normoxia. However, hypoxia did not change the reporter gene expression with pSPM. Statistics (mean ± S.E.) were based on three independent experiments.

FIG. 7. Effect of Sp1 and Sp3 on COX-2 promoter activity in hypoxic HUVECs. HUVECs were co-transfected with 0.3 μg of pCMV-Sp1 or pCMV-Sp3 along with 3 μg of pWT. Total amount of transfected DNA was kept constant with control plasmid pCMV5. Data are expressed as mean ± S.E. of LUC/PAP ratio representing three different experiments (* \( p < 0.05 \) versus normoxia control). Overexpression of Sp1, but not Sp3, up-regulates the expression of COX-2 promoter under hypoxic conditions.

is increased by hypoxia, facilitating transactivation of the cox-2 promoter. In this study, we demonstrated that Sp1 (and possibly Sp3) also participates in the regulation of cox-2 gene expression by hypoxia. Sp1 content was increased by hypoxia in HUVEC nuclear (but not cytoplasmic) protein, whereas Sp3 was not changed. Both Sp1 and Sp3 bound specifically to a Sp1 consensus sequence just upstream of the NF-kB-3’ element that we previously demonstrated to enhance the COX-2 transcription in hypoxia. Overexpression of sp1 synergistically increased cox-2 promoter activity and protein expression with hypoxia. We conclude that hypoxia increases the nuclear localization of the Sp1 and thereby activates expression of cox-2 in vascular endothelium.

The Sp family includes Sp1, Sp2, Sp3, and Sp4. All four members contain highly conserved DNA-binding zinc finger domains close to the C terminus and glutamine-rich and serine/threonine-rich domains in the N-terminal region. In most promoters, Sp1 and Sp3 recognize the classical Sp1 consensus element with comparable affinity and specificity (20). In our study, both Sp1 and Sp3 bind to the same Sp1 consensus sequence (−250 to −231) in the COX-2 promoter. However, they contribute differentially to cox-2 promoter activity. In HUVEC, Sp1 not only activated cox-2 promoter in a dose-dependent manner but also synergistically enhanced the promoter with hypoxia. In contrast, Sp3 acted as a repressor in HUVECs. Co-transfection of a higher dose of Sp3 resulted in decreased cox-2 promoter activity. It has been reported elsewhere that down-regulation of sp3 in hypoxic C2C12 myocytes removes the associated transcriptional repression of muscle specific pyruvate kinase-M and β-enolase, thereby enabling expression of these glycolytic enzyme genes (21). In our experiment, hypoxia activated the COX-2 promoter by increasing the activator Sp1 and not changing the repressor Sp3. In both cases, the Sp1/Sp3 ratio is elevated. It appears that an elevated Sp1/Sp3 ratio is critical to the gene activation involved with Sp1 and Sp3 as both compete for the same binding site. Both Sp1 and Sp3 are expressed at high levels in endothelial cells, and the Sp1/Sp3 ratio in these cells is much higher than that in non-endothelial cells (22). In Sp-deficient Drosophila SL2 cells, we noticed that Sp3 acted as a weak activator of COX-2 promoter instead of a repressor. This is consistent with the concept that Sp3 is a bi-functional regulator (repressor/activator) depending on the promoter or cellular context, as reported previously (20, 23). An inhibitory domain has been identified at the 5’ end of the zinc finger DNA-binding domain of Sp3; our findings support previous data indicating that the inhibitory domain of Sp3 functions only in mammalian cells (24).

FIG. 8. Effect of overexpression of Sp1 on COX-2 protein in hypoxic HUVEC. HUVEC were transfected with 0.3 μg of total DNA (pCMV-Sp1 or a control plasmid pCMV5). COX-2 protein was increased by hypoxia and Sp1 overexpression in the representative blot illustrated. The bar graph demonstrates the relationship of hypoxia, Sp1, and COX-2 immunoreactive protein (mean ± S.E., corrected for α-tubulin) from three separate experiments (* \( p < 0.05 \) versus normoxia control).

HIE-1 is not found adjacent to cox-2 (15, 25). Given the sum of our published data, it would appear that there are hypoxia-inducible enhancers other than HIE-1 that regulate cox-2 transcription. Recently an endothelial PAS domain protein was
identified in embryonic mouse endothelium which appears to be a new basic helix-loop-helix/PAF domain transcription factor (26). This protein can induce transcription of the gene for endothelial tyrosine kinase Tie-2, and its activity is stimulated by hypoxia. It shares a 48% sequence identity with HIF-1α. The HIF-1α-like factor (27) and MOP-2 (28) are among other candidate hypoxia-inducible factors that regulate transcription and have been partially characterized. ORP150, an oxygen-regulated protein, appears to be regulated by hypoxia and is found in atherosclerotic blood vessels (29). Our studies show that the Sp1 element just upstream the NF-κB-3′ element is critical to the regulation of Cox-2 promoter activity by Sp1/Sp3 and hypoxia. The interaction between this element and nuclear protein is Sp1/Sp3-specific. Both Sp1 and Sp3 antibodies supershifted the DNA-protein complex. This element is distinct from the adjacent NF-κB-3′ element as NF-κB p65 antibody has no effect on the DNA-protein complex. Sp1-mediated induction of the COX-2 promoter reflects interaction with the general transcription machinery, including the TATA box-binding protein TBP (30) and components of the TFIID complex (31–33). Recently it was noted that a transcriptional complex ing protein TBP (30) and components of the TFIID complex (31–33). Recently it was noted that a transcriptional complex

HIF-1α cofactor (CRSP) is required for Sp1-mediated transcription (31–33). Recently it was noted that a transcriptional complex

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...vascular wall (50). PGE_2 can also experimentally inhibit vascul ar smooth muscle cell proliferation (51). COX-2 is found in atherosclerotic lesions and those associated with transplant allograft vasculopathy (52), as well as in cerebrovascular hypoxia (53). We have published data suggesting that COX-2 enzymatically facilitates endothelial production of PGE_2 in hypoxia (8). Both COX-2 expression and NF-κB activation appear in heart failure (4). We believe that NF-κB-mediated transcription in hypoxia and other forms of oxidative stress will eventually be shown to be an important pathway in the pro-inflammatory environment that leads to modulation of vascular endothelial and smooth muscle cellular proliferation.

Epidemiologic data suggest that patients with elevated plasma C-reactive protein, a marker for systemic inflammation, are the ones most likely to benefit from aspirin as a preventive therapy for myocardial infarction and stroke (54). We may hope in the not-too-distant future to learn whether COX-2 is a "friend or foe" (55) in cardiovascular disease. This may lead to development of a “better aspirin” (56) that will be useful in cardiovascular disease by learning how to control the regulation of COX-2 in disease states such as those associated with tissue hypoxia. The regulatory role of Sp1 in the transactivation of COX-2 by hypoxia in this cell culture model of human vascular endothelium suggests a link between cellular hypoxia and the mediators of inflammatory responses associated with aortic aneurysm and heart failure.

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