Regulation of Cyclooxygenase-2 Expression in Aortic Smooth Muscle Cells

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Abstract  Activation of the gene for inducible cyclooxygenase (cyclooxygenase-2 [Cox-2]), prostaglandin endoperoxide synthase is an early response to injury in vascular smooth muscle cells. We used in vitro and in vivo models to demonstrate that activation of quiescent smooth muscle cells by mitogens leads to a rapid, short-term rise in mRNA for Cox-2, followed by synthesis of new Cox-2 enzyme protein and a marked increase in prostaglandin production that depends on new enzyme synthesis. Moreover, the Cox-2 mRNA response observed in smooth muscle cells differs in both time and degree, depending on the particular mitogenic stimulus. Serum, platelet-derived growth factor, epidermal growth factor, and thrombin are strong inducers of Cox-2 mRNA, whereas acidic and basic fibroblast growth factors and interleukin-1α are weak inducers. In contrast to the transient activation of Cox-2 in vitro after introduction of a mitogenic stimulus, the Cox-2 response after in vivo vascular injury extends over many days and may represent protracted cellular activation. During induction of Cox-2 message and protein, expression of constitutive cyclooxygenase (Cox-1) remains unchanged, however. These data suggest a pathophysiologic role for Cox-2 in the early modulation of vascular responses to injury. (Arterioscler Thromb. 1994;14:1021-1031.)

Key Words  • cyclooxygenase-2 expression • smooth muscle cells • response to injury

Vascular smooth muscle cells (SMCs) respond to injury by changing from a contractile, nongrowing phenotype to a secretory, growing phenotype, a process termed activation. SMC activation occurs early in the development of lesions in arteriosclerosis and hypertension and after balloon angioplasty and has been observed both in vitro and in vivo. The activation process includes expression of numerous genes; release of enzymes, growth factors, and extracellular matrix components; and secretion of prostaglandins (PGs).

Mediators of SMC activation include serum, growth factors, and cytokines. One early event in SMC activation is transcriptional activation of primary response genes that are key regulators of SMC activation. For example, migration and proliferation of carotid arterial SMCs after experimental injury can be suppressed by administration of antisense oligonucleotides to c-myc or c-myb mRNA, the products of primary response genes. As SMCs are activated they also release PGs. This pattern of PG production by stimulated SMCs suggests that protein synthesis is required and that the key protein synthesized is the enzyme cyclooxygenase (PG endoperoxide synthase [Cox]), which itself is the product of an early response gene.

For many years only a single constitutive form of Cox (Cox-1) was recognized, but recently a second, inducible form of the enzyme, Cox-2, has been detected in a variety of cells, including 3T3 fibroblasts, endothelial cells, monocytes, and neurons. cDNA clones for Cox-1 and Cox-2 have been isolated and sequenced from the murine and human genomes. The gene products of Cox-1 and Cox-2 display about 60% homology at the amino acid level in a single species, whereas Cox-2 proteins show 80% amino acid homology between species. Both genes encode transmembrane proteins of similar molecular weight (70 kD) and conserve aspirin-sensitive, heme ligand, glycosylation, and active sites. Important differences between these Cox enzymes include an 18-amino acid sequence near the carboxyl terminal of Cox-2 that is absent in Cox-1 and a 17-amino acid sequence close to the amino terminal of Cox-1 that is lacking in Cox-2.

The focus of the present work was to understand the mechanisms that regulate Cox-2 expression in vascular SMCs in relation to injury and to the action of mediators of SMC activation. We studied Cox-2 induction in cultured rat aortic SMCs by examining gene expression, protein translation, Cox enzyme activity, and PG release. To complement these in vitro studies, we also examined vascular Cox-2 induction in an animal model of arterial injury that leads to intimal hyperplasia. Our studies show that Cox-2 gene expression at both the mRNA and protein level is negligible in quiescent SMCs but is rapidly and transiently induced when quiescent SMCs are exposed to serum or mitogens. The level and duration of Cox-2 mRNA that are induced varies with the mitogen employed. Enhanced spontaneous production of PGs follows shortly after induction of the Cox-2 message, and this production requires synthesis of Cox-2 protein. During this sequence of events expression of the Cox-1 gene and the level of the Cox-1 enzyme remain unaltered.
Methods

Materials

Medium 199 (M199) and antibiotics were from Whittaker and fetal bovine serum (FBS) was from Gemini Bioproducts Inc. Antibody to α-SMC actin, protein assay kits, fatty acid-free bovine serum albumin (BSA), insulin/transferrin/selenium (ITS), phorbol myristate acetate (PMA), epidermal growth factor (EGF), and actinomycin D were from Sigma. Enzyme immunoassay (EIA) reagents for PG assays were from Cayman Co. Platelet-derived growth factor (PDGF) was from RD Systems. Crude endothelial cell growth factor (ECGF) was prepared from whole bovine brain and titered for growth and fetal bovine serum (FBS) was from Gemini Bioproducts. Riboprobe was prepared from whole bovine brain and titered for growth and free bovine serum albumin (BSA), insulin/transferrin/sele-
mide, 5x saline-sodium phosphate-EDTA buffer (SSPE), 5x Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 100 μg/mL single-stranded salmon sperm DNA. Probes were allow to hybridize to filters for 36 hours at 42°C. The following probes were used: a murine Cox-1 cDNA probe, a gift of Dr. David Dewitt (Michigan State University); a murine Cox-2 cDNA probe (TIS 10) kindly provided by Dr. Harvey Herschman (UCLA); and c-fos and an 18S rRNA probe, the latter of which was used to normalize RNA loading. Cox-2 and 18S rRNA probes were labeled with [32P]dCTP by random priming. The Cox-1 probe was labeled with [32P]UTP by using a riboprobe kit. Filters were washed for 20 minutes at room temperature in 2xSSPE-0.1% SDS, twice for 20 minutes in the same solution at 55°C, and twice for 20 minutes in 0.1xSSPE-0.1% SDS at 55°C. Washed filters were exposed to Kodak XAR-2 film at —70°C with intensifying screens. Quantitation of Northern and slot blots was done using a digital scanner (Umax) and IMAGE 1.41 software to analyze the digital images.

Determination of Cox-2 mRNA Half-life

SMCs were stimulated to induce Cox-2 mRNA, and 5 μg/mL actinomycin D was added at the time of peak Cox-2 mRNA induction. RNA was isolated from replicate flasks of treated cells every 15 minutes thereafter, and slot blots were prepared and hybridized with either the Cox-2 or 18S rRNA probe. The blots were scanned and mRNA half-life was determined from the slope of a semilogarithmic plot of mRNA concentration versus time.

Western Blotting

Lysates of stimulated SMCs were prepared at specific times by treating washed SMCs with lysis buffer consisting of 0.15 mol/L NaCl, 0.1 mol/L Tris-buffered saline with 50 mmol/L diethylthiopyrocarbonate, 1% Tween-20, and 10 μmol/L phenylmethylsulfonyl fluoride. Lysates were sonicated for 10 minutes to solubilize the enzyme and centrifuged at 9000g to sediment the particulate material. Protein concentration was measured and supernatant samples were stored at —70°C until used. Lysate samples (25 or 100 μg protein per lane) were applied to 7.5% SDS polyacrylamide gels (Bio-Rad), electrophoresed, and transferred to nitrocellulose membranes overnight at 30 V constant voltage at room temperature with use of a Western blot transfer apparatus (Yale University instrument shop). Immunostaining was performed with a rabbit polyclonal antibody raised against the unique 18-amino acid sequence from the carboxy-terminal portion of Cox-2 that does not react with Cox-1. Development was performed with a peroxidase-labeled second antibody and diaminobenzidine.

Immunofluorescence in Cultured Cells

Quiescent SMCs cultured in multwell chamber slides (LabTek) as detailed above were subjected to the identical stimulation protocol for 0 to 24 hours. After exposure to serum for different times, the SMCs were washed with PBS and fixed in acetone:methanol (1:1, vol/vol). Fixed SMCs were incubated

Isolation and Culture of Rat Aortic SMCs

Adult male Fisher 344 rats (2 to 3 months old) were killed while under CO2 inhalation anesthesia and the aortas were dissected free, cut into small pieces, and explanted. Outgrowth of which time at least 90% of the cells were in the G0/G1 phase as characterized by staining with antibodies to α-SMC actin. Cells were cultured in M199 containing 20% FBS and 50 μg/mL gentamicin at 37°C in a humidified atmosphere of 5% CO2 in air. At confluence, cells were passaged by trypsinization. Cells from passages 3 to 12 gave consistent doubling times.

Stimulation Protocol

Preconfluent SMC cultures were incubated in serum-free M199 supplemented with 50 μg/mL BSA for 48 hours, at which time at least 90% of the cells were in the G0/G1 phase as demonstrated by fluorescence-activated cell sorting (FACS) analysis. These cells were considered quiescent. SMCs were then stimulated by replacing the serum-free medium with fresh medium containing 20% FBS or serum-free M199 supplemented with ITS plus different individual SMC mitogens. Mitogens were used at the concentrations that have been reported to produce maximal growth effects (as indicated in each manufacturer's specifications), ie, 10 ng/mL for PDGF, 10 ng/mL for EGF, 150 μg/mL for ECGF, 1 to 10 ng/mL for aFGF, 1 to 10 ng/mL for bFGF, 50 ng/mL for PMA, and 1 U/mL for thrombin. The effects of each mitogen on SMC transition from G0 to G1 were tested by documenting the appearance of c-fos mRNA by Northern blotting.

PG Production

SMCs were plated in 24-well plates, made quiescent by the procedure described above, and stimulated with different mitogens for up to 48 hours. Postculture medium was collected at different times after mitogen addition from these 24-well plates or from similarly treated flasks of SMCs destined for RNA studies. The samples of media were centrifuged to remove cell debris and the supernatants frozen at —70°C until assay. Supernatants were assayed for spontaneously released PGE2 and PGH1 by EIA as described.
with antibodies against Cox-1 or Cox-2 at a 1:250 dilution in PBS for 30 minutes at 37°C, washed, reincubated with a fluorescein-labeled secondary antibody for 30 minutes at room temperature, washed extensively, and mounted in Vectashield. Stained slides were photographed under a Nikon fluorescence microscope with Kodak 400 or 1200 ASA print film.

Cox Activity

Cox activity was measured by direct oxygen consumption assay as well as by EIA measurement of PGs released into the incubation medium. For oxygen consumption measurement, lysates were prepared from quiescent SMCs or SMCs treated for 6 hours with serum. Protein concentrations of these preparations were measured by a colorimetric assay. Immunoprecipitates were prepared from SMC lysates by using a specific anti-Cox-2 antibody. Oxygen consumption after addition of arachidonic acid to aliquots of whole-cell lysates or immunoprecipitated cellular protein fractions was measured in a Yellow Springs Instrument model 53 oxygen monitor with a micro Clark oxygen probe fitted with a Hitachi recorder. Assays were performed at 37°C in a 3-mL chamber containing 100 mmol/L Tris-HCl, pH 8.0, 500 μmol/L phenol, and 1 μmol/L hematin. After equilibration of SMC lysates or immunoprecipitates, 100 μmol/L sodium arachidonate was added to start the reaction, and oxygen consumption was measured. Oxygen consumption was inhibited by pretreatment of the lysate or immunoprecipitate for 15 minutes with 1 mmol/L aspirin. Oxygen consumption by SMC lysates was standardized against oxygen consumption rates of ram seminal vesicle microsomes, a major source of Cox activity, at equal protein concentrations and expressed as nanomoles of oxygen per minute per milligram of protein.

Vascular Injury Model

Fisher 344 rats were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/mL). The left carotid artery was exposed and one of the following procedures performed.

Balloon Angioplasty of the Common Carotid Artery

A 2F Fogarty balloon catheter was inserted through the external carotid artery into the left common carotid artery, and the balloon was inflated three times in the latter vessel. Animals were killed 1, 4, 7, and 14 days after injury. The contralateral noninjured carotid artery served as a control.

Balloon Angioplasty of the Aorta

Following the same procedure as above, we inserted the balloon into the common carotid artery and advanced it into the aorta to the level of the iliac bifurcation. The balloon was inflated and passed three times along the entire aorta. Animals were killed 14 days after injury. Control animals were those that underwent surgery but did not receive balloon angioplasty.

Pinching Injury of the Carotid Artery

This lesion, a modification of the model described by Banai et al27 and Barbee et al,28 was performed on the rat carotid artery. A section of the left common carotid artery (~2.5 cm) was pinched five times with a small clamp for 30 seconds each. Animals were killed 1, 4, 7, 12, and 14 days after the procedure. The contralateral right common carotid artery served as the noninjured control in each animal. Both balloon angioplasty and pinching injuries induced similar intimal hyperplasia (Fig 9).

At specified times the carotid arteries or aortas were removed and fixed in 10% buffered formalin. Samples were embedded in paraffin, and sections were made and stained with hematoxylin-eosin or specific antibodies against Cox-1 or Cox-2 followed by a second antibody labeled with immunoperoxidase. The detection system for immunoperoxidase was diaminobenzidine.

Statistical Analysis

Samples from EIA and oxygen consumption experiments were compared by ANOVA and a modification of the t test for multiple comparisons. Differences were considered significant at P<.05.

Results

Induction of Cox-2 mRNA by Serum in Quiescent SMCs

Entry of quiescent SMCs into the cell cycle was examined by FACS analysis and the appearance of message for c-fos, an early response gene known to be expressed at the G0/G1 transition. The steady-state level of Cox-2 mRNA in SMCs was determined by Northern blot analysis of total cellular RNA prepared at zero time and at different times after addition of serum. The level of Cox-1 mRNA was also examined under the same conditions. When quiescent rat aortic SMCs were exposed to medium containing 20% FBS, more than 90% of cells entered the cell cycle, as assessed by FACS (data not shown).

No Cox-2 mRNA was detectable at time zero in quiescent SMCs. Cox-2 mRNA appeared 45 to 60 minutes after addition of serum, peaked between 1.5 and 3 hours, and fell to trace or undetectable levels by 6 hours. Under stringent conditions, a major mRNA species of 4.2 kb hybridized with the murine Cox-2 probe (Fig 1A). In addition, two minor bands of 7 and 9.2 kb consistently appeared within the same time frame and at roughly the same relative ratios to the 4.2-kb band. These minor bands may represent precursors of mature Cox-2 mRNA. Comparable results were obtained by slot blot analysis, so this latter method was used for subsequent studies involving time courses and dose responses (Fig 1B). c-fos mRNA was detectable 45 minutes after serum addition but disappeared after 1.5 hours. Under these conditions, Cox-2 mRNA was rapidly and transiently induced (Fig 1A).

In contrast to the changes in steady-state mRNA for Cox-2 after serum stimulation of SMCs, mRNA for Cox-1 was expressed at a very low level in quiescent cells, and this level did not change with SMC entry into the cell cycle or during the first 24 hours after stimulation (Fig 1C).

Suprression of Cox-2 mRNA by Cycloheximide

When SMCs were serum stimulated in the presence of 10 μg/mL cycloheximide, a concentration that inhibits protein synthesis in these cells, the steady-state level of Cox-2 mRNA was greatly increased over that observed in the absence of cycloheximide. Furthermore, mRNA levels remained elevated for at least 12 hours (Fig 2). This superinduction of Cox-2 mRNA, a feature characteristic of many primary response genes, appears to represent increased stability of the normally transiently expressed message. The precise mechanism by which this increased stability is maintained has not been determined, but message size remained unchanged during induction of Cox-2, suggesting that sequential cleavage of the 3' untranslated region is an unlikely mechanism of degradation.29 Again, in contrast to Cox-2 mRNA, no superinduction of Cox-1 mRNA in the presence of cycloheximide was observed.
**Induction of Cox-2 mRNA by SMC Mitogens**

The effect of serum on SMC proliferation is a net result of the mixture of mitogens, cytokines, and inhibitors in serum. To identify factors that regulate Cox-2 mRNA levels, we stimulated quiescent SMCs without serum but with individual mitogens, including the following: PDGF, EGF, ECGF, aFGF, bFGF, IL-1α, thrombin, and the tumor promoter PMA. After preparation of total cellular RNA from treated SMCs, Cox-2 mRNA expression was determined as a function of time after exposure to each mitogen. Each of these growth factors was used at concentrations that are known to induce mitogenesis; furthermore, c-fos mRNA expression was used as an indicator of the transition from Go to G1 for each separate test mitogen and was observed to be induced in each case.

Representative slot blots were prepared to examine the time course of Cox-2 mRNA induction in SMCs after stimulation with individual mitogens (Fig 3). Clearly, various mitogens induced different levels of Cox-2 mRNA but with different kinetics. The highest Cox-2 mRNA induction in SMCs was observed with PDGF, thrombin, EGF, and PMA, but the kinetics of induction were not identical with these four mitogens. PDGF produced the most intense and rapid response, peaking between 45 minutes and 1.5 hours, compared with that of thrombin, whose effect peaked at about 3 hours. EGF and PMA showed roughly the same kinetics, peaking at 1.5 hours, and resulted in mRNA levels about 30% of that due to serum or PDGF. ECGF, bFGF, and aFGF also showed a peak mRNA level at 1.5 hours, but the mRNA signal was only about 1% to 5% of the maximal PDGF response. A dose-response curve was constructed for each mitogen to verify that the Cox-2 mRNA response was maximal at the mitogen concentration used. IL-1α induced even less Cox-2 mRNA over this time frame.

To determine the half-life of Cox-2 mRNA induced by these mitogens, we stimulated quiescent SMCs with PDGF or serum, and at the peak of Cox-2 expression, as determined by our previous time-course study, we added actinomycin D (5 μg/mL) to inhibit transcription. The level of Cox-2 mRNA was measured every 15 minutes thereafter via slot blotting. Calculated half-life of Cox-2 mRNA was 25 minutes.

A representative slot blot of c-fos mRNA induction is also shown in Fig 3; the change in c-fos mRNA followed
Immunofluorescence was demonstrated by immunofluorescence with a specific signal on both Western blots and immunofluorescence. Cox-2 protein in serum-stimulated SMCs by Western blotting. Absorption of this anti-peptide antibody raised against the unique carboxy-terminal 18-amino acid peptide of Cox-2. This antibody does not cross-react with Cox-1, which lacks this peptide.20 The same antibody was used to demonstrate specific induction of immunoreactive Cox-2 in SMC lysates by Western blotting. Absorption of this anti-Cox-2 with the specific C-terminal peptide abrogated the signal for Cox-2 mRNA (data not shown).

**Immunofluorescence**

The same stimulation protocol was followed to evaluate induction of Cox-2 protein in previously quiescent SMCs stimulated by medium containing 20% FBS. In stimulated SMCs, the presence of Cox-2 protein antigen was demonstrated by immunofluorescence with a specific antipeptide antibody raised against the unique carboxy-terminal 18–amino acid peptide of Cox-2. This antibody does not cross-react with Cox-1, which lacks this peptide.20 The same antibody was used to demonstrate specific induction of immunoreactive Cox-2 in SMC lysates by Western blotting. Absorption of this anti-Cox-2 with the specific C-terminal peptide abrogated the signal on both Western blots and immunofluorescent assay.

Cox-2 protein is not detectable in quiescent SMCs by immunofluorescence (Fig 4A) but appears 3 hours after serum stimulation and is first localized in the nuclei of stimulated SMCs (Fig 4B). Cytoplastic staining then develops, and this immunoreactivity peaks after 6 hours, while changing to a perinuclear and granular cytoplasmic distribution (Fig 4C). By 12 hours only a few cells remain immunoreactive (Fig 4D). Similar results were seen at 24 hours. In contrast, Cox-1 immunostaining is cytoplasmic, diffuse, and weak, with a few scattered cells (<2%) clearly more positive than others over the same time course. There was no change in the subcellular location or pattern of Cox-1 immunoreactivity over time. No induction of Cox-1 immunostaining was observed.

**Western Blotting**

We also evaluated time-dependent production of Cox-2 protein in serum-stimulated SMCs by Western blotting of cell lysates. Results are shown in Fig 5. In quiescent cells no protein of the expected size, ie, 70 kD, was detected (lane 0). In contrast, after 3-hour exposure of SMCs to serum, the anti-Cox-2 antibody reacted strongly with a 70-kD protein doublet that gradually disappeared with time, reaching very low levels by 12 hours; Cox-1 gave either a faint or no band over the same 12 hours (data not shown).

**Cox Activity in Quiescent and Mitogen-Stimulated SMCs**

To further investigate induction of an active Cox in serum- or mitogen-treated cells compared with quiescent cells, we measured Cox activity by both oxygen consumption and PG release into the assay buffer. Total-cell lysates of quiescent or serum-stimulated SMCs were used to measure enzyme activity by an oxygen consumption assay. Lysates prepared from SMCs stimulated with serum for 6 hours showed higher oxygen consumption than did lysates from quiescent cells: 43.4±4.5 nmol O$_2$/mg SMC protein per minute for stimulated SMCs versus 13.4±1.5 nmol O$_2$/mg per minute for quiescent SMCs (P<.01). Furthermore, immunoprecipitates prepared from SMC lysates with Cox-2–specific antibodies showed more oxygen consumption when prepared from serum-treated SMCs (18.3±1.5 nmol O$_2$/mg per minute) than when prepared from quiescent SMCs (8.1±1.1 nmol O$_2$/mg per minute). Elevation of Cox activity after serum stimulation was corroborated by measurement of PGE$_2$ production. Equal amounts of cell lysate protein (500 μg in 1 mL final incubation volume) from quiescent or 6-hour serum-stimulated SMCs were tested for their ability to transform arachidonic acid into PGE$_2$. Control SMC lysates possessed minimal Cox activity, as measured by PGE$_2$ release into the assay buffer (1557±356 pg/mL). In contrast, lysates from serum-stimulated SMCs showed a 9.3-fold increment of released PGE$_2$ (14 486±1104 pg/mL, P<.01). Lysates from serum-stimulated SMCs that had been treated with cycloheximide during serum
Fig 4. Subcellular localization of induced cyclooxygenase-2 (Cox-2) protein in smooth muscle cells (SMCs), as demonstrated by immunofluorescence. SMCs grown on chamber slides were fixed at different times after stimulation with serum and stained by a specific antibody against Cox-2 protein. A, Unstimulated, quiescent cells. No specific immunoreactivity is detected (x 100). B, 3 hours after addition of serum, Cox-2 is first detected in the nucleus, with staining throughout the nucleus (x 100). C, 6 hours after addition of serum, Cox-2 immunoreactivity reaches a maximum; in addition to nuclear staining, reactivity is seen in the perinuclear membrane and cytoplasm. The entire population of SMCs stains strongly positive (x 400). D, 12 hours after addition of serum, cellular sublocalization of immunoreactivity is mainly cytoplasmic and perinuclear. Staining intensity has diminished (x 100). All magnifications are original.

stimulation did not show any increment over baseline PG production (data not shown).

In addition to the aforementioned studies, the specific antibody against Cox-2 selectively precipitated Cox activity from total-cell lysates of serum-stimulated SMCs, as measured by PG production (Fig 6), whereas control nonimmune serum did not precipitate any specific PG-producing activity.

PG Production After Serum Stimulation

PGs were released into the culture medium after stimulation of SMCs by serum. After exposure of quiescent SMCs to serum, large amounts of PGE₂ and PGF₁α appeared in the medium after 3 hours, with peak release at 6 hours (100-fold increase over baseline). Because PGs are not stored in cells but released immediately after their synthesis, this result indicates continual de novo synthesis of these metabolites. Peak release of PGs was followed by a slow decline over the next 18 hours, although levels remained above baseline (Fig 7). As a control for the mechanical stimulation of SMCs that occurred during hourly harvesting of the medium from serum-stimulated cells, cultures maintained in medium that lacked added growth factors were also harvested at intervals for up to 24 hours. Negligible PG release into control media was observed (Fig 7). Moreover, continued production of Cox-2 metabolites requires continuous protein synthesis, since cycloheximide ablated most of the mitogen-stimulated PG production (data not shown). The presence of serum mitogens may be a crucial factor for sustained release of PGs by SMCs by upregulating Cox-2 mRNA and enzyme levels.

In Vivo Induction of Cox-2 After Vascular Injury

Three methods of vascular injury were used to evaluate Cox-2 induction in the vessel wall. Two well-established models, balloon angioplasty of the common carotid artery and balloon angioplasty of the aorta, and
FK3 5. Induction of cyclooxygenase-2 (Cox-2) protein in smooth muscle cells (SMCs), as measured by Western blot. Total-cell lysates of SMCs were prepared at different times after addition of 20% fetal bovine serum to quiescent cultures. Lysate protein (30 μg/lane) was loaded onto a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis minigel, electrophoresed, and electroblotted onto nitrocellulose. Incubation with antibody specific for Cox-2 (see "Methods") revealed a 70-kD doublet band that was not detected at time 0 but was induced with time, peaking 3 to 6 hours after stimulation of SMCs by serum and gradually diminishing thereafter.

Ballooning of the Common Carotid Artery

Fig 8A shows that balloon angioplasty–induced injury of the carotid artery resulted in the appearance of Cox-2 protein 24 hours after injury, whereas the uninjured contralateral carotid artery from the same animal was negative for Cox-2, as evaluated by immunoperoxidase staining with antibodies specific for Cox-2 (Fig 8B).

Ballooning of the Aorta

Fig 8C depicts an intimal lesion 14 days after balloon injury of the rat aorta. Intimal SMCs show strong Cox-2 immunoreactivity, and medial cells are weakly positive.

Pinching Model

With this novel method of carotid injury, Fig 9A depicts a carotid artery 4 days after injury, with Cox-2 staining in medial SMCs. The contralateral noninjured control is negative for Cox-2 (Fig 9B). Furthermore, by 14 days a substantial intimal lesion had developed (Fig 9C). This lesion is comparable to that produced by balloon angioplasty of the common carotid artery. A higher-magnification view of this lesion demonstrates that Cox-2 staining is present in intimal cells (Fig 9D). It is interesting to note that cells in the adventitia are also positive for Cox-2 (Fig 9C, arrow e). This may be due to the nature of the injury: the injury is made from the outside and the adventitia is consequently affected; mononuclear infiltration and monocytes are also Cox-2–positive. Staining of vessels by all three injury models

![Image](http://atvb.ahajournals.org/)

Fig 5. Induction of cyclooxygenase-2 (Cox-2) protein in smooth muscle cells (SMCs), as measured by Western blot. Total-cell lysates of SMCs were prepared at different times after addition of 20% fetal bovine serum to quiescent cultures. Lysate protein (30 μg/lane) was loaded onto a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis minigel, electrophoresed, and electroblotted onto nitrocellulose. Incubation with antibody specific for Cox-2 (see "Methods") revealed a 70-kD doublet band that was not detected at time 0 but was induced with time, peaking 3 to 6 hours after stimulation of SMCs by serum and gradually diminishing thereafter.

![Image](http://atvb.ahajournals.org/)

Fig 6. Immunoprecipitation of cyclooxygenase-2 (Cox-2) enzymatic activity by specific antibody to a unique Cox-2 peptide. Whole-cell lysates containing equal amounts of cellular protein were prepared from serum-stimulated smooth muscle cells (SMCs) and either tested directly or immunoprecipitated with polyclonal rabbit anti-Cox-2 antibody or nonimmune rabbit serum, followed by staphylococcal protein A–Sepharose beads. Approximately 50% of total Cox activity was precipitated with anti-Cox-2 antibody or nonimmune rabbit serum, as demonstrated by measurement of Cox activity, whereas nonimmune precipitate did not show prostanoid-producing activity. A, total-cell lysate; B, immunoprecipitate with rabbit anti-Cox-2 antisera; C, normal rabbit serum and protein A–Sepharose beads. PGE2 indicates prostaglandin E2.

![Image](http://atvb.ahajournals.org/)

Fig 7. Prostaglandin (PG) release after serum stimulation. Quiescent smooth muscle cells (SMCs) were stimulated with 20% serum, media were collected at different times, and PG release was measured by enzyme immunoassay. Production of both PGE2 (e) and PGI2 (c) increased rapidly between 3 and 6 hours. PG release was sustained for up to 24 hours. As a control for mechanical stimulation induced by hourly changes of medium, medium lacking serum was also changed hourly in parallel SMC cultures and PG production was measured. Release of PGE2 into the latter medium was 60±30 pg/mL per hour and did not change significantly during sequential collections; PGI2 values were in the same range as PGE2 in serum-free medium (e) and were also unaltered over time.
FIG 8. In vivo induction of cyclooxygenase-2 (Cox-2) in arteries after vascular injury. A and B, Balloon injury of the rat carotid artery. The left carotid artery was injured by passage of a Fogarty balloon catheter; the right carotid artery served as the noninjured control. A, Carotid artery 24 hours after injury. Smooth muscle cells (SMCs) in the media are positive for Cox-2 (arrows). B, Noninjured control artery stained with antibodies against Cox-2. Note that mononuclear cells are positive (arrows) while medial SMCs are negative (bars=50 μm). C, Balloon injury of the rat aorta. An intimal lesion that developed 14 days after balloon injury demonstrates Cox-2-positive cells (arrow). Cells in medial layer are faintly positive. (Bar=50 μm).

did not show induction of Cox-1, as assessed by a specific antibody to Cox-1 (data not shown). As suggested by our in vitro studies, Cox-2 expression appears to be a G₁ function, because staining for the S-phase cell-cycle marker (PCNA) was not observed until 24 hours after injury: PCNA staining peaked at 4 days in the media and between 10 and 14 days in the intimal lesion (data not shown).

Discussion

In the present work, we have demonstrated that the gene for inducible cyclooxygenase, i.e., Cox-2, is activated during the early response to injury of the vessel wall. A key feature of this study was the use of quiescent SMCs, which do not demonstrate the presence of Cox-2 mRNA or protein. Our in vitro model of Cox-2 induction in quiescent vascular SMCs parallels the situation in arteries, where SMCs are normally maintained in a quiescent state with a very low rate of cell renewal. When quiescent SMCs are stimulated in vitro by exposure to growth factors such as PDGF, EGF, or thrombin, Cox-2 mRNA is transiently induced. The pattern of Cox-2 mRNA induction is that of a primary response gene, as was first noted in 3T3 fibroblasts; that is, mRNA induction occurs at the G₀/G₁ transition of the cell cycle, does not require protein synthesis, and is superinduced in the presence of the protein synthesis inhibitor cycloheximide. Once induced, Cox-2 mRNA is translated into an active enzyme that mediates synthesis of PGs. Under the same conditions in which Cox-2 mRNA is strongly upregulated, mRNA for Cox-1, the constitutive enzyme, remains unchanged and occurs at relatively low levels compared with mRNA for Cox-2.

We observed differences in the steady-state level of Cox-2 mRNA induced in vitro by different growth factors. Not all SMC mitogens are strong inducers of Cox-2. For example, within 1.5 hours serum stimulates Cox-2 mRNA steady-state expression 20-fold over the quiescent, serum-starved level. Under serum-free conditions, PDGF or thrombin increases the Cox-2 mRNA steady-state level in SMCs to the same extent as serum, although thrombin does so with different kinetics (see below). PMA and EGF induce only about 30% as much Cox-2 mRNA as serum. In contrast, aFGF, bFGF, and IL-1α, all of which induce c-fos mRNA expression equally as well as serum, are only weak early inducers of Cox-2 message and increase its level only 1% to 5% as much as does serum. The steady-state level of Cox-2 mRNA rises more slowly and remains elevated longer in SMCs after exposure to thrombin than after exposure to serum or PDGF. Because thrombin is known to induce PDGF and FGF in SMCs, thrombin treatment may stimulate Cox-2 expression through induction of these growth factors, thus resulting in prolonged elevation of Cox-2 mRNA levels.

These results suggest that Cox-2 gene activation may require a regulator(s) that is not induced by all SMC mitogens and further suggest that the pathways leading to proliferation and PG production may diverge. For example, IL-1 induces PG secretion in WI 38 fibroblasts and chondrocytes mainly by stimulating transcription of phospholipase A₂, with only minor stimulation of Cox-2; it
is also clear that IL-1 has different effects on these two genes in different types of mesenchymal cells.\textsuperscript{34}

We determined that the half-life of Cox-2 mRNA in PDGF, EGF, and serum-stimulated SMCs was 25 minutes, similar to the half-lives of other primary response genes such as c-fos. Recently, it has been shown that Cox-2 gene expression is transcriptionally regulated after exposure of quiescent 3T3 fibroblasts to serum.\textsuperscript{35} However, as with c-fos and other cytokine-induced genes, Cox-2 mRNA contains multiple AUUUA consensus sequences in its 3' untranslated region. Such sequences confer increased mRNA instability due to degradation by specific mitogen-regulated RNAses.\textsuperscript{36} This finding, plus the striking increase in Cox-2 mRNA level and peak duration in the presence of cycloheximide, suggests that posttranscriptional mechanisms also contribute to the regulation of Cox-2 expression.
These findings suggest that the previously reported rapid stimulation of PG synthesis in SMCs exposed to growth factors reflects Cox-2 induction. Moreover, both PDGF and EGF have been documented to enhance synthesis of PGE\textsubscript{2} and PGH\textsubscript{2} in a time-dependent, protein-synthesis-dependent manner\textsuperscript{12} that requires de novo synthesis of Cox. Furthermore, these same mitogens have been shown to induce rapid recovery of PG synthesis in fresh medium after irreversible inhibition of preexisting Cox by aspirin.\textsuperscript{37} This pattern closely resembles the appearance of Cox-2 described above.

Cox-2 protein appears first in the nucleus of SMCs stimulated in vitro with serum, PDGF, or PMA, as assessed by immunohistochemistry with a specific anti-Cox-2 antibody. Nuclear staining becomes prominent in the entire cell population 3 hours after stimulation. With increasing time after serum stimulation, localization of immunoreactive Cox-2 protein shifts slowly from the nucleus to the cytoplasm. The pattern becomes granular and localizes at the perinuclear membrane and in the endoplasmic reticulum. Such time-dependent shifts of Cox-2 protein from the nucleus to the cytoplasm have not been reported, although cytoplasmic localization of Cox-2 in fibroblasts has been published.\textsuperscript{28} However, because metabolites of PGE\textsubscript{2} (PGH\textsubscript{2} and PGG\textsubscript{2}) are cytotatic and localize in the nucleus, the presence of Cox-2 in the nucleus may provide the means for localized production of these PGs. In future studies we plan to assess Cox enzymatic activity in the nuclear fraction at different times after stimulation.

PG secretion increases markedly when quiescent SMCs are stimulated with serum. The kinetics of PG release closely follows that of induction of Cox-2 protein and enzymatic activity. PG production increases very rapidly in the first 3 to 4 hours after mitogen stimulation and remains high for up to 24 hours. In this study, Cox-2 mRNA induction by various stimuli was followed by a rapid de novo synthesis of cyclooxygenase. This sustained PG production may involve other factors in addition to Cox-2 induction, such as augmented phospholipase activity. The eicosanoid products of Cox influence many cellular processes. In the vasculature they affect vascular tone, cellular growth, and thrombogenesis. For example, PGE\textsubscript{2} can decrease proliferation of aortic SMCs\textsuperscript{7} and in some cases alter cytokine responses of SMCs; eg, IL-1 stimulates SMC proliferation only if PG synthesis is blocked.\textsuperscript{39}

In our in vivo experimental models, ie, pinching injury or balloon angioplasty of the carotid artery or aorta, we have shown that Cox-2 protein is induced in arterial SMCs of the injured but not uninjured vessels soon after vascular injury. SMC activation in arterial vessel walls after balloon injury is known to be prolonged, with rounds of migration and division of SMCs lasting up to 2 weeks.\textsuperscript{40,41} Because SMC activation leads to Cox-2 expression, this process may result in sustained production of PGs with resulting effects on the outcome of the response to injury. For example, we have shown that vascular SMCs in the neointima can restore thromboresistance to the vessel wall after deendothelialization, in part through induction of PGH\textsubscript{2} production.\textsuperscript{42} This effect likely results from protracted Cox-2 induction in the SMC-rich neointimal layer. Because localized generation of thrombin, which prolongs induction of Cox-2 message in vitro, might also be a continuing factor during in vivo injury, thrombin may be important in the continued expression of Cox-2. This possibility may accord with our present findings, that neointimal lesions persistently express large amounts of Cox-2.

Our study points to a modulatory role for Cox-2 in the response of SMCs to injury. In order to vitally in vivo models we observed substantial increases in Cox-2 message, protein, and enzyme activity after induction by SMC activators. This Cox-2 induction results in increased production of PGs that can affect injury outcome by influencing platelet-vascular interactions, vascular tone, or SMC proliferation. The early activation of Cox-2 in this context may act as a negative autocrine loop to limit SMC proliferation via PG products. Activation of Cox-2 may modify multiple contributions of SMCs to the formation of arteriosclerotic lesions. The expression of Cox-2 during SMC activation may be part of a homeostatic process that limits the potentially deleterious effects of continuous or repeated minimal damage to blood vessels.

Acknowledgments

This study was supported by National Institutes of Health SCOR in Thrombosis grants HL-18828 and NIH ROI-HL 35724 and program project grant AG 00541. Dr Rimarachin is supported in part by a National Institutes of Health HL supplement to SCOR for underrepresented minorities and the American Federation of Aging Research.

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Arterioscler Thromb Vasc Biol, 1994;14:1021-1031
doi: 10.1161/01.ATV.14.7.1021

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