Cyclooxygenase Isozyme Expression and Intimal Hyperplasia in a Rat Model of Balloon Angioplasty

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ABSTRACT

Prostaglandin formation is enhanced in vascular disease, in part through induction of cyclooxygenase (COX-2) in vascular smooth muscle cells. Because COX regulates cell growth and migration, we examined whether the COX expression plays a role in the development of intimal hyperplasia after vascular injury. Rats undergoing balloon angioplasty of the carotid artery were randomized to receive a selective COX-2 inhibitor (SC-236), a selective COX-1 inhibitor (SC-560) or a combination of the two. Normal, uninjured vessels showed COX-1, but no COX-2 expression. Fourteen days after balloon injury, both COX-1 and COX-2 were expressed in the neointima. Balloon angioplasty resulted in a marked increase in the urinary excretion of prostaglandin (PG) E_2, PGF_2alpha, and thromboxane (TX) B_2. Both the COX-1 inhibitor SC-560 and the COX-2 inhibitor SC-236 suppressed the generation of PGE_2 and PGF_2alpha, particularly when combined, suggesting a role for both isozymes in the generation of prostaglandins in this model. In contrast, TXA_2 was markedly suppressed by the COX-1 inhibitor SC-560. COX-2 inhibition with SC-236 had no effect on intimal hyperplasia at day 14 (0 versus 8.5%; n = 7 in controls). In contrast, intimal hyperplasia was reduced by SC-560 when administered alone (by 42%; n = 7, p < 0.05) or in combination with SC-236 (by 40%; n = 7, p < 0.05). COX-1 may play a role in the development of intimal hyperplasia, potentially through the inhibition of platelet TXA_2. Despite being expressed in the neointima, COX-2 does not play a role in the development of intimal hyperplasia after vascular injury.

Prostaglandin generation is enhanced in patients with atherosclerosis and after balloon angioplasty (Braden et al., 1991; Belton et al., 2000). The products include prostacyclin (PGI_2) from vascular endothelium and thromboxane A_2, which is largely derived from platelets. Prostaglandins are synthesized from arachidonic acid by the enzyme cyclooxygenase (COX), of which there are two isoforms, COX-1 and COX-2 (Hla and Neilson, 1992; Xi et al., 1994). COX-1 is constitutively expressed in most tissues, including platelets and is largely responsible for TXA_2 formation. COX-2 is normally undetectable or is expressed in low amounts (Masferrer et al., 1994). Nevertheless, COX-2 is largely responsible for the biosynthesis of PGI_2 in normal subjects (Cullen et al., 1998; Catella-Lawson et al., 1999; McAdam et al., 1999). COX-2 is induced in the vascular smooth muscle and inflammatory cells of human atherosclerotic plaque (Baker et al., 1999; Schonbeck et al., 1999; Belton et al., 2000) and we have shown that both isozymes are responsible for the increase in PGI_2 generation in patients with severe atherosclerosis (Belton et al., 2000). The expression of COX-2 in vascular injury is not unexpected, because COX-2 is readily induced by growth factors (Lin et al., 1989), cytokines (Jones et al., 1993), and free radicals (Adderley and Fitzgerald, 1999).

COX isozymes and prostaglandins may modify the response to vascular injury. Prostaglandins regulate the expression of genes involved in cell growth (Brown et al., 1999), apoptosis (Bornfeldt et al., 1997), and migration (Attiga et al., 2000), processes that have been implicated in the development of lesions in atherosclerosis (Ross, 1993) and after balloon angioplasty (Clowes and Reidy, 1991). Alternatively, vasodilator prostaglandins such as PGI_2 may limit the injury response because overexpression of prostacyclin synthase in the vessel wall decreases neointimal hyperplasia, potentially by accelerating regeneration of the endothelium (Harada et al., 1999). Other products such as PGJ_2 and its metabolites suppress the expression of inflammatory genes (Straus et al., 2000). COX-2 may also limit disease progression or the development of thrombotic complications through PGI_2-mediated inhibition of platelet function. Indeed, inhibition of COX-2-derived PGJ_2 may undermine the reports of thrombosis in patients treated with selective COX-2 inhibitors (Bombar-

ABBREVIATIONS: PG, prostaglandin; COX, cyclooxygenase; RT-PCR, reverse transcription-polymerase chain reaction; TX, thromboxane; I/M, ratio of intima to media areas.
dier et al., 2000; Crofford et al., 2000). To explore the functional role of COX isozymes in lesion development after vascular injury, we examined the expression of COX isozymes in the rat model of balloon injury to the carotid artery and explored their role in the subsequent development of restenosis.

Materials and Methods

Animal Model of Intimal Hyperplasia. Eighty-five male Sprague-Dawley rats weighing 250 to 300 g were studied. Animals were maintained on normal diet and were allowed free access to chow and water throughout this study. Animal care and treatment were conducted in conformity with institutional guidelines in compliance with international laws and policies. A license and permission for the study were obtained from the Department of Health.

Anesthesia was induced and maintained with inhalational halothane. The method of balloon denudation was adapted from that described by Clowes et al. (1983). The bifurcation of the left common carotid artery was exposed and the left common, internal and external carotid arteries were controlled with surgical ligatures. A 2Ch Fogarty balloon embolectomy catheter (Baxter Edwards, Horw, Switzerland) was introduced through an arteriotomy in the external carotid artery and advanced to the proximal edge of the omohyoid muscle. The balloon was inflated with saline and drawn three times up and down the common carotid artery. All procedures were performed by a single operator. Sham-operated animals where the animals were anesthetized and the left external carotid artery exposed were used as controls (n = 17).

The animals were randomized into four groups to receive intravenous vehicle (saline and dimethyl sulfoxide (v/v) 3 mg/kg SC-236 (n = 17), 10 mg/kg SC-580 (n = 17) (both kind gifts from Dr. P. Isakson, Monsanto, St. Louis, MO), or a combination of SC-236 3 mg/kg and SC-560 10 mg/kg (n = 17). The first dose of drug was administered through the internal jugular vein before the balloon injury. The same dose was administered by intraperitoneal injection 24 h after surgery, and this was repeated on alternate days for the duration of the study. All treatment regimens were for 14 days. Twenty-four-hour urine samples were collected preoperatively and on day 3 postoperatively.

Fourteen days after arterial injury, the animals were anesthetized by halothane inhalation and the left carotid artery removed. After inflation with saline, samples for immunohistochemistry (n = 5 for each group) were fixed in 10% formal saline for 24 h and embedded in paraffin blocks. Samples for RT-PCR analysis (n = 5 for each group) were fixed in 10% formal saline for 24 h and embedded in paraffin blocks. Samples for RT-PCR analysis (n = 5 for each group) were placed in Tri-Reagent and analyzed for COX-1, COX-2, and GAPDH mRNA expression. Tissue samples for quantification of COX-1, COX-2, and GAPDH mRNA expression. Tissue samples for quantification of intimal hyperplasia (n = 7 for each group) were placed in formalin and fixed for 24 h before being embedded in paraffin. Blood was obtained by cardiac puncture at time of sacrifice for measurement of COX-1 activity.

Immunohistochemistry Analysis. Left carotid artery samples were fixed in formal saline for 24 h. The tissues were paraffin embedded (Shandon Citadex 200; Shandon Lipshaw Inc., Pittsburgh, PA) and 5-μm sections were cut (Leitz 1512 microtome; Wetzlar GmbH, Wetzlar, Germany). The sections were incubated in primary antibody against COX-1 or COX-2 (both polyclonal antibodies, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. After washing in phosphate-buffered saline the slides were incubated in the secondary biotinylated antibody and the immunocomplex visualized using the diaminobenzidine chromagen (ABC Complex, Vectastain Elite kit; Vector Laboratories, Burlingame, CA).

RT-PCR Analysis. COX-1, COX-2, and GAPDH mRNA were extracted and detected by RT-PCR as described previously (Belton et al., 2000). Each primer pair was designed to span at least one intron of the gene. The primers used were as follows: COX-2 sense: 5’-AACACGAGACTGCTCCTTTT-3’; COX-2 antisense: 5’-TACTG-TAGGTTAATGTC-3; COX-1 sense: 5’-TCAACAGATCACGCTAT-3’; COX-1 antisense: 5’-TGCGTGGCACTTCTCCA-3’; GAPDH sense: 5’-ACCCATACATTTCCAGGACC-3’; and GAPDH antisense: 5’-CACAGTCTTCTGAGTGCGATGAT-3’.

Urinary Eicosanoid Excretion. Urine was collected from the animals over 24 h before surgery and on the third day after surgery. Urinary TXB2 was determined by enzyme immunoassay (R & D Systems, Abingdon, UK). Five milliliters of urine was spiked with deuterated internal standards for 1 ng/ml PGE2, 1 ng/ml PGE3, and 1 ng/ml isoprostane 8-iso-PGF2α. The sample was purified and concentrated by solid phase extraction by using a C18 Sep-Pak cartridge.

The methoxylamine derivative was formed and the samples were further purified by thin layer chromatography. The samples were derivatized to the pentafluorobenzyl ester. After further separation by thin layer chromatography, the samples were converted to the trimethylsilyl ether. Gas chromatography/mass spectrometry was performed on a Varian 3400 gas chromatograph linked to a Finnigan Incos XL mass spectrometer operated in the negative ion, chemical ionization mode, by using methane as the reagent gas. Analyses were monitored by selected monitoring of the mass ion and quantitation was based on the ratio of analyte to internal standard.

COX-1 Activity in Whole Blood. Serum TXB2 was assayed by allowing whole blood to clot in nonsiliconized glass tubes at 37°C for 1 h (Panara et al., 1995). Serum was separated by centrifugation at 1000g for 10 min. TXB2 was measured by enzyme immunoassay (R & D Systems).

Quantification of Intimal Hyperplasia. The intimal area and medial area of each artery (n = 7 for each group) were obtained by computerized planimetry (Samba software; Alcatel, Grenoble, France). Neointimal thickening was quantified as the ratio of intima to media areas (I/M) from transverse sections of hematoxylin- and eosin-stained arterial sections. Two to four sections were examined for each section by an analyst blind to the treatment regimen and the average I/M calculated. The data are expressed as mean ± S.E.M.

Statistical Analysis. The data are expressed as mean ± S.E.M. For comparison between groups, the data were analyzed by analysis of variance followed by paired or unpaired tests as appropriate, or in instances where data distribution deviated from normality, by using the Kruskal-Wallis nonparametric analysis of variance and subsequent Mann-Whitney U test.

Results

Immunohistochemistry. Analysis of COX-1 and COX-2 protein expression was performed on sections of rat carotid artery 14 days after balloon injury. In the balloon injury group, a neointima encircling the lumen was clearly visible (Fig. 1). COX-1 was diffusely expressed throughout the intima and media of the normal vessel (Fig. 1A). COX-1 was also expressed in the cells of the neointima after carotid angioplasty (Fig. 1C). There was COX-2 expression in the adventitia of the carotid arteries from both the sham-operated animals and the animals undergoing balloon injury (Fig. 1, B and D). This probably reflects damage and inflammation triggered during isolation of the carotid artery because no COX-2 was detected in the absence of surgery (data not shown). In addition, there was a marked increase in COX-2 expression in the neointima in the animals that underwent balloon injury (Fig. 1D). The increase in COX-2 expression was confined to the neointima and was not evident in the media. The specificity of the COX-2 expression was confirmed by preabsorbing the COX-2 antibody with a peptide against which it was raised (Fig. 1E).

RT-PCR. In animals that were not subjected to anesthesia or any surgical procedure, there was no COX-2 mRNA ex-
pression in the carotid rat artery. In all samples from animals undergoing surgery with or without balloon injury (sham-operated animals), there was expression of COX-1 and COX-2. The results suggest that isolation of the vessel alone, even in the absence of balloon injury, induced COX-2 mRNA expression, consistent with the adventitial expression of COX-2 seen on immunohistochemistry in the sham-operated animals. COX-2 mRNA expression was unaffected by either of the COX inhibitors (data not shown).

**Urinary Eicosanoid Production.** Urinary TXB₂, PGE₂, PGF₂α, and 8-iso-PGF₂α were measured before surgery and at 3 days after angioplasty (Fig. 2). TXB₂, PGE₂, and PGF₂α (ng/mg creatinine) were unaltered in sham-operated controls after surgery compared with levels seen preoperatively (TXB₂ 7.2 ± 0.5 versus 5.5 ± 0.7 (n = 8), p = 0.19; PGF₂α 10.6 ± 1.4 versus 8.9 ± 1.2 (n = 8), p = 0.39; and PGE₂ 21.8 ± 3.6 versus 23.5 ± 2.6 (n = 8), p = 0.21). However, after balloon angioplasty there was a marked increase in the their excretion compared with sham-operated controls [TXB₂: 19.8 ± 1.7 (n = 8), p < 0.001; PGF₂α: 24.8 ± 2.7 (n = 8), p < 0.001; and PGE₂: 66.4 ± 4.9 (n = 8), p < 0.001].

The selective COX-2 inhibitor SC-236 inhibited urinary thromboxane [TXB₂ to 11.1 ± 0.8 (n = 8), p < 0.01] and prostaglandin excretion [PGF₂α to 11.0 ± 0.9 (n = 8), p < 0.01 and PGE₂ to 15.7 ± 1.9 (n = 8) p < 0.01]. Similarly the selective COX-1 inhibitor reduced TXB₂ [3.7 ± 0.6 (n = 8) p < 0.001] and prostaglandin excretion [PGF₂α, 4.03 ± 0.4 (n = 8) p < 0.001 and PGE₂ 16.6 ± 1.3 (n = 8), p < 0.01]. Administration of the COX-1 and COX-2 inhibitor together induced a further reduction in prostaglandin generation [PGE₂ to 7.2 ± 0.9 ng/mg creatinine (n = 8) p < 0.001 and PGF₂α to 1.5 ± 0.3 (n = 8) p < 0.001], suggesting that a combination of both selective inhibitors are better inhibitors of prostaglandin generation than either drug alone.

There was no difference in 8-iso-PGF₂α excretion between the sham and the injury groups after surgery. Moreover, generation of 8-iso-PGF₂α was unaltered by the COX inhibitors.

**Serum TXB₂.** Serum TXB₂, an assay of COX-1 activity, was reduced by SC-560 alone and when combined with SC-236. SC-236 alone had no effect on serum TXB₂ formation (Fig. 3).

**Quantification of Intimal Hyperplasia.** Intimal hyperplasia developed over the 14 days after balloon injury in the group that received no treatment (I/M ratio 1.76 ± 0.2; n = 7). SC-236, the selective COX-2 inhibitor, had no effect on the development of intimal hyperplasia (I/M ratio 1.61 ± 0.2; n = 7, p = 0.92). Administration of SC-560 significantly reduced intimal hyperplasia (I/M ratio 1.03 ± 0.2; n = 7, p < 0.05) as
did the combination of SC-560 and SC-236 (I/M ratio 1.06 ± 0.1; n = 7, p < 0.05) (Fig. 4).

**Discussion**

Our study demonstrates expression of both COX-1 and COX-2 in the neointima after balloon injury of the carotid artery. The expression of the two COX isoforms in the neointima of vascular lesions is not surprising. COX-2 is induced by several factors implicated in the development of vascular proliferation and restenosis, including growth factors, free radicals, and cytokines (Lin et al., 1989; Jones et al., 1993; Adderley and Fitzgerald, 1999). COX-1 is also inducible (McAdam et al., 2000) and in any event is constitutively expressed in virtually all cells. There was also a marked increase in prostaglandin formation and thromboxane. Both PGE₂ and thromboxane are relevant, because they are major products of vascular smooth muscle and inflammatory cells.

![Fig. 2. Urinary prostaglandin generation in sham animals and after balloon angioplasty. Data are mean ± S.E.M.; $, p < 0.001 versus before surgery; **, p < 0.01; and ***, p < 0.001 versus balloon injury on no treatment.](image)

![Fig. 3. Measurement of COX-1 activity. Serum TXB₂ from whole blood after balloon angioplasty. SC-560 and combination of SC-560 with SC-236 reduced serum TXB₂ levels. *, p < 0.05 versus no treatment.](image)
Moreover, COX-2 colocalizes with PGE synthase in human atherosclerotic plaque (Cipollone et al., 2001). Although the source of the increase was not defined, because there was no increase in sham-operated animals, it is reasonable to infer that the rise in urinary product excretion derived from the site of vascular injury. However, it should be noted that these are primary prostaglandins and we cannot exclude a renal origin.

To explore the functional roles of COX-1 and COX-2 in this model, we examined the effect of SC-236 and SC-560. SC-236 is highly selective for COX-2 (Kishi et al., 2000). Consistent with this, SC-236 had no effect on serum TXB2, an assay of COX-1 activity, despite marked suppression of urinary prostaglandin metabolites. SC-560 is selective for COX-1 (Smith et al., 1998) and as expected inhibited serum TXB2, although only by 60%. Other studies have described an 85% reduction in TXB2 production in calcium ionophore-stimulated whole blood with 10 mg/kg SC-560 (Smith et al., 1998). The incomplete suppression of both serum TXB2 and whole blood-stimulated TXB2 is probably due to the fact that SC-560, although selective for COX-1, is a competitive inhibitor.

Both compounds reduced PGE2 and TXB2 excretion. This is not surprising, because the two COX isozymes are capable of generating all prostaglandins, and although there may be quantitative differences in product formation, no one product discriminates between the two isozymes. The findings are similar to human studies of atherosclerosis, where both isozymes are responsible for the increase in prostaglandin generation (Belton et al., 2000). SC-560 markedly inhibited TXA2 generation based on a marked reduction in TXB2 excretion in animals. This is to be expected, because platelet COX-1 is a major source of thromboxane generation in vivo (Clarke et al., 1991). The selective COX-2 inhibitor SC-236 also inhibited TXA2, although to a smaller degree. Tissue COX-2 may contribute to TXA2 in some settings, for example, in patients with unstable angina where there is persistent formation of TXA2 despite treatment with aspirin (Cipollone et al., 1997). Indeed, several cell types found in vascular lesions express COX-2 and generate TXA2, including vascular smooth muscle cells and monocytes (Penglis et al., 2000; Young et al., 2000).

Smooth muscle cell proliferation is a key event in the development of atherosclerosis, restenosis, and intimal hyperplasia (Clowes and Reidy, 1991; Ross, 1993). Prostaglandins may influence the process in several ways. TXA2 and PGE2, and the isoprostane 8-iso-PGF2α (a free radical-derived product of arachidonic acid) induce proliferation of vascular smooth muscle cells through activation of G protein-coupled transmembrane receptors (Craven et al., 1996; Pakala et al., 1997; Zucker et al., 1998). Platelets that adhere at the site of injury may also contribute to lesion formation through the release of growth factors or by triggering thrombosis (Clowes and Reidy, 1991; Ross, 1993). Therefore, in addition to a direct effect on vascular tissue, TXA2 may contribute to lesion formation by amplifying platelet activation at the site of vascular injury.

Alternatively, prostaglandins may limit lesion development. Overexpression of COX-2 or prostacyclin synthase suppresses the development of vascular lesions and the growth of vascular smooth muscle cells (Hara et al., 1995; Harada et al., 1999). These effects may be mediated through peroxisome proliferator-activated receptors (Kilewer, 1997; Staels et al., 1998), a series of nuclear membrane receptors for prostaglandins that heterodimerize with other transcription factors and regulate the expression of genes in vascular smooth muscle cells, including those involved in cell growth and/or apoptosis.

In the model of balloon injury to the carotid artery, COX-1 inhibition reduced lesion development and no additional effect was seen when both isozymes were inhibited by combining the two drugs. The results suggest a role for COX-1 alone in the development of the neointima. Although this could reflect suppression of tissue prostaglandin generation, overexpression of COX-1 in vascular tissue by using an adenoviral vector protects against lesion development by preventing the local formation of thrombus (Zoldhelyi et al., 1996). Alternatively, the effects of SC-560 may be due to the suppression of platelet and/or vascular TXA2, a potent platelet activator and mitogen for vascular smooth muscle cells. Similar inhibition of the vascular response to injury has been seen with selective TXA2/prostaglandin endoperoxide receptor antagonists, supporting the hypothesis (Cayatte et al., 2000).

No inhibition of neointimal hyperplasia was detected using the selective COX-2 inhibitor despite a marked reduction in prostaglandin formation. Because COX-2 is a major source of endogenous PGI2, concern has been raised that COX-2-selective inhibitors may allow unopposed TXA2-mediated effects on platelets and place patients at risk of thrombosis (Cullen et al., 1998; Catella-Lawson et al., 1999; McAdam et al., 1999). Reports of thrombosis in patients receiving selective COX-2 inhibitors are consistent with this hypothesis (Bouabdellier et al., 2000; Crofford et al., 2000). We saw no increase in lesion development and no thrombosis, although it should be emphasized that we did not measure PGI2 biosynthesis in vivo in this model, a limitation given that PGI2 may influence lesion development by inhibiting platelet activity at the site of vascular damage (Zoldhelyi et al., 1996). Moreover, thrombosis is not a major feature of animal models. Thus, further studies are required to explore the effect of COX-2 on PGI2 formation and the development of thrombus in vivo.

In conclusion, selective inhibition of COX-1 reduced the development of the neointimal hyperplasia that follows balloon injury of the carotid artery, whereas inhibition of COX-2 had no effect. Our findings are consistent with findings show-
ing evidence of a role for COX-1 but not COX-2 in a murine model of atherosclerosis (Pratico et al., 2001).

References


