COX-2 Inhibition and Retinal Angiogenesis in a Mouse Model of Retinopathy of Prematurity

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PURPOSE. The prostaglandin-cyclooxygenase (COX) pathway influences new blood vessel growth in a variety of tissues. This study was conducted to determine the cellular location of COX-2 in the retina and whether the inhibition of COX-2 would reduce retinal angiogenesis in a rodent model of retinopathy of prematurity (ROP).

METHODS. ROP was induced in C57BL/6 mice by exposing 7-day-old mice to 75% oxygen (hyperoxia) for 5 days followed by 5 days in room air (relative hypoxia and retinal angiogenesis). Normal mice were those with a normally developing retinal vasculature exposed to room air from birth until postnatal day (P)17. The COX-2 inhibitor, rofecoxib (15 mg/kg body weight intraperitoneally) was administered to normal and ROP mice from P12 to P17. Immunohistochemistry for COX-2 was performed on retinas from all groups by the avidin-biotin method. Histologic methods were used to count blood vessel profiles (BVPs) in the inner retina (inner limiting membrane, ganglion cell layer, and inner plexiform layer) with a masked approach.

RESULTS. Intense COX-2 immunolabeling was specifically localized to ganglion cells and blood vessels of all mice retinas. In ROP mice, COX-2 immunolabeling was detected on blood vessels extending into the vitreous cavity. Quantitation of BVPs in the inner retina revealed an increase in untreated ROP mice compared with untreated normal mice (P < 0.001). Rofecoxib decreased BVPs by approximately 45% in normal mice and 37% in ROP mice.

CONCLUSIONS. COX-2 is localized to sites associated with retinal blood vessels. The finding that the selective COX-2 inhibitor, rofecoxib, attenuated the retinal angiogenesis that accompanies ROP, and normal retinal development indicates that COX-2 plays an important role in blood vessel formation in the retina. (Invest Ophthalmol Vis Sci. 2003;44:974–979) DOI: 10.1167/iovs.02-04592

Angiogenesis is a hallmark feature of proliferative diabetic retinopathy and contributes to the severe visual loss that accompanies this disease. Investigation into the causative factors involved in the progression of diabetic retinopathy is limited by the lack of a diabetic rodent model that progresses from early background retinal changes, such as loss of pericytes and thickening of the basement membrane, to frank retinal angiogenesis. Retinopathy of prematurity (ROP) in laboratory animals is a widely used method to study diabetic retinal microvascular complications, because, as is proliferative diabetic retinopathy, it is characterized by hypoxia-induced retinal angiogenesis.

Several pathogenic factors are implicated in the development of both proliferative diabetic retinopathy and ROP and include vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), connective tissue growth factor, and angiotensin II. However, despite blockade of these cytokine pathways in ROP, complete prevention of retinal angiogenesis does not always occur. This has led to a search for other factors that may participate in formation of new blood vessels in diabetic retinopathy and other angiogenesis-associated retinal diseases. The prostaglandin-cyclooxygenase (COX) pathway has recently been implicated in the formation of new blood vessels. Prostaglandins are liberated from arachidonic acid by the action of the enzyme COX. There are two COX enzymes, COX-1 and COX-2, that catalyze identical reactions. COX-1 is constitutively expressed in most tissues and cells, whereas COX-2 is inducible by a number of factors, including cytokines, inflammatory mediators, and tumor promoters.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin, and ibuprofen inhibit the formation of both COX-1 and COX-2 and are commonly used for the treatment of pain and arthritis. Evidence that NSAIDs produce gastrointestinal ulcers in approximately 25% of users and that this side effect may be due to the blockade of COX-1 rather than COX-2 prompted the development of selective COX-2 inhibitors.

The angiogenic properties of NSAIDs are mainly linked to COX-2 activity, with reports of COX-2 inhibitors attenuating angiogenesis in cancer, granulation tissue, arthritis, and experimental models of angiogenesis. A limited number of studies have examined COX in the retina, although nonselective COX inhibitors have been reported to have varying effects on ROP in children and rodents. The novelty of the present study is the cellular localization of COX-2 to sites associated with retinal blood vessels and the finding that selective inhibition of COX-2 elicits an antiangiogenic response in a rodent model of ROP.

METHODS

Animals

Pregnant female C57BL/6 mice were provided by The Animal Resource Centre, Western Australia, and housed in the Biological Research Facility, Department of Physiology, The University of Melbourne. The mothers were randomly divided into four experimental groups with eight pups per group. These groups consisted of group 1: normal untreated; group 2: normal treated with the COX-2 inhibitor rofecoxib; group 3: untreated ROP; and group 4: ROP treated with rofecoxib.

In the first group (normal untreated), the mother and her newborn pups were housed in room air from postnatal day (P90 to P17). In group 2 (normal treated), normal mice were housed in room air from birth until P17 and treated daily with rofecoxib (15 mg/kg body weight).
intraperitoneally [IP]) between P12 and P17. In group 3 (untreated ROP), ROP was induced in C57BL/6 mice by placing 7-day-old pups with their mother in sealed chambers, containing 75% ± 5% O2 and 2% CO2, using medical grade O2 and industrial grade air. Gas levels in the chamber were monitored twice daily with a gas analyzer (Model ML 205; AD Instruments, Pty., Ltd., Castle Hill, New South Wales, Australia) and chart recorder (Chart, ver. 5.5, on the MacLab/2E System; AD Instruments, Pty., Ltd.). An airflow rate of approximately 2.5 L/min assisted in maintaining adequate levels of metabolically produced CO2 and decreases in O2 tension. Mice remained in the chamber for 5 days (hypoxic period, postnatal day [P]17–P12) and were then housed in room air for a further 5 days (hypoxia-induced angiogenesis, P12–P17).

In group 4 (treated ROP), the ROP-induction protocol was used, and rofecoxib (15 mg/kg body weight, IP) was administered daily during the time in room air (P12–P17). Before administration, rofecoxib was dissolved in a 0.5% aqueous methyl cellulose solution.

During the experiment, mothers were provided with water and standard mice chow (GR2; Clark-King and Co., Gladesville, Victoria, Australia) ad libitum and exposed to normal 12 hour light-dark cycles. Pups received nutrition from their mothers. To avoid respiratory distress, mother and pups were removed each day from the chamber and placed in room air for 2 hours. Experimental procedures were consistent with the guidelines set by the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Tissue Collection and Histology**

After the 17-day experimental period, mice were killed by an IP injection of pentobarbital (120 mg/kg body weight; Nembutal; Rhone Merieux, Queensland, Australia) and decapitated. Both eyes were removed from each mouse and fixed for 5 hours in Bouin fixative. Eyes were processed in graded alcohol baths before being embedded in paraffin wax, serially sectioned at 3 μm at 90° to the optic nerve, and placed on 3-amino-propyl-triethoxysilane (Sigma, St. Louis, MO)-coated slides. Approximately 120 sections per eye were collected and incubated overnight at 37°C.

**Immunohistochemistry for COX-2**

Randomly chosen sections of eye from each mouse (n = 8 mice per group) were deparaffinized and then incubated for 20 minutes with normal goat serum (NGS; Zymed, South San Francisco, CA), diluted 1:10 with 0.1 M phosphate buffered-saline (PBS; pH 7.4). The sections were then incubated overnight at 4°C with polyclonal COX-2 antisera (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:5000 with 0.1 M PBS. Sections incubated with NGS instead of primary antiserum were used as the negative control. Because COX-2 is located in the macula densa of the kidney, sections of rat and mouse kidney were used as a positive control. All sections were then washed with 0.1 M PBS (three 5-minute washes) and then incubated with biotinylated rabbit anti goat IgG (Dako Corp., Glostrup, Denmark), diluted 1:200 with 0.1 M PBS, for 20 minutes at room temperature. The sections were then rinsed with 0.1 M PBS (once for 5 minutes) and incubated with avidin-biotin peroxidase complex (Vector Laboratories, Inc. Burlingame, CA) prepared by placing 1 drop of each component (A and B) in 5 mL 0.1 M PBS. After one last rinse with 0.1 M PBS, sections were incubated with 0.05% diaminobenzidine (Dako Corp.) for approximately 1 minute before rinsing in tap water for 5 minutes. The sections were then stained with Mayer hematoxylin, differentiated in Scott tap water, dehydrated in alcohol, cleared in a paraffin solvent and clearning agent (Fronine Pty. Ltd., Riverton, New South Wales, Australia) and mounted in di-butyl phthalate xylene (BDH Laboratory Supplies, Poole, UK).

**Blood Vessel Profiles in the Inner Retina**

Three sections of one eye of each animal were randomly chosen, deparaffinized, and stained with Mayer hematoxylin (5 minutes) and cosin (5 minutes) (Amber Scientific Laboratories, Belmont, New South Wales, Australia), and coverslipped. By an established technique,7 blood vessel profiles (BVPs) were counted in the inner retina and included vessels adherent to the inner limiting membrane (ILM). The inner retina comprised the ILM, ganglion cell layer (GCL), and inner plexiform layer (IPL). Four fields per section were evaluated in a masked manner. A BVP was defined as an endothelial cell (stained blue) or a blood vessel with a lumen. Counting was performed by photomicroscope (BH-2; Olympus, Tokyo, Japan) at a magnification of ×40, and images were captured on a digital camera connected to an IBM computer (Spot; SciTech Pty., Ltd., Preston, Victoria, Australia).

**Statistical Analysis**

Data were analyzed on computer (StatView for Windows, ver. 5.0.1; SAS Institute Inc, Cary, NC). A two-way ANOVA with the Fisher post hoc comparison was applied, with P < 0.05 considered to be statistically significant.

**RESULTS**

**Histopathology**

In normal untreated mice, the vasculature of the inner retina appeared normal, with blood vessels confined to the inner retina (Fig. 1A). In contrast, in untreated ROP mice, large clusters of blood vessels were adherent to the ILM, and many blood vessels were observed in the inner retina (Fig. 1B). In normal mice treated with rofecoxib, the density of blood vessels in the inner retina appeared similar to or slightly less than in untreated mice (Fig. 1C). In ROP mice treated with rofecoxib, fewer blood vessels were attached to the ILM and observed in the inner retina (Fig. 1D).

**COX-2 Immunohistochemistry**

In all groups, intense immunolabeling for COX-2 was observed in the cytoplasm of ganglion cells and in retinal blood vessels (Fig. 2). The intensity of immunolabeling for COX-2 was similar among all groups. In untreated and treated ROP mice, specific COX-2 immunolabeling was also observed on vessels protruding from the inner retina into the vitreous cavity (Fig. 2). Sections of rat and mouse kidney immunolabeled for COX-2 showed the expected distribution of immunolabeling in macula densa cells of the distal convoluted tubule (not shown).

**Quantification of Blood Vessel Profiles in the Inner Retina**

Evaluation of BVPs in the inner retina confirmed the histopathologic findings (Fig. 3). Significantly more BVPs were observed in untreated ROP mice than in all other groups (P < 0.001). In ROP mice treated with rofecoxib, BVPs in the inner retina were reduced by approximately 37% compared with those in untreated ROP mice. Rofecoxib reduced BVPs in normal treated mice by approximately 45% compared with normal untreated mice.

**DISCUSSION**

The present study provides new evidence that COX-2 is present in ganglion cells and retinal blood vessels of the mouse...
The antiangiogenic effect of the selective COX-2 inhibitor, rofecoxib, indicates the importance of COX-2 in the formation of new retinal blood vessels. The ability of rofecoxib to reduce angiogenesis in situations of retinal hypoxia such as ROP and the normal developing retina is consistent with findings in other tissues where COX-2 has a key role in the angiogenic process.

Despite the presence of COX-2 enzymatic activity within ocular tissues, little is known about its cellular location in the retina. Our finding of COX-2 protein on new blood vessels in the inner retina and protruding into the vitreous of ROP mice is consistent with previous reports of COX-2 expression on angiogenic endothelial cells in rheumatoid arthritis and granuloma.

COX-2 immunolabeling was also present on retinal blood vessels of mice that had just completed normal developmental angiogenesis, indicating that COX-2 protein is not confined to proliferating vessels. The observation of COX-2 protein in ganglion cells of normal and ROP mice is also important in understanding retinal angiogenesis. Ganglion cells contribute to formation of new blood vessels in the retina, and we and other investigators have shown them to contain a number of proangiogenic cytokines, including VEGF, insulin-like growth factor (IGF), secreted protein acidic and rich in cysteine (SPARC), and renin. Macroglial Müller cells are also implicated in retinal angiogenesis, because, like ganglion cells, they synthesize numerous growth factors, contribute to the integrity of the blood vessel wall, and also participate in new vessel formation in diabetic retinopathy. Relevant to the present study is that Müller cells also express the proangiogenic growth factor FGF-2 in response to prostaglandin E2. The finding of COX-2 in cellular sites associated with blood vessels has important implications for normal developmental angiogenesis and diseases such as proliferative diabetic retinopathy.

The concept that COX-2 participates in angiogenesis is supported by studies showing that cells overexpressing COX-2 produce prostaglandins and angiogenic cytokines and induce migration of endothelial cells and tube formation. Interventional studies with selective COX inhibitors have been key to further understanding the antiangiogenic effects of COX-2, with selective COX-2 inhibitors such as rofecoxib, celecoxib, and NS-398, attenuating vessel density in tumors, sponge implants, and granulation tissue. In the eye, the most well-known vascular effect of the prostaglandin-COX pathway is vasodilation. A limited number of studies have investigated COX and ocular angiogenesis, with an early report showing that the nonselective COX inhibitor indomethacin reduces corneal angiogenesis. More recently, COX-2 was identified as the enzyme responsible for the attenuation of formation of blood vessels in the cornea. Indomethacin has been reported to reduce growth of new blood vessels in the inner retina of ROP mice. The present study reproduced this result.

**Figure 1.** Histopathology of the inner retina of C57BL/6 mice with retinopathy of prematurity treated with the COX-2 inhibitor rofecoxib. Sections are counterstained with Mayer hematoxylin and eosin. (A) Normal untreated. (B) Untreated ROP. (C) Normal treated with rofecoxib. (D) ROP treated with rofecoxib. Arrows: blood vessels in the inner retina (A, C, D) and protruding into the vitreous (B). Magnification, ×200.
with the selective COX-2 inhibitor rofecoxib and further demonstrated that COX-2 is likely to be the COX enzyme involved in an angiogenic response in ROP. Furthermore, contrary to the results of Nandgaonkar et al.,26 we found that selective inhibition of COX-2 had antiangiogenic effects in animals undergoing normal developmental retinal angiogenesis. The reasons for the discrepancies between the two studies are uncertain but may be due to different stereologic methods used to quantitate growth of retinal blood vessels and the timing of drug administration, which in the present study occurred during the retinal hypoxic and angiogenic period, in contrast with Nandgaonkar et al.,26 who administered indomethacin concurrently with exposure to hyperoxia.

The mechanisms by which COX-2 is upregulated in ROP and diabetic retinopathy are unknown. However, hypoxia and cytokines have been reported to induce COX-2.15,31,32 For instance, bFGF increases the expression of COX-2 mRNA and angiogenesis in rat sponge implants,15 and bFGF-driven angiogenesis in the mouse corneal micropocket assay can be blocked with a selective COX-2, but not COX-1, inhibitor.56 VEGF is also implicated in induction of COX-2, with VEGF increasing COX-2 in human umbilical vein endothelial cells, an effect that may occur through the transcription factor, nuclear factor of activated T cells.19 The roles of tissue hypoxia and VEGF in retinal angiogenesis in ROP and proliferative diabetic retinopathy are well known.4,57,58 Indeed, we and other investigators have shown VEGF to be upregulated in ganglion cells and blood vessels of rodents with ROP and diabetes,7,8,59,60 sites which correspond with the location of COX-2 protein. Future studies are needed to determine whether an interaction between angiogenic cytokines and COX-2 in these cells elicits retinal angiogenesis in response to local tissue hypoxia.

In summary, we report for the first time the presence of COX-2 in the retina, and demonstrate the antiangiogenic effects of selective COX-2 inhibition in both developmental and pathologic retinal angiogenesis. These findings encourage the investigation of the therapeutic potential of COX-2 inhibitors for the treatment of angiogenic retinal diseases such as proliferative diabetic retinopathy.

References


Figure 2. Immunolabeling for COX-2 in 3-µm paraffin-embedded sections of inner retina from C57BL/6 mice with retinopathy of prematurity. Counterstain, hematoxylin. (A) Normal untreated. (B) Untreated ROP. (C) Normal treated with rofecoxib. (D) ROP treated with rofecoxib. Intense COX-2 immunolabeling was observed in ganglion cells (single arrow) and blood vessels (double arrows). Magnification, ×250.

Figure 3. Blood vessel profiles in the inner retina of C57BL/6 mice after retinopathy of prematurity and COX-2 inhibition. Data are the mean ± SEM of results in eight mice per group. *P < 0.01 compared with untreated normal mice and ROP + rofecoxib mice; #P < 0.001 compared with all groups; †P < 0.01 compared with normal untreated mice.


