Increases in Retinovascular Prostaglandin Receptor Functions by Cyclooxygenase-1 and -2 Inhibition

Pierre Hardy,1 Mousumi Bhatbacharya,2 Daniel Abran,1 Krishna G. Peri,1 Pierre Asselin,1 Daya R. Varma,2 and Sylvain Chemtob1,2

PURPOSE. To determine the relative contribution of cyclooxygenase (COX)-1 and COX-2 in regulating prostaglandin (PG) E2 and PGF2α receptors (EP and FP, respectively) and their functions in retinal vasculature of neonatal pigs.

METHODS. Newborn pigs were treated intravenously every 8 hours for 48 hours with saline, 40 mg/kg nonselective COX inhibitor ibuprofen, 80 mg/kg COX-1 inhibitor valeryl salicylate, or 5 mg/kg DuP697 and 5 mg/kg NS398, COX-2 inhibitors. Retinal microvessel EP and FP receptor densities were measured by radioligand binding and receptor-coupled effects by determining second-messenger inositol 1,4,5-trisphosphate (IP₃) and vasomotor responses. Retinal blood flow (RBF) response to incremental increases in blood pressure (BP) was measured by a microsphere technique.

RESULTS. Valeryl salicylate, DuP697, and NS398 reduced retinal PGE2 and PGF2α concentrations in the newborn by approximately half, whereas ibuprofen caused further reduction to levels observed in adults. Retinal vessel EP₁, EP₃, and FP receptor densities increased approximately threefold after treatments with COX-1 or COX-2 inhibitors, and five- to sixfold after ibuprofen treatment. EP and FP receptor upregulation was associated with corresponding increases in IP₃ production and retinal vasoconstriction in response to PGF2α, fenoprostanol (an EP agonist), PGE2, 17-phenyl trinor PGE2 (an EP agonist), and M&B28,767 (an EP3 agonist) and with enhanced RBF autoregulation of high BP (≥125 mm Hg). Conversely, EP₂ receptor density and coupled functions were minimally affected by COX inhibition.

CONCLUSIONS. Data suggest that increased COX-1- and COX-2-catalyzed prostaglandin synthesis contribute equivalently to the downregulation of retinovascular EP₁, EP₃, and FP receptors and their vasoconstrictor functions in newborn pigs; the EP₂ receptor was not significantly influenced by ontogenic alterations in prostaglandin levels. (Invest Ophthalmol Vis Sci. 1998;39:1888-1898)

Prostaglandins (PGs) are produced by the enzymatic activities of two isofoms of cyclooxygenase (COX), COX-1 and COX-2. There is evidence for a tissue-specific increase in the activity of one or both of these isozymes in the perinatal period.1-3 In retina, activities of COX-1 and COX-2 are high in the newborn,1 and each isozyme contributes to approximately 50% of total COX activity.2

Prostaglandins (PGs) play an important role in the control of retinal blood flow (RBF),4 particularly in the newborn.5 Prostaglandin F2α and PGE2 elicit minimal retinal vasoconstriction in the newborn,6 compared with their effects in the adult.6,7 We have recently reported that these ontogenic differences in the actions of PGF2α and PGE2 on retinal vasculature could be caused by a relative deficiency in receptors for PGF2α (FP) and PGE2 (EP) and receptor-coupled second messengers that mediate vasoconstriction.8 It has been shown in brain vasculature that a reduction in the high concentrations of PGs in the newborn animal to levels observed in the adult, increases EP₁, EP₃, and FP receptors to adult values9 and raises the upper limit of cerebral blood flow autoregulation.4 These observations suggest that PG receptors are downregulated by high perinatal PG levels.9,10 Contrary to brain vasculature, retinal vessels contain EP₂ receptors8 that elicit vasodilation.6 Therefore, upregulation of the EP₂ receptor may diminish PGE2-induced contraction.9

Of the four EP receptor subtypes so far described, the retinal microvasculature contains EP₁, EP₂, and EP₃.9 EP₁ and FP are coupled to inositol 1,4,5-trisphosphate (IP₃) formation and EP₂ to 3',5'-cyclic adenosine monophosphate (cAMP) synthesis. Signaling of EP₃ receptors in retinal vessels remains undefined.9 At present, the role of PGs in downregulating FP and EP receptor density (mainly EP₁, EP₂ and EP₃)9 and receptor-coupled functions in retinal microvasculature of the new-
born and the relative contributions of COX-1 and COX-2 in this process are unknown.

We tested the hypothesis that selective inhibition of COX-1, COX-2, or both in vivo in newborn animals would increase retinovascular PGF$_{2\alpha}$ and PGE$_2$ receptors and receptor-coupled vasoconstriction in accordance with their contribution to PG synthesis. For this purpose, newborn pigs were treated with saline, nonselective COX inhibitor ibuprofen, selective COX-1 inhibitor valeryl salicylate, or selective COX-2 inhibitors DuP697 and NS398. We determined retinal microvascular EP and FP receptor densities, second-messenger production, and vasomotor responses. In addition, because PGs affect hypertension-elicited RBF autoregulatory responses, which is diminished in the newborn, compared with that in the adult, we also investigated the impact of the upregulation in EP and FP receptors on RBF autoregulation.

**MATERIALS AND METHODS**

### Animals

Newborn pigs (1 day old) were purchased from a local breeder (Les Fermes Menard, Quebec, Canada) and used according to the guidelines of the Animal Care Committee of Ste-Justine Hospital Research Center and of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were maintained at 25°C and 50% to 70% humidity on a 12-hour-light (7 AM-7 PM), 12-hour dark schedule and were fed milk and tap water ad libitum. Eyes from adult pigs (5-7 months old) were obtained from a local abattoir (Bienvenue-Olympia, St. Valérien, Québec, Canada), immediately after the animals were killed and were transported to the laboratory on ice.

### Treatments

Newborn pigs were anesthetized with 2% halothane. A polyethylene catheter (PE-90) was placed into the right femoral vein, exteriorized, and attached with tape to the back of the animal. Animals were randomly assigned to receive intravenous (IV) saline, 40 mg/kg ibuprofen, 80 mg/kg COX-1 inhibitor valeryl salicylate, 5 mg/kg COX-2 inhibitor DuP697, or 5 mg/kg NS398 every 8 hours for a total of 48 hours. Doses of COX-1 and COX-2 inhibitors used had been shown to be effective in reducing PG levels, and peak changes in receptor density were reported to occur after 48 hours of treatment with COX inhibitor. At the end of the 48-hour period, animals were either killed (120 mg/kg IV pentobarbital) to obtain eyes or were subjected to a protocol to determine RBF autoregulation.

### Preparation of Retinal Microvessels

Retinal microvessels were prepared as previously described. Briefly, retinas were gently homogenized with a Wheaton pestle in 5 mM Tris-HCl buffer (pH 7.4) containing 1.1 mM acetylsalicylic acid (ASA), 0.5 mM EGTA, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 μg/ml soybean trypsin inhibitor. The homogenate was mixed with 40% Ficoll 400 (1:1 vol/vol; Pharmacia Biotech, Baie d’Urfe, Quebec, Canada) and centrifuged at 20,000 g for 20 minutes at 4°C, which allowed separation of neural tissue from microvessels. The pellet, which contained pure microvessels not contaminated by photoreceptor outer segments and other retinal cell membranes, was washed in the above buffer three times and subsequently filtered through a 120-μm filter. The filtrate that contains microvessels was kept and the residue on the filter discarded. The filtrate fraction was centrifuged at 1000 g for 15 minutes and stored at −80°C until used for experiments. The purity of the microvessel preparation was confirmed by high-power microscopy (Fig. 1) and consisted of capillaries and small arterioles that are considered important in the control of vasomotor tone. In addition, a more than 10-fold higher γ-glutamyl transpeptidase activity in microvessels than in retinal parenchyma was used to show purity of microvessel preparation. γ-Glutamyl transpeptidase activity in retinal microvessels and parenchyma was 53 U/mg to 85 U/mg protein and 2.8 U/mg to 3.6 U/mg protein, respectively.

### Assay of Prostaglandins

Prostaglandins in whole retinal tissue were assayed to account for their paracrine actions. Retinal tissue was homogenized as described and centrifuged at 1000 g for 10 minutes at 4°C, and the supernatant was homogenized again and recentrifuged at 50,000 g for 30 minutes at 4°C. Prostaglandins in the supernatant were extracted on octadecylsilyl silica columns and measured by radioimmunoassay, as described previously.

[**[^3H]PGE$_2$ and [^3H]PGF$_{2\alpha}$ Binding Assays**](#)

Microvessels were homogenized with a tissue grinder and then centrifuged at 1000 g for 15 minutes to remove nuclei and undisrupted cells. The supernatant that contained the membranes was re centrifuged at 100,000 g for 45 minutes and the pellet was used for binding and second-messenger-stimulation experiments. For binding assays aliquots of microvessel membranes (150 μg protein) were incubated at 37°C for 30 minutes in 100 μl 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl, 2 mM MgCl$_2$, 1 mM benzamidine, 0.5 mM EGTA,
Vasomotor Responses of Retinal Vessels

without 30 JLIM AH23848B (an EP4 antagonist), butaprost (an EP2 agonist), M&B28.767 (an EP3 agonist) and AH23,848B (an EP3 agonist). Receptor densities (Bmax) and affinity constants (KD) were determined from the saturation isotherms and displacement curves, by using a computer program (Prism 2.1; GraphPad). Pilot experiments showed that acute treatment (1 hour) with COX inhibitors did not affect the receptor densities, as previously reported.

IP3 and cAMP Assays

For IP3 stimulation retinal membrane preparations (200 µg protein) were incubated at 37°C for 5 minutes in 50 mM HEPES buffer (pH 7.4) containing (in millimolar) 108 NaCl, 4.7 KCl, 2.5 CaCl2, 1.18 MgSO4, 10 LiCl, 0.045 disodium EDTA, 1 dithiothreitol, and 1 benzamidine in the absence or presence of PGE2 (with or without 10 µM AH6809 [an EP1 antagonist]), 17-phenyl trinor PGE2 (an EP1 agonist), PGF2α, or fenprostanol (an FP agonist). The reaction was stopped with ice-cold 20% (vol/vol) HClO4. The IP3 was extracted and then measured by radioimmunoassay.

For cAMP determination, retinal membranes were incubated at 37°C for 5 minutes in an assay mixture containing (in millimolar) 10 Tris-HCl buffer (pH 8.0), 1 adenosine triphosphate, 7.5 MgCl2, 15 creatine phosphate, 0.5 EGTA, 0.5 isobutyl methylxanthine, 1 dithiothreitol, 1 benzamidine, 0.1 PMSF, 185 U/ml creatine phosphokinase and 100 µg/ml soybean trypsin inhibitor, and varying concentrations of [3H]PGE2 or [3H]PGF2α, to determine total and nonspecific binding, as previously described by us. Specific binding was calculated by determining the difference between total and nonspecific binding. To determine specificity and proportion of PGE2 receptor subtypes, displacement of specific [3H]PGE2 binding was performed by increasing concentrations of unlabeled PGE2, PGE2, 16,16-dimethyl PGE2, AH6809 (an EP1 antagonist), butaprost (an EP1 agonist), M&B28,767 (an EP3 agonist) and AH23,848B (an EP3 agonist). Receptor densities (Bmax) and affinity constants (KD) were determined from the saturation isotherms and displacement curves, by using a computer program (Prism, GraphPad, CA). Each measurement was repeated three times and had a variability of less than 10%.

Vasomotor Responses of Retinal Vessels

Eye cups were prepared as described previously. The eye cup was pinned to a wax support in a bath containing 20 ml Krebs-Ringer buffer equilibrated with 21% O2, 5% CO2, and 74% N2 and maintained at 37°C. Eye cups were washed two to three times with fresh Krebs buffer and allowed to equilibrate for 30 to 45 minutes before the experiments were begun.

Cumulative concentration–vasomotor response curves to PGE2, 17-phenyl trinor PGE2, butaprost, M&B28,767, PGF2α, and fenprostanol were determined on tissues pretreated (30 minutes) with 0.1 µM L670,596 to minimize nonspecific effects through the thromboxane receptors. All measurements were made on vessels 80 µm to 120 µm in diameter. Vasodilation to butaprost (an EP1 agonist) was determined on tissues preconstricted with 50 nM phenylephrine, corresponding to approximately 50% of maximum constriction. Response to phenylephrine was unaffected by treatment of animals with COX inhibitors. In addition, acute treatment with COX inhibitors did not elicit a vasomotor response and did not alter the responses to PGs and analogues tested. Vascular diameter was recorded with a video camera (Dage-MTI, Michigan City, IN) before and after topical application of an increasing concentration of the agent every 7 minutes (the time required to reach a stable response). Digital images were analyzed using commercial software (Sigma Scan; Jandel Scientific, Corte Madera, CA). Each measurement was repeated three times and had a variability of less than 10%.

Preparation of Animals to Study RBF Autoregulation

Animals were anesthetized with 2% halothane for tracheostomy and placement of catheters into the left subclavian artery for the withdrawal of blood samples, including reference samples; into the left ventricle through the right subclavian artery for the injection of radiolabeled microspheres; and into the femoral artery for continuous BP recording, using a pressure transducer (Statham, Glen Burnie, MD) connected to a multichannel recorder (TA240; Gould Inc., Valley View, OH), as described elsewhere. A silicone-coated balloon-tipped catheter was placed in the distal thoracic descending aorta through a femoral artery and inflated to produce hypertension in the aortic arch.

Halothane was discontinued after surgery, and animals were maintained on α-chloralose (a bolus IV injection of 50 mg/kg followed by infusion of 10 mg/kg per hour) and were paralyzed with 0.1 mg/kg IV pancuronium. Body temperature was maintained at 38°C with an overhead bright lamp, and the animals were allowed to recover from the surgery for 2 hours before the experiments were begun. Intraocular pressure, measured through a needle inserted into the anterior chamber, remained constant throughout the experiment at 15 ± 3 mm Hg and was not modified by COX inhibitors.

### Table 1. Retinal Concentrations of PGE2 and PGF2α in Newborn Pigs Treated with Nonselective and Selective COX Inhibitors and in Adult Pigs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE2 (pg/mg protein)</th>
<th>PGF2α (pg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>398 ± 22*</td>
<td>746 ± 47*</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>70 ± 4</td>
<td>181 ± 7</td>
</tr>
<tr>
<td>Valeryl salicylate</td>
<td>188 ± 13†</td>
<td>357 ± 29†</td>
</tr>
<tr>
<td>DuP697</td>
<td>204 ± 21†</td>
<td>370 ± 38†</td>
</tr>
<tr>
<td>NS398</td>
<td>211 ± 9†</td>
<td>358 ± 25†</td>
</tr>
<tr>
<td>Adult</td>
<td>82 ± 6</td>
<td>194 ± 16</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of four to five experiments, each performed in duplicate. Newborn animals were treated for 48 hours with saline or COX inhibitors, as described in the Methods section. Adults were untreated.

PG, prostaglandin; COX, cyclooxygenase.

* P < 0.01 compared with all other values in the same column.
† P < 0.01 compared with saline- and ibuprofen-treated animals in the same column.
Measurement of RBF

Because our previous observations suggested that diminished vasoconstrictor response to PGE$_2$ and PGF$_{2\alpha}$ in the newborn curtails RBF autoregulation, we focused on the upper end of the RBF autoregulatory range, which requires appropriate constriction to maintain constant blood flow. Therefore, we investigated whether upregulation of EP and FP receptors by selective COX inhibition is associated with improved autoregulation. Using the radiolabeled microsphere technique, described in detail elsewhere, RBF was measured at preselected mean BP (MBP) values at and above the upper limit of the RBF autoregulation range of the newborn pig. Mean BP was increased stepwise to 90 mm Hg (upper limit of RBF autoregulation of neonatal pigs), 105 mm Hg, and 125 mm Hg. These values varied by 5 mm Hg or less in the animals; baseline MBP was 69 ± 4 mm Hg for all animals and was unaffected by the treatments. Once MBP remained steady for 30 seconds after inflation of the balloon, approximately $10^6$ microspheres (15-μm diameter) labeled with $^{141}$Ce, $^{113}$Sn, $^{95}$Nb, and $^{85}$Sr were injected in a random sequence into the left ventricle. Withdrawal of reference blood samples from the left subclavian artery catheter was started 10 seconds before the injection of each type of microspheres and was continued for 70 seconds at a rate of 2 ml/min, using a Harvard infusion-withdrawal pump. Immediately after injection of microspheres, blood samples were withdrawn from the left subclavian artery to determine blood gases, which were normal and remained stable.

After the experiment, animals were killed with 120 mg/kg pentobarbital, the locations of catheters were verified, and the eyes were removed. The retinas were isolated and weighed. Radioactivity in the retinas and the reference blood samples...
TABLE 2. Maximum Binding of [3H]PGE₂ and [3H]PGF₂α in Retinal Microvessels of Newborn Pigs Treated with Nonselective and Selective COX Inhibitors and in Microvessels of Adult Pigs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE₂ (fmol/mg protein)</th>
<th>PGF₂α (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5.6 ± 0.6*</td>
<td>3.0 ± 0.3*</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>32.8 ± 3.9</td>
<td>14.1 ± 1.9</td>
</tr>
<tr>
<td>Valeryl salicylate</td>
<td>17.1 ± 1.4†</td>
<td>9.8 ± 1.2†</td>
</tr>
<tr>
<td>DuP697</td>
<td>14.2 ± 0.8†</td>
<td>9.2 ± 1.1†</td>
</tr>
<tr>
<td>NS398</td>
<td>14.7 ± 0.5†</td>
<td>8.8 ± 0.9†</td>
</tr>
<tr>
<td>Adult</td>
<td>35.8 ± 4.1</td>
<td>13.9 ± 1.0</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of four to five experiments, each performed in duplicate. Newborn animals were treated for 48 hours with saline or COX inhibitors as described in the Methods section. Adults were untreated.

PG, prostaglandin; COX, cyclooxygenase.
* P < 0.01 compared with all other corresponding values.
† P < 0.05 compared with other values in the same column.

were counted in a gamma scintillation counter (Cobra II, Canberra Packard, Meriden, CT). Retinal blood flow (in milliliters per minute per 100 g) was calculated using the formula: (counts per minute per gram tissue X reference blood withdrawal rate)/(counts per minute in the reference blood) using an on-line computer program with the counter (PCGERDA, Charlottesville, VA).

Materials

M&B28,767 was a gift from Rhone-Poulenc Rorer (Dagenham, United Kingdom), butaprost from Miles (West Haven, CT), DuP697 from DuPont-Merck (Wilmington, DE), and L670,596 from Merck-Frosst (Pointe-Claire, Quebec). The following agents were purchased: NS398 (Biomol, Plymouth Meeting, PA); 191 Ci/mmol [3H]PGE₂, 219 Ci/mmol [3H]PGF₂α enhanced chemiluminescence kit, and radioreceptor assay kits for IP₃ (Amersham, Mississauga, Ontario, Canada); ibuprofen, soybean trypsin inhibitor (type II-S), acetyllysylc acid, benzanilide, piroxicam, ibuprofen, pimethesin, adenosine triphosphate, creatine phosphate, creatine phosphokinase, β-mercaptoethanol, EDTA, EGTA, and isobutyl methylxanthan (Sigma, St. Louis, MO); PGE₂, PGF₂α, 17-phenyl trinor PGE₂, and valeryl salicylate (Cayman, Ann Arbor, MI); fenoprostone (Syntex, Mississauga, Ontario, Canada); radioimmunoassay kits for PGE₂ and PGF₂α (Advanced Magnetics, Boston, MA); radiolabeled microspheres (DuPont NEN, Boston, MA); all other chemicals (Fisher Scientific, Montréal, Québec, Canada).

Statistical Analysis

Data were analyzed by analysis of variance factoring for treatment and age group and by a comparison among means test (Tukey-Kramer method). Retinal blood flow data were analyzed by regression analysis, as previously described in detail.4,14,21 The Pearson's product moment coefficient (r) was calculated. Linear regressions were compared by regression equality test using the method of least squares. Statistical significance was set at P < 0.05. Data were expressed as mean ± SEM with the exception of RBF as a function of MBP.

RESULTS

Effects of COX-1 or COX-2 Inhibition on Retinal Prostaglandins

Treatment of piglets with the nonselective COX inhibitor, ibuprofen, caused a marked reduction in retinal PGE₂ and PGF₂α concentrations to levels observed in adults (Table 1). The effect of the COX inhibitor was dose dependent (Fig. 2). The COX-1 inhibitor, valeryl salicylate and the COX-2 inhibitors, DuP697 and NS398, caused comparable decreases in retinal PGE₂ and PGF₂α concentrations, which were less than those observed after ibuprofen treatment (Table 1).

[3H]PGE₂ and [3H]PGF₂α Binding in Retinal Microvessels

Specific binding of [3H]PGE₂ and [3H]PGF₂α to retinal microvessel membranes was saturable (Fig. 3). The effect of COX inhibition on maximum specific binding was dose dependent and inversely related to PG concentrations, as shown in the effect of ibuprofen on [3H]PGE₂ binding and PGE₂ concentrations (Fig. 2). Prostaglandin E₂ and PGE₂ receptors densities increased significantly in retinal microvessels of newborn pigs

TABLE 3. Competitive Inhibition by Different Agents of [3H]PGE₂ Binding to Retinal Microvessel Membranes from Newborn Pigs Treated with Saline, Ibuprofen, Valeryl Salicylate, or DuP697 and from Adult Pigs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE₂</th>
<th>AH6809 Butaprost M&amp;B28,767 AH23848B</th>
<th>PGE₂</th>
<th>AH6809 Butaprost M&amp;B28,767 AH23848B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>100 ± 0 42 ± 3* 42 ± 2* 15 ± 2*</td>
<td>&lt;1</td>
<td>31 ± 12 219 ± 71 187 ± 45 971 ± 201 ND</td>
<td></td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>100 ± 0 53 ± 3 22 ± 2 23 ± 3</td>
<td>&lt;1</td>
<td>34 ± 9 227 ± 89 200 ± 58 888 ± 211 ND</td>
<td></td>
</tr>
<tr>
<td>Valeryl salicylate</td>
<td>100 ± 0 48 ± 4 32 ± 3 19 ± 3*</td>
<td>&lt;1</td>
<td>30 ± 11 190 ± 68 196 ± 52 943 ± 197 ND</td>
<td></td>
</tr>
<tr>
<td>DuP697</td>
<td>100 ± 0 46 ± 4 33 ± 2 19 ± 3*</td>
<td>&lt;1</td>
<td>33 ± 12 208 ± 75 188 ± 57 891 ± 191 ND</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>100 ± 0 55 ± 4 21 ± 4 24 ± 2</td>
<td>&lt;1</td>
<td>51 ± 16 272 ± 71 265 ± 61 1071 ± 226 ND</td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ± SEM of three experiments, each performed in duplicate. Newborn animals were treated for 48 hours with saline or cyclooxygenase inhibitors, as described in the Methods section. Adults were untreated. The concentration of [3H] PGE₂ was 10 nM. ND determined (ND) indicates inhibition of binding was too low for accurate calculation of IC₅₀.

PG, prostaglandin; VSA, valeryl salicylate; IC₅₀; inhibitory concentration of 50%.
* P < 0.05 compared with corresponding value in adults.
Inhibition of specific binding of $[^{3}H]$PGE$_2$

(Expressed as percent of the binding without ligand)

![Graphs showing inhibition of specific binding of $[^{3}H]$PGE$_2$](image)

FIGURE 4. Competitive inhibition by prostaglandins (PGs) and analogues of specifically bound $[^{3}H]$PGE$_2$ on retinal microvessel membranes from newborn pigs treated with saline (A, B) or ibuprofen (C, D) and from adult pigs (E, F). Retinal microvessel membranes were incubated with 10 nM $[^{3}H]$PGE$_2$ at 37°C for 30 minutes in the presence or absence of unlabeled PGs at concentrations indicated. One hundred percent specific binding was determined in the presence of 25 μM unlabeled PGE$_2$. Each point is the mean of three experiments, each performed in duplicate. Displacement of $[^{3}H]$PGE$_2$ was performed with PGE$_2$ (•), 16,16-dimethyl PGE$_2$ (○), PGF$_2\alpha$ (▲), the prostaglandin receptor (EP)$_1$ antagonist AH6809 (▲), the EP$_2$ receptor agonist butaprost (■), the EP$_3$ receptor agonist M&B28,767 (●), and the EP$_4$ receptor antagonist AH23848B (△).

Treatment with ibuprofen, valeryl salicylate, DuP697, and NS398 (Fig. 2; Table 2). Ibuprofen caused a greater increase in EP and FP receptor densities than selective COX-1 and COX-2 inhibitors, reaching values comparable to those in adults (Table 2). The $K_d$ values for PGE$_2$ and PGF$_2\alpha$ in the saline-treated newborn were 7.6 ± 1.5 nM and 8.1 ± 1.5 nM and were similar in adults and treated newborn animals.

In adult preparations, the EP$_1$ receptor antagonist AH6809 displaced specifically bound $[^{3}H]$PGE$_2$ by 55%, and the EP$_2$ receptor agonist butaprost and the EP$_3$ receptor agonist M&B28,767 each caused approximately 20% to 25% displacement of bound $[^{3}H]$PGE$_2$ (Table 3). In contrast, on retinal microvessel membranes of saline-treated newborn animals, the EP$_1$ receptor antagonist AH6809 and the EP$_2$ receptor agonist butaprost displaced specifically bound $[^{3}H]$PGE$_2$ by 40% to 45%, and the EP$_3$ receptor agonist M&B28,767 displaced it by approximately 15% (Table 3). The EP$_4$ receptor antagonist AH23848B did not displace $[^{3}H]$PGE$_2$ in newborn or adult retinal microvessel membranes. The proportion of EP$_1$ and EP$_3$ receptors in ibuprofen-treated animals was raised to adult values (Table 3, Fig. 4), whereas in newborn pigs treated with valeryl salicylate and DuP697, the proportion of EP$_1$ and EP$_3$
Thus, because COX inhibitors caused an increase in the total density (Bmax X EP proportion) to values that correspondingly, the proportion of EP2 receptors was reduced by those observed in saline-treated newborns and adults. Consequently, the proportion of EP receptors was intermediately increased to values between newborns and adults. Correspondingly, the proportion of EP2 receptors was reduced by COX inhibitors to values approaching those of adults (Table 3).

**Table 4. Net IP3 and cAMP Synthesis (Expressed as Picomoles per Milligram Protein per Minute) Elicited by PGs and PG Analogues in Retinal Microvessels of Newborn Pigs Treated with COX Inhibitors and in Microvessels of Adult Pigs**

<table>
<thead>
<tr>
<th>Agents</th>
<th>Saline</th>
<th>Ibuprofen</th>
<th>VSA</th>
<th>DuP697</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE2</td>
<td>5.8 ± 0.5*</td>
<td>21.3 ± 1.3</td>
<td>12.5 ± 0.9†</td>
<td>11.9 ± 1.4†</td>
<td>21.9 ± 2.2</td>
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<tr>
<td>17-phenyl trinor PGE2</td>
<td>5.1 ± 1.5*</td>
<td>26.2 ± 1.8</td>
<td>20.1 ± 2.7†</td>
<td>18.3 ± 1.9†</td>
<td>25.6 ± 2.6</td>
</tr>
<tr>
<td>PGE2 + AH6809</td>
<td>1.8 ± 0.3†</td>
<td>5.9 ± 0.9</td>
<td>4.2 ± 1.3</td>
<td>3.6 ± 0.7</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td>PGF2α</td>
<td>6.2 ± 1.9*</td>
<td>16.3 ± 1.1</td>
<td>11.3 ± 0.8†</td>
<td>10.5 ± 0.6†</td>
<td>15.8 ± 1.0</td>
</tr>
<tr>
<td>Fenprostalene</td>
<td>6.1 ± 1.8*</td>
<td>17.1 ± 1.1</td>
<td>10.8 ± 0.9†</td>
<td>10.9 ± 1.0†</td>
<td>16.9 ± 1.2</td>
</tr>
<tr>
<td>cAMP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE2</td>
<td>5.5 ± 0.3</td>
<td>5.2 ± 0.4</td>
<td>4.7 ± 0.2</td>
<td>4.5 ± 0.4</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>Butaprost</td>
<td>5.1 ± 0.7</td>
<td>5.0 ± 0.9</td>
<td>5.3 ± 0.7</td>
<td>5.4 ± 0.7</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>M&amp;B28,767</td>
<td>-1.1 ± 0.4</td>
<td>-3.0 ± 0.8</td>
<td>-2.3 ± 0.3</td>
<td>-2.5 ± 0.4</td>
<td>-2.9 ± 0.2</td>
</tr>
<tr>
<td>PGE2 + AH23848B</td>
<td>5.3 ± 0.3</td>
<td>4.7 ± 0.4</td>
<td>4.9 ± 0.4</td>
<td>5.0 ± 0.3</td>
<td>5.1 ± 0.2</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of four to six experiments, each performed in duplicate. Newborn animals were treated for 48 hours with saline or COX inhibitors, as described in the Methods section. Adults were untreated. Concentrations of all agents were 1 μM, except for AH23848B, which was 30 μM. The net production of IP3 and cAMP stimulated by the PGs and PG analogues was corrected for basal (unstimulated) synthesis of IP3 and cAMP. Basal IP3 synthesis in newborn and adult tissues was 2.6 ± 0.6 pmol/mg and 3.7 ± 0.9 pmol/mg protein per minute, respectively, and basal cAMP production was 39 ± 1.2 pmol/mg protein/min and 4.8 ± 0.5 pmol/mg protein per minute, respectively. Negative values represent a net reduction in synthesis.

IP3, inositol 1,4,5-trisphosphate; cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; VSA, valeryl salicylate; PG, prostaglandin.

* P < 0.01 compared with all other values in the same row.
† P < 0.05 compared with other values in the same row.

**Receptors**

Receptors was immediately increased to values between those observed in saline-treated newborns and adults. Correspondingly, the proportion of EP2 receptors was reduced by COX inhibitors to values approaching those of adults (Table 3).

Thus, because COX inhibitors caused an increase in the total number of EP receptors (Table 2), results indicate that COX inhibitors induced a marked rise in EP1 and EP3 receptor density (Bmax X EP proportion) to values that approach those in adults, but there were marginal changes in EP2 density.

**IP3 and cAMP Production in Retinal Microvessels**

Treatment of newborn animals with ibuprofen, valeryl salicylate, and DuP697 caused a significant increase in the effects of PGF2α, fenprostalene (an FP agonist), PGE2, and 17-phenyl trinor PGE2 (an EP1 agonist) on IP3 production by retinal microvessels (Table 4). AH6809 (an EP1 antagonist) nearly abolished IP3 production by these PGs and analogues, suggesting that almost all IP3-induced production is caused by EP1 stimulation. AH6809 did not modify effects of PGF2α on IP3 synthesis (data not shown). Consistent with its upregulation of EP3 receptors, ibuprofen caused a greater increase in IP3 production than did selective COX inhibitors. In contrast, PGE2 and butaprost (an EP2 agonist) caused similar stimulation of cAMP production that was unaffected by treatments with COX inhibitors (Table 4). Concordant with the absence of EP3 in retinal vasculature, AH23848B (an EP3 antagonist) did not affect cAMP production by PGE2. Also, M&B28,767 (an EP3 agonist) did not elicit changes in IP3 or cAMP formation in retinal vasculature, as previously reported. An increase in basal cAMP production using 1 mM NaF was equally unaffected by M&B28,767 (data not shown). Basal IP3 production in newborn and adult tissues was 2.6 ± 0.6 pmol/mg protein/min and 3.7 ± 0.9 pmol/mg protein/min, respectively; basal cAMP production was 3.9 ± 1.2 pmol/mg protein/min and 4.8 ± 0.5 pmol/mg protein/min in newborn and adult tissues, respectively.

**Retinal Vasomotor Response to EP and FP Receptor Agonists**

Retinal vasorestriction induced by PGF2α, fenprostalene (an FP agonist), PGE2, 17-phenyl trinor PGE2 (an EP1 agonist), and M&B28,767 (an EP3 agonist) was significantly increased (P < 0.05; two-way analysis of variance factoring for agonist concentration and treatment group) in newborn pigs treated with valeryl salicylate, DuP697, and NS398 (NS398 not shown), and to a greater extent in animals treated with ibuprofen (Fig. 5).

Piglets that received ibuprofen showed maximum retinal vasorestriction similar to that of adults: For instance, the maximum response to PGE2 was 22% ± 3% in ibuprofen-treated newborns and 22% ± 3% in adults. In contrast, the effects of butaprost (an EP2 agonist) were not modulated by COX inhibitors and did not differ from those in adult tissues (Fig. 5).

The reduced response of saline-treated newborns and the upregulation of constrictor responses in ibuprofen- and DuP697-treated newborns were specific to constrictor PGs and their analogues, because 1 μM phenylephrine (maximum response) elicited comparable constriction in vessels from saline-treated newborns, ibuprofen-treated newborns, and untreated adult animals (22% ± 2%, 23% ± 4%, and 23% ± 3%, respectively; n = 3 in each group).

**Effects of Selective COX Inhibition on RBF Autoregulation**

We sought to determine whether the increase in retinal microvascular constrictor-coupled EP and FP receptors after inhibition of COX-1 or COX-2 (Tables 2, 4; Fig. 5) is associated with
Figure 5. Vasomotor responses to prostaglandin E2, 17-phenyl trinor PGE2 (a prostaglandin receptor [EP] agonist), butaprost (an EP2 agonist), M&B 28,767 (an EP3 agonist), PGF2a, and fenprostalene (an FP agonist) in retinal microvessels from adult pigs treated with saline (•) and from newborn pigs treated intravenously with saline (•), 40 mg/kg ibuprofen (ibu; O), 80 mg/kg valeryl salicylate (Δ), or 5 mg/kg DuP697 (△) every 8 hours for 48 hours. Dilator responses to butaprost were determined on vessels preconstricted with 50 nM phenylephrine (~50% of maximum constriction). Vessel diameters were recorded using a video camera and quantified by an image analyzer. Vasomotor responses in newborn animals treated with 5 mg/kg NS398 were also tested and were similar to responses of those treated with DuP697 (data not shown). Values are mean ± SEM of four to five experiments; positive values refer to vasoconstriction and negative ones to dilation. *P < 0.05 in saline-treated newborn compared with all other groups; † P < 0.05 in valeryl salicylate- or DuP697-treated newborn compared with all other groups (by two-way analysis of variance).

enhanced autoregulatory RBF response to an acute rise in perfusion pressure in the newborn animal. Basal RBF was 0.37 ± 0.03 ml/min per gram in saline-treated pigs and did not differ significantly from that of animals treated with COX inhibitors. In saline-treated animals, RBF increased linearly with MBP (r = 0.93–0.99; P < 0.001; Fig. 6). In contrast, treatment of animals with nonselective COX inhibitor ibuprofen, selective COX-1 inhibitor valeryl salicylate, and selective COX-2 inhibitor DuP697 prevented the change in RBF as a function of MBP (r = 0.01–0.12; P ≥ 0.4). Regression coefficients for saline-treated animals differed significantly from those treated with COX inhibitors (P < 0.05; regression equality test).

**DISCUSSION**

The retina of the newborn contains high activities of COX-1 and COX-2. Increased concentrations of PGs in the newborn have been suggested to result in a downregulation of PG receptors and their functions in brain. However, the types of PG receptors in brain and retinal vessels differ. Among the different PGE2 receptors, EP1 predominates in brain, whereas in retina EP1, EP2, and EP3 are all present. Furthermore, EP receptors do not uniformly exhibit homologous regulation, as observed for EP3b compared with EP3a. Other PG receptors, such as PGE2, that exhibit homologous downregulation in certain tissues in vitro are not regulated by their ligand in vivo during the perinatal period. We therefore investigated the role of high perinatal PGs in the downregulation of FP and EP receptor density and receptor-coupled functions in retinal vasculature and the relative roles of COX-1 and COX-2 in this process. The present results provides strong evidence that COX-1 and COX-2 contribute to the downregulation of retinal microvascular PGE2 and PGF2α receptors and EP1, EP2, and FP receptor-coupled vasoconstrictor functions and to the limited RBF autoregulatory response to increased perfusion pressure in the newborn. In contrast, EP2 levels are minimally affected by PG levels during development.

The effects of selective COX inhibitors on PG levels in retina are consistent with the nearly equal contribution of COX-1 and COX-2 in catalyzing PG synthesis in this tissue. The decreases in retinal PGE2 and PGF2α levels after the inhibition of COX-1 and COX-2 were associated with an increase in retinal vascular PGE2 and PGF2α receptor densities. Inhibition of COX-1 and COX-2 by ibuprofen reduced concentrations of PGs in retina to levels found in adults, and caused an upregulation of PGE2 and PGF2α receptors to levels comparable to...
those in adults. In contrast, the alterations in PG concentrations and PGF$_{2\alpha}$ and PGF$_{2\alpha}$ receptor densities after selective COX-1 or COX-2 inhibition were intermediate compared with the combined inhibition of both these isozymes. These observations were also found to apply to the PGE$_2$ receptor subtypes: Ibuprofen caused an increase in EP$_1$ and EP$_3$ (and a relative decrease in EP$_2$) receptor proportions equivalent to that of adults, whereas selective COX-1 and COX-2 inhibition caused intermediate changes in EP receptor number. These observations are in accordance with our previous findings, which showed that COX inhibitor-induced upregulation of PGE$_2$ and PGF$_{2\alpha}$ receptors is specifically modulated by PGs,$^9$,$^{10}$ and is supported by the inverse correlation between PG levels and receptor density observed in the present study.

We have previously shown that retinal microvessels contain the FP, EP$_1$, EP$_2$, and EP$_3$ receptors (but not EP$_4$).$^8$ In porcine retinal parenchyma the proportions of EP receptor subtypes on retinal microvessels and IC$_{50}$ for the selective ligands are consistent with those previously reported$^4$; only EP$_3$ has been detected in retinal parenchyma.$^{25}$ Prostaglandin F$_{2\alpha}$ receptor and EP, are linked to IP$_3$ production, EP$_2$ to cAMP generation. Stimulation of EP$_3$ does not cause a significant change in either of these messengers in either of these messengers in retinal vasculature.$^9$ An increase in IP$_3$ is usually associated with vasoconstriction,$^{26}$ a rise in cAMP with vasodilation.$^{27}$ The vasoconstrictor response to stimulation of the EP$_3$ isoform on retinal microvessels$^6$ seems to be coupled to other transduction pathways, but these mechanisms remain to be defined in this tissue.$^6$,$^{28}$ Retinal microvessel IP$_3$ production in response to PGF$_{2\alpha}$, fenprostalene (FP agonist), and 17-phenyl trinor PGE$_2$ (EP$_1$ agonist) was significantly increased in newborns treated with COX-1 and COX-2 inhibitors. Increased retinal vasoconstriction in-

**FIGURE 6.** Retinal blood flow (RBF) as a function of increases in mean blood pressure (MBP) in newborn pigs treated intravenously with saline, 40 mg/kg ibuprofen, 5 mg/kg DuP697, or 80 mg/kg valeryl salicylate every 8 hours for 48 hours. Each animal was subjected to stepwise acute increases in MBP preset at 90 mm Hg, 105 mm Hg, and 125 mm Hg, which varied by 5 mm Hg or less on different animals. Baseline MBP was 69 ± 4 mm Hg in all animals and was unaffected by the treatments. The faint lines correspond to the regressions for individual animals and the thick lines the mean regressions for all animals in the group. In saline-treated animals, RBF increased linearly with MBP ($r = 0.93-0.99; P < 0.001$). In animals treated with ibuprofen, valeryl salicylate, and DuP697, RBF remained constant across the MBP range studied ($r = 0.01-0.12; P ≥ 0.4$).
duced by FP and EP<sub>1</sub> agonists in newborn animals treated with COX inhibitors was related to increments in FP and EP, receptor density and IP<sub>3</sub> synthesis. These data provide additional evidence that PG synthesis by COX-1 and COX-2 contribute to the downregulation of retinal microvessel FP, EP<sub>1</sub>, and EP<sub>3</sub> receptors and receptor-mediated functions in the perinate.

In contrast to FP, EP<sub>1</sub>, and EP<sub>3</sub>, EP<sub>2</sub> receptor density and its function in retinal vasculature do not seem to be significantly modulated by the changes in PG concentrations that occur during development. These findings differ from those in brain, where EP<sub>2</sub> is regulated by PGE<sub>2</sub> levels. However, in choroid EP<sub>2</sub> is not homologously regulated. Therefore, it is likely that tissue specificity, which accounts for various factors, may cause discrepancies observed in the regulation of EP<sub>2</sub>. It thus appears that constrictor-coupled PGF<sub>2α</sub> and PGE<sub>2</sub> receptors are prone to homologous regulation during maturation, whereas the dilator-coupled receptor EP<sub>2</sub> is not significantly affected (Figs. 4, 5; Tables 3, 4); this inference concurs with age-dependent decrease in the relative proportion of EP<sub>2</sub> receptors (Table 3).

It has been proposed that insufficient RBF autoregulatory vasocostractor response to increased perfusion pressure in the newborn animal is, to a significant extent, caused by minimal retinal vasoconstrictor activity of PGs. Particularly PGE<sub>2</sub> and PGF<sub>2α</sub>. In the present study, an upregulation of the constrictor EP and FP receptors and receptor-coupled vasoconstrictor functions after COX inhibition was found to be associated with enhanced RBF autoregulatory response to acute hypertension in the newborn. Valeryl salicylate, DUP697, and ibuprofen prevented an increase in RBF at MBP of 125 mm Hg or more (the uppermost MBP studied; Fig. 6). The relative efficacy of selective versus nonselective COX inhibitors in raising the upper MBP limit of RBF autoregulation could not be determined, because steady MBP values higher than 130 mm Hg to 140 mm Hg are difficult to reproduce in the neonate (personal observation). Nonetheless, the results indicate that the RBF autoregulation range in the newborn could be enhanced by inhibition of COX-1 or COX-2, consistent with upregulation of PGE<sub>2</sub> and PGF<sub>2α</sub> receptors and receptor-coupled IP<sub>3</sub> production and vasoconstriction. However, the association between EP and FP receptor upregulation and enhanced RBF autoregulation can only be inferred. A direct link would require selective antagonists to these receptors in retinal vasculature, which, other than for EP<sub>1</sub>, are currently unavailable.

In summary, COX-1 and COX-2 exert a significant and comparable effect in the downregulation of constrictor PGE<sub>2</sub> and PGF<sub>2α</sub> receptors and receptor-coupled functions in retinal microvasculature of the newborn, according to the equivalent roles of these isozymes in PG synthesis in retina. Consequently, inhibition of either COX isoform causes comparable reduction in PG levels in retina and an increase in EP<sub>1</sub>, EP<sub>3</sub>, and FP receptor densities and receptor-coupled constriction in the retinal vasculature of the newborn, associated with enhanced RBF autoregulation at high perfusion pressures, which approaches that the adult. Observations also provide new information on the physiologic role of the inducible COX-2 enzyme during development.

**Acknowledgment**

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**References**


