

# Expression of Prostaglandin PGE<sub>2</sub> Receptors under Conditions of Aging and Stress and the Protective Effect of the EP2 Agonist Butaprost on Retinal Ischemia

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**PURPOSE.** To localize different prostaglandin E<sub>2</sub> receptors in rat retinas of varying age, deduce how they are affected by acute stress insult, and determine whether the negative effect of ischemia/reperfusion is attenuated by the EP2 agonist butaprost.

**METHODS.** Ischemia was induced by the elevation of intraocular pressure. Butaprost was injected intravitreally immediately after ischemia. Standard methods were used for recording of electroretinograms (ERGs) and processing of immunohistochemistry. Extracts of whole retinas were analyzed for specific proteins by Western blotting or by RT-PCR for defined mRNAs.

**RESULTS.** The localization of different EP receptor types is similar in retinas of all aged rats. However, differences exist in the monomer/dimer ratios in retinas of different age. Acute stress insult (48 hours after ischemia) affects the ratio of monomer/dimer of all EP receptor types and increases EP2 and EP3 immunoreactivities in Müller cells of the adult retina. Ischemia and 5 to 7 days of reperfusion to the retina caused the normal ERG and the localization of nNOS and ChAT immunoreactivities to be affected. Certain proteins and mRNAs were lowered in content, whereas other proteins and mRNAs were upregulated. In addition, specific optic nerve proteins were drastically reduced. Most of these changes induced by ischemia/reperfusion were significantly blunted by butaprost.

**CONCLUSIONS.** All subtypes of EP receptors exist primarily in the inner retina at different ages, but their monomer/dimer ratios vary. Stress affects the monomer/dimer ratio and EP2 and EP3 immunoreactivities in Müller cells. Butaprost injected intravitreally significantly blunts the detrimental influence of ischemia/reperfusion to the retina. (*Invest Ophthalmol Vis Sci* 2009;50:3238–3248) DOI:10.1167/iov.08-3185

Inflammation plays a part in the pathogenesis of various central nervous system disorders and is mediated at least in part by prostaglandins (PGs), which are produced through the cyclooxygenase (COX) pathway. Significantly, the inhibition of

COX has been shown to reduce cell injury from ischemia, Alzheimer disease, amyotrophic lateral sclerosis, and inflammatory neuronal death,<sup>1–7</sup> suggesting that specific PG agonists/antagonists might mimic the inhibition of COX. COX-deficient mice are known to be less susceptible to ischemia and NMDA-mediated neurotoxicity,<sup>8</sup> both processes implicated in the pathogenesis of glaucoma.<sup>9,10</sup>

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is secreted by various cells in response to inflammatory stimuli and elicits actions through binding to membrane-specific receptors (EP1–EP4 receptors).<sup>11,12</sup> The rat EP1 receptor and EP2 receptor consist of 405 and 357 amino acids with molecular masses of 42 kDa and 52 kDa, respectively.<sup>13,14</sup> The EP3 receptor has multiple spliced variants that encode proteins with a molecular mass between 40 and 45 kDa.<sup>15</sup> The EP4 receptor has a long intracellular C terminus and an intracellular third loop, giving a predicted molecular mass of 53 kDa.<sup>16</sup> All four receptors have the characteristic seven-hydrophobic-transmembrane segment architecture typical of G-protein-coupled receptors.<sup>12</sup> Stimulation of these receptors coupled to heterotrimeric guanosine triphosphate-binding proteins leads to the activation of adenylyl cyclase (EP2 and EP4) or phospholipase C (EP1 and EP3), resulting in the formation of cyclic adenosine monophosphate or in increased intracellular calcium concentrations.<sup>12,17</sup> Although EP2 and EP4 receptors share a common signaling pathway, butaprost is a specific agonist for EP2 receptors that subsequently contributed to the discovery of the EP4 receptor.<sup>18,19</sup> EP4 receptors have been linked to other signaling pathways, such as PI3K and PKA.<sup>20</sup>

EP receptor localization in the retina is restricted to studies carried out in porcine,<sup>21</sup> mouse,<sup>22</sup> and human retinas.<sup>22–24</sup> In the mouse retina, EP1 immunoreactivity is associated with blood vessels and the ganglion cell layer, whereas in the human retina it is also present in the inner nuclear layer. EP2 immunoreactivity in human and mouse retinas are in all the retinal layers and are prominent in the ganglion and nerve fiber layers.<sup>22,24</sup> EP3 receptor subtype is localized to most cell types of the porcine retina.<sup>21</sup> In the human retina, EP3 receptor immunoreactivity is associated with Müller cells and the nerve fiber layer<sup>24</sup> and is not detected in the mouse. EP4 receptor immunoreactivity in mouse and in human retinas is associated mainly with plexiform and ganglion cell layers. Relatively little is known about the functional roles for individual PGE<sub>2</sub> receptors in the retina, though Simminoff and Bito<sup>25</sup> demonstrated that intravitreal injection of PGE<sub>2</sub> affects the physiology of the flash electroretinogram. The only other study of which we are aware is that of Najarian et al.,<sup>26</sup> who reported that, unlike EP1 and EP3 agonists, the EP2 agonist butaprost, when administered intravenously, nullified, for example, hypoxia-induced changes in the b-waves of electroretinograms in newborn pigs.

There are two forms of COX, COX-1 and COX-2. COX-1 is constitutively present in many cell types and is involved in homeostatic functions, whereas COX-2 is undetectable in most cells but is readily inducible by inflammatory stimuli and mitogenic agents.<sup>27,28</sup> The activity of COX in the retina increases

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under certain conditions,<sup>29,30</sup> as in retinal PGE<sub>2</sub> content after injury.<sup>31,32</sup> COX-2 inhibitors reduce PGE<sub>2</sub> secretion in diabetic retinas<sup>30</sup> and attenuate the effects of ischemia to the retina.<sup>29</sup> It remains unknown whether defined PGE<sub>2</sub> agonists/antagonists function like selective COX-2 inhibitors at, for example, reducing the impact of an ischemic insult to the retina.

Impressive data by McCullough et al.<sup>3</sup> show that the EP2 agonist butaprost attenuates the effects of ischemia to the rat forebrain in situ and insults of NMDA and oxygen-glucose deprivation to slices of hippocampal tissue in vitro. These studies are supported by the report of Liu et al.,<sup>33</sup> who used a stroke animal model to show that the effect was enhanced in rats with a genetic lesion of EP2 receptors and confirmed that butaprost attenuated the influence of NMDA toxicity to hippocampal neurons in vitro. A number of additional studies on cell cultures<sup>34–37</sup> support the contention that butaprost functions as a neuroprotective agent, though one group<sup>38,39</sup> has reported that butaprost induces apoptosis and contributes to neuronal death in culture. Such studies highlight the importance of the impact of concentration, namely, the avoidance of potential cytotoxic concentrations in culture.

One aim of this study was to compare the expression of the various EP receptors in the rat retina derived from animals at different ages, including the stage before eye opening. Another aim was to determine how an acute stress insult to the retina caused by elevated IOP affects the expression of the different EP receptors in the adult rat retina. A final goal was to deduce whether butaprost could reduce dysfunction of the adult retina after an insult of ischemia/reperfusion. Ganglion cells are particularly susceptible to ischemia/reperfusion insult,<sup>40</sup> and any substance found to reduce the death process of ganglion cells could be of potential use in the treatment of diseases such as glaucoma, in which ganglion cell loss leads to blindness.

## MATERIALS AND METHODS

Animal procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Home Office in the United Kingdom. Rats were housed in a 12-hour light/12-hour dark cycle and were provided food and water ad libitum. Experiments were conducted on Wistar (postnatal [P] day 4, P8, P16, P24, and adult) rats. Adult animals were anesthetized with intramuscular injection of fentanyl/fluanisone (Hypnorm [0.3 mL/kg]; Janssen, Grove, UK), and diazepam (2.5 mg/kg; CP Pharmaceuticals Ltd., Wrexham, UK), and electroretinograms (ERGs) were recorded of both eyes.

### Induction of Retinal Ischemia

Ischemia was induced by the insertion of a 30-gauge needle, connected to a sterile saline reservoir at a certain height, into the anterior chamber of an eye to produce intraocular pressure of 120 mm Hg. Ischemia was confirmed by immediate whitening of the fundus and was maintained for 50 minutes. Normal body temperature was maintained and monitored during ischemia and during the recovery period before transfer to animal house conditions. Animals were killed after 2 days (defined as an acute stress insult) or 7 days (defined as an ischemia/reperfusion insult). In rats killed 7 days after ischemia, 5  $\mu$ L butaprost (4 nmol) or vehicle was injected into the vitreous humor directly after ischemia.

### Electroretinography

Electroretinograms (ERGs) from both eyes were recorded 2 to 3 days before and 5 days after ischemia in animals subjected to 7 days of reperfusion. Animals were initially dark adapted for at least 6 hours before the recording of their flash ERGs. Pupils were dilated with 1% tropicamide (Smith and Nephew Pharmaceuticals Ltd., Romford, UK) and 2.5% phenylephrine (Smith and Nephew Pharmaceuticals Ltd.). A platinum electrode was placed in contact with the cornea, and a

reference electrode was placed through the tongue; a grounding electrode was attached to the scruff of the neck. All procedures were performed in dim red light, and the rats were kept warm during and after the procedure. Responses to a 2500 cd/m<sup>2</sup> white light flash (1–2 seconds, 0.1 Hz) from a photic stimulator (model PS33-plus; Grass Instrument Divisions, West Warwick, UK) were amplified and averaged (1902 Signal Conditioner/1401 Laboratory Interface; CED, Cambridge, UK). Amplitude of the a-wave was measured from the baseline to the maximum a-wave trough, and that of the b-wave was measured from the maximum a-wave trough to the maximum b-wave peak. Data were analyzed by one-way ANOVA and Bonferroni test, and  $P < 0.05$  was considered significant.

### RT-PCR Procedure

Levels of GAPDH, Thy-1, NF- $\kappa$ B, PARP, GFAP, caspase-3, and caspase-8 mRNA were determined in the retina from rats 7 days after ischemia using semiquantitative RT-PCR, as described previously.<sup>41,42</sup> Briefly, total RNA was isolated, and first-strand cDNA synthesis was performed on 2  $\mu$ g DNase-treated RNA. Individual cDNA species were amplified in a reaction containing aliquots of cDNA, PCR buffer, MgCl<sub>2</sub> (5 mM for NF- $\kappa$ B, caspase-3, and caspase-8; 4.5 mM for GAPDH; and 4 mM for all other primers), deoxynucleotide triphosphates, relevant primer pairs, and DNA polymerase. Reactions were initiated by incubation at 94°C for 10 minutes and PCR (94°C, 15 seconds; 52°C, 55°C or 56°C, 30 seconds; 72°C, 30 seconds) performed for a suitable number of cycles, followed by a final extension at 72°C for 3 minutes. Interexperimental variation was avoided by performing all amplifications in a single run. PCR reaction products were separated on 1.2% agarose gels with ethidium bromide for visualization. Relative abundance of each PCR product was determined by quantitative analysis of digital photographs of gels using analysis software (Labworks; UVP Products, Upland, CA). Sequences and annealing temperatures of the primers used (Gibco Life Technologies, Paisley, Scotland) are shown in Table 1.

Before semiquantitative amplification of the experimental samples, the amount of cDNA in all the samples was equalized. In addition, the optimal conditions (e.g., Mg<sup>2+</sup> concentration, annealing temperature) for each set of primers were determined. Subsequently, cycle-dependent reactions were performed for each mRNA species to determine the linear range of detection by ethidium bromide. Once the linear range was established, PCRs were performed at the lowest cycle number that gave a reliably detectable product. To minimize variability, duplicate runs were performed for each mRNA amplified, and the data were averaged.

For assessment of the levels of the various mRNAs in the retina, all values were normalized to that of the housekeeping gene *GAPDH*, which thus acted as an internal standard to correct for any variations in RNA isolation or cDNA synthesis.

### Electrophoresis and Western Blotting

Optic nerves (6–8 mm long) or retinas from rats 7 or 2 days, respectively, after ischemia were sonicated in 100  $\mu$ L homogenization buffer (20 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 50  $\mu$ g/mL aprotinin, 50  $\mu$ g/mL leupeptin, and 50  $\mu$ g/mL pepstatin A), to which an equal volume of sample buffer (62.5 mM Tris/HCl, pH 7.4, containing 4% sodium dodecyl sulfate, 10% glycerol, 10% mercaptoethanol, and 0.002% bromophenol blue) was added. Optic nerve and retinal samples were boiled for 3 minutes, and an aliquot was analyzed for total protein content using a bicinchoninic acid protein kit (Sigma-Aldrich, St. Louis, MO).

Retinal proteins from tissues 7 days after ischemia were separated and isolated from RNA according to the standard tri-reagent procedure. Proteins from individual retinas were then solubilized in 200  $\mu$ L homogenization buffer/protease inhibitor solution, to which was added an equal volume of sample buffer.

Equal amounts of protein were fractionated by electrophoresis with the use of 10% polyacrylamide gels containing 0.1% SDS, as

TABLE 1. Primer Sequences Used for RT-PCR Analysis of mRNAs

Primers	Sequences	Annealing Temperature (°C)	Accession Number
Caspase-3	GTTAACACGAGTGAGGATGTG ACTGGCTGCCCTCAGTTCCTGTGC	52	BC081854
Caspase-8	ACTGGCTGCCCTCAAGTTCCTGTGC TCCCTCACCATTTCCTCTGGGCTGC	60	AF279308
GAPDH	CATCAAGAAGGTGGTGAAGCAGG CCACCACCCTGTTGCTGTAGCCA	56	AF106860
GFAP	ATCCCGCGCCTCTCCCTGTCTC GCTTCATCCGCTCCTGTCTGT	55	U03700
NF-L	ATGCTCAGATCTCCGTGGAGATG GCTTCGCAGCTCATTCTCCAGTT	52	AF031880
Opsin	CAGTGTTCATGTGGGATTGACT ATGATTGGGTTGTAGATGGAGG	52	Z46957
Thy-1	CGCTTTATCAAGTCTTACTC GCGTTTTGAGATATTTGAAGGT	52	X03150

described by Laemmli.<sup>43</sup> Proteins were transferred to nitrocellulose, and blots were incubated for 3 hours at room temperature with various primary antibodies. Detection was then performed with appropriate biotinylated secondary antibodies. The final nitrocellulose blots were developed with a 0.016% wt/vol solution of 3-amino-9-ethylcarbazole (AEC) in 50 mM sodium acetate (pH 5.0) containing 0.05% (vol/vol) Tween-20 and 0.03% (vol/vol) H<sub>2</sub>O<sub>2</sub>. The colorimetric reaction was stopped with 0.05% sodium azide solution, and the blot was scanned at 800 dpi (Perfection 1200u scanner; Epson, Torrance, CA). Quantitative analysis of the detected proteins was performed with analysis software (UVP Products).

Primary antibodies used were as follows: rabbit anti-EP1, anti-EP2, anti-EP3, and anti-EP4 polyclonal antibodies (1:500; Cayman, Ann Arbor, MI); mouse anti-PARP-1 (1:1000, Becton-Dickinson, Oxford, UK); mouse anti-actin (1:2000; Chemicon, Edinburgh, UK); tubulin and NF-L (1:2000; Chemicon); and primary rabbit antibody to GFAP (1:2000; Dako Scientific, Glostrup, Denmark).

### Immunohistochemistry

Retinas were freshly dissected from pup and adult retinas and fixed in 2% paraformaldehyde (0.1 M phosphate buffer) for 30 minutes at room temperature, after which 10- $\mu$ m frozen retinal sections were cut. Subsequently, sections were thawed and incubated overnight at 4°C with rabbit anti-EP1, anti-EP2, anti-EP3, or anti-EP4 (all at 1:100; Cayman) polyclonal antibody, mouse anti-choline acetyltransferase monoclonal (ChAT; 1:100; Sigma, Poole, UK) antibody, or sheep anti-neuronal nitric oxide synthase (nNOS; 1:2000; gift from Piers Emson, University of Cambridge, Cambridge, UK) antibody. Development was with appropriate secondary antibodies conjugated to Cy3 or fluorescein. Sections were photographed with epifluorescence optics.

## RESULTS

### Expression of EP Receptors in Retinas at Different Ages

EP1 receptor immunoreactivity was associated with cells in the ganglion cell layer at all retinal stages (P4, P8, P16, P24, and adult; Fig. 1A). Immunoreactivity was also present in the inner part of the neuroblastic layer and the inner plexiform layer in the P4 retina. The outer plexiform layer became apparent only at P8. At P8 and all subsequent retinal stages, EP1 receptor immunoreactivity had a similar distribution; it was present in the ganglion cell layer and appeared as three bands in the inner plexiform layer. Immunoreactivity was also associated with putative amacrine cell bodies in the

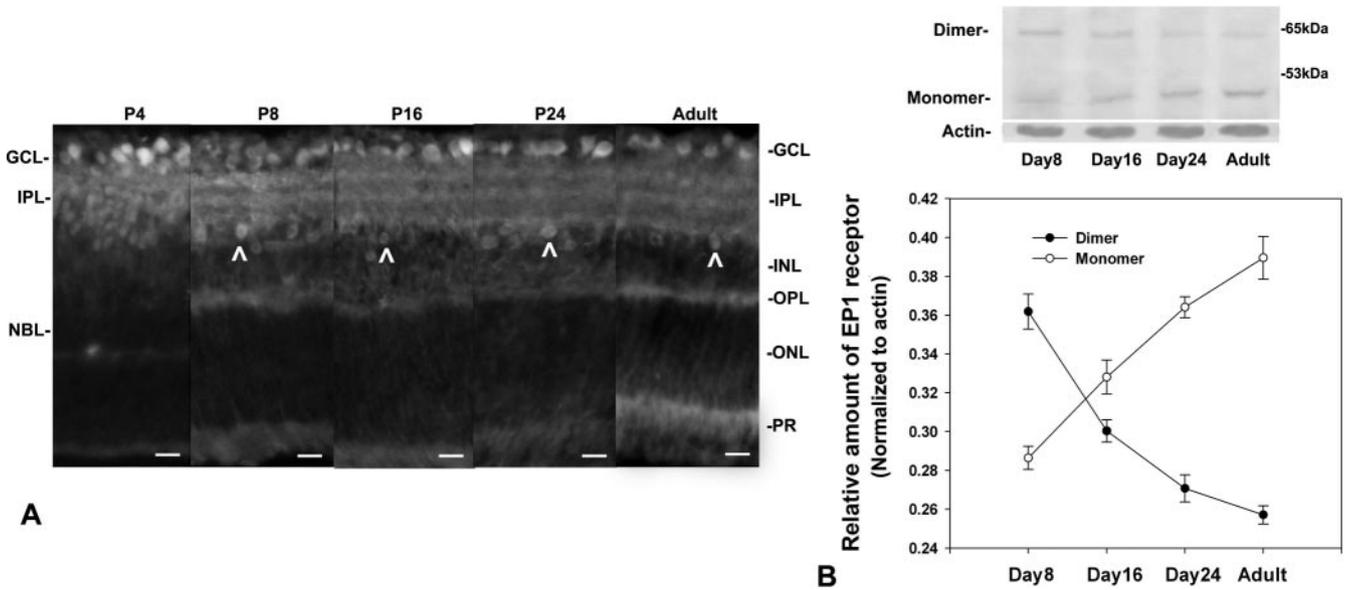
inner nuclear layer, inner segments of the photoreceptor, and outer plexiform layer.

Staining for EP2 receptor immunoreactivity in the P4 retina was weak compared with the retinas at other stages (P8, P16, P24, and adult) and was associated with the ganglion cell layer and, to a lesser extent, with the inner portion of the neuroblastic layer (Fig. 2A). EP2 receptor immunoreactivity was similarly distributed in retinal sections from P8 to adult and was associated strongly with cells in the ganglion cell layer and less strongly with cells in the inner nuclear layer and the inner segments of photoreceptors.

EP3 receptor immunoreactivity was associated primarily with the inner plexiform, ganglion cell, and nerve fiber layers of the retina at all stages of development (Fig. 3A). Moderate EP3 receptor immunoreactivity appeared to be located to putative amacrine cells at all stages. Positive staining appeared to be associated with the outer plexiform layer at all stages.

EP4 receptor immunoreactivity was associated primarily with varying numbers of cells in the ganglion cell and inner nuclear layers in retinas of rats of different ages. EP4 immunoreactivity appeared to be absent from the inner plexiform and outer plexiform layers (Fig. 4A).

Western blot analysis shows each EP receptor existed as two bands corresponding to homodimers (monomers) and heterodimers (dimers; Figs. 1B, 2B, 3B, 4B). The lower molecular weight band for the EP1 receptor had a molecular weight of 42 kDa. For the EP2, EP3, and EP4 receptors, the lower molecular weight bands were approximately 53 kDa. The higher molecular weight band for the EP1 receptor had a molecular weight of 65 kDa. For the EP2, EP3, and EP4 receptors, the higher molecular weight bands were approximately 98 kDa. The relative amount of dimer/monomer varied for EP receptor type and retina age. There was a clear shift in the ratio dimer/monomer for EP1 receptors in retinas from P8 to adult so that the monomer dominated in the adult but not at P8. The EP2 receptor, in contrast, existed primarily as a monomer in retinas of all ages, with the dimer just detectable at P8 but slightly upregulated in the adult retina, in which there was also a slight downregulation of the monomer. Unlike the EP2 receptor, the EP3 receptor existed primarily as a dimer in P16, P24, and adult retinas, whereas in the P8 retina, approximately equal amounts of dimer and monomer were present. The EP4 receptor also existed primarily as a dimer and at all retinal stages. The EP4 receptor monomer existed in low amounts at all retinal stages.

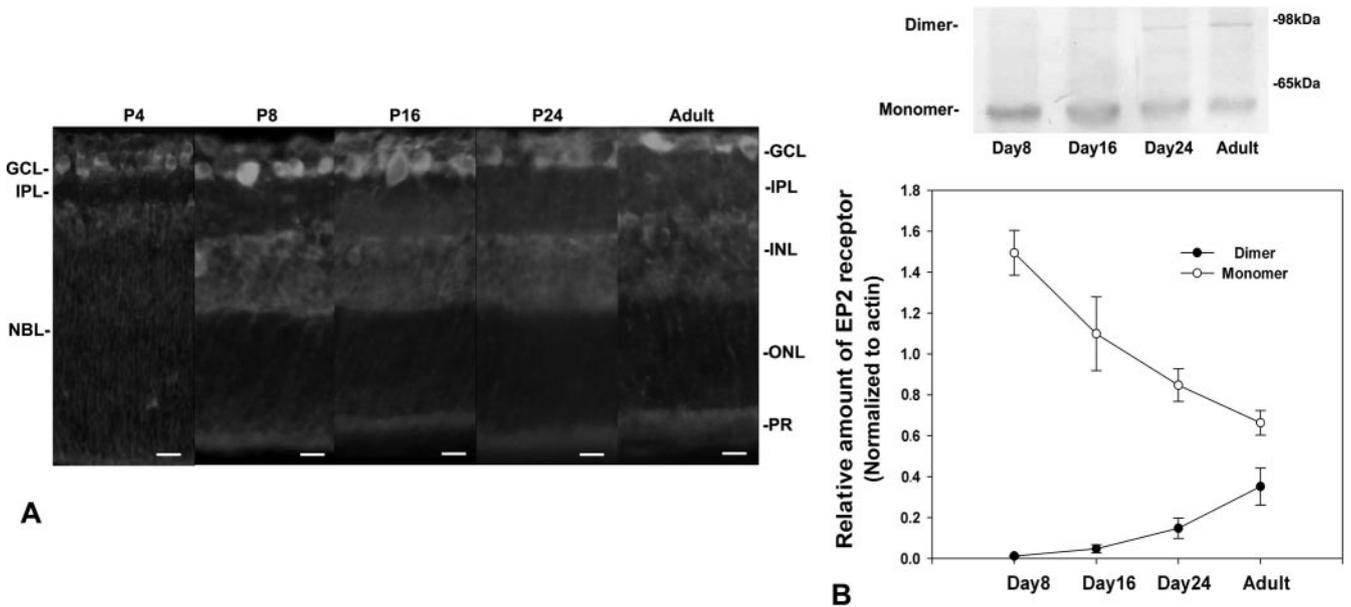


**FIGURE 1.** (A) Localization of EP1 receptor immunoreactivity in retinal sections from P4, P8, P16, P24, and adult rat retinas. Positive staining in P4 retina is associated with the ganglion cell layer (GCL) and inner plexiform layer (IPL). Similar patterns of staining occurred in retinas from P8 to adult, associated with the GCL, IPL (as three bands), and putative amacrine cells (*arrowheads*) in the inner nuclear layer (INL). OPL, outer plexiform layer; ONL, outer nuclear layer. Some positive staining was also associated with the inner segments of the photoreceptors (PR). Scale bars, 20  $\mu$ m. (B) Western blot studies show EP1 receptors exist as a dimer and a monomer in retinas from all aged rats, though the ratios varied when related to actin. Results are mean  $\pm$  SEM of three separate experiments.

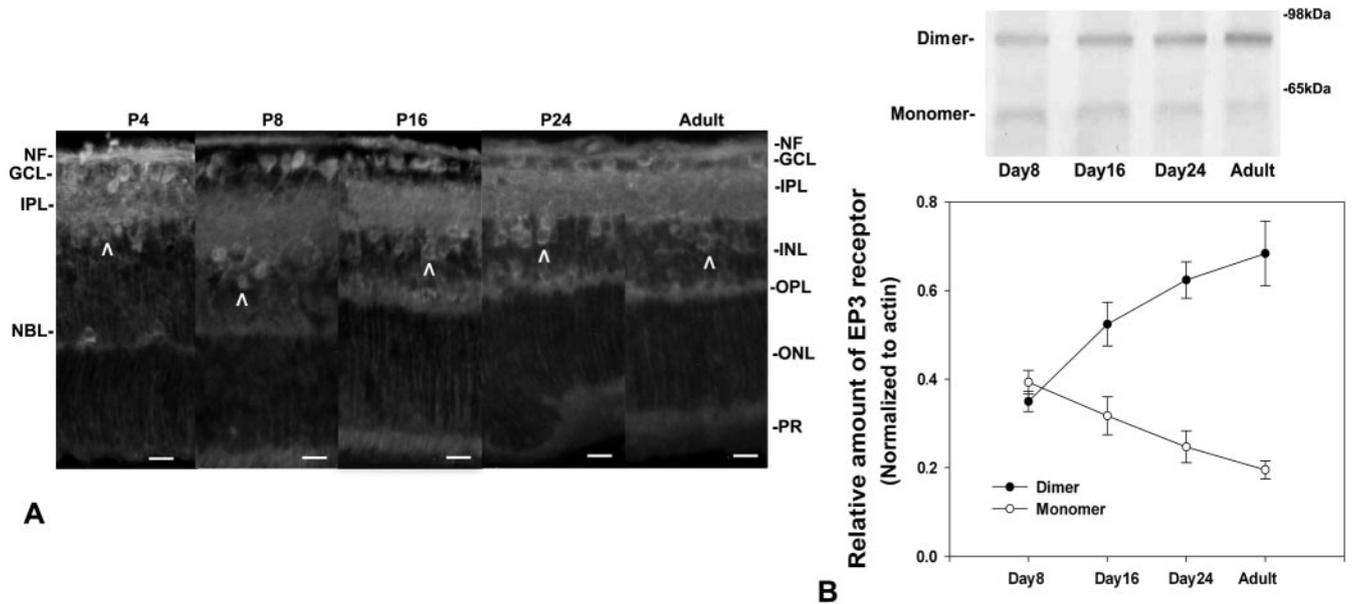
**Effect of Stress-Induced Ischemia on the Expression of Adult EP Receptors**

In these studies, ischemia was initiated by raising the intraocular pressure (120 mm Hg for 50 minutes) and then analyzing the retina 48 hours later. This insult does not result in any morphologic changes to the retina<sup>40</sup> and was, therefore, defined as a stress insult. Stress-induced ischemia caused some change in the

localization of certain EP immunoreactivities in the adult retina (Fig. 5). The most obvious effect of the insult on EP1 immunoreactivity was a distinct enhanced expression of immunoreactivity located to the outer segments of the photoreceptors. In contrast, the most obvious effect of the insult on the localization of EP2 and EP3 receptor immunoreactivities was strong staining of Müller cells. Stress-induced ischemia did not appear to have any obvious effect of the localization of EP4 receptors.



**FIGURE 2.** (A) Localization of EP2 receptor immunoreactivity in rat retinas of different ages. Positive staining in P4 retina is associated with the ganglion cell layer (GCL) and the inner portion of the neuroblastic layer (NBL). EP2 receptor immunoreactivity is similarly distributed in retinas from P8 to adult, associated with the GCL, the inner nuclear layer (INL), and inner segments of the photoreceptors (PR). Scale bars, 20  $\mu$ m. IPL, inner plexiform layer; ONL, outer nuclear layer. (B) Western blot studies show EP2 receptors exist as a dimer and a monomer in retinas from all aged rats, with the ratios varying and the monomer always predominating, when related to actin. Results are mean  $\pm$  SEM of three independent experiments.

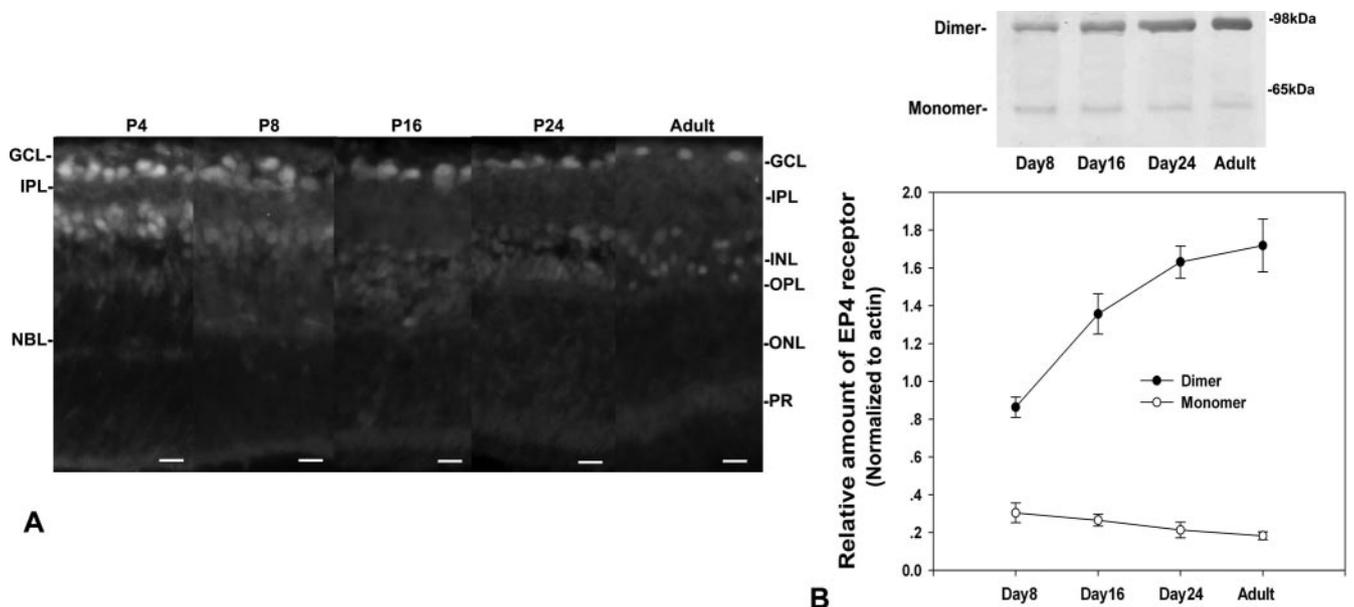


**FIGURE 3.** (A) Localization of EP3 receptor immunoreactivity in rat retinas of different ages. Positive EP3 receptor immunoreactivity is associated with the ganglion cell layer (GCL), nerve fiber (NF) layer, inner plexiform layer (IPL), and cell bodies (*arrowheads*) in the neuroblastic layer (NBL) in the P4 retina. This pattern of EP3 receptor immunoreactivity was also associated with retinas from P8 to adult, when it was clear that certain amacrine cells (*arrowheads*) stained positively. There was also an indication that EP3 immunoreactivity was associated with the outer plexiform layer (OPL) at all stages. Scale bars, 20  $\mu$ m. INL, inner nuclear layer. (B) Western blot studies show EP3 receptors to exist as a dimer and a monomer in retinas from all aged rats with the ratios varying and where the dimer predominated except at the P8 stage when related to actin. Results are mean  $\pm$  SEM of three independent experiments.

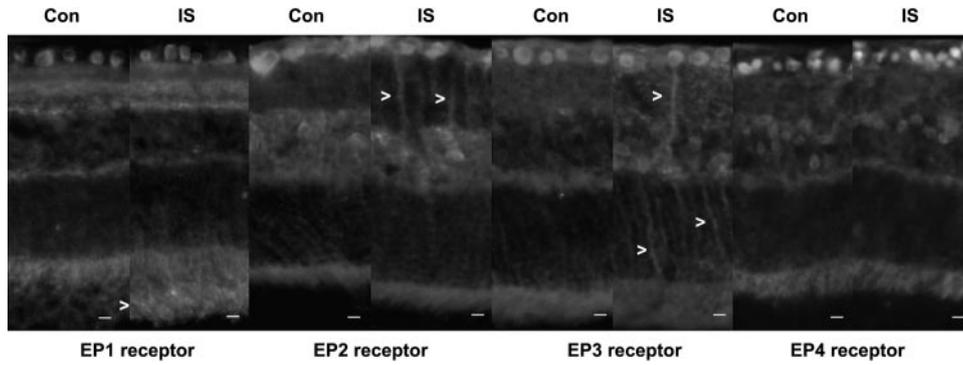
Western blot analysis also revealed that stress-induced ischemia affected the ratio of dimer/monomer of all EP receptors (Fig. 6). In the EP1 receptor, the insult caused an upregulation of the dimer and a downregulation of the monomer. For EP2, EP3, and EP4 receptors, the insult caused a downregulation of dimers and an upregulation of monomers.

### Effect of Butaprost on Retinal Ischemia/Reperfusion

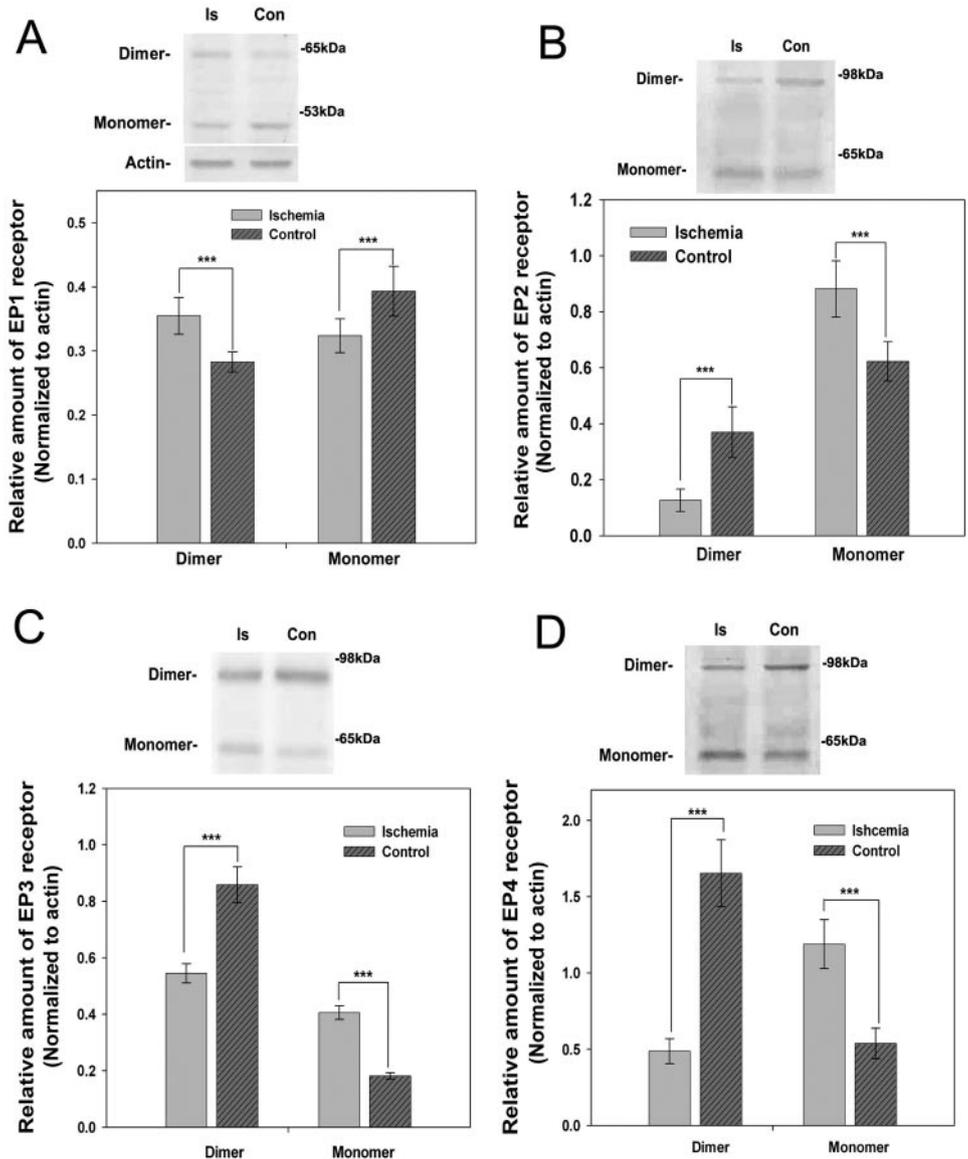
Elevated intraocular pressure-induced ischemia for 50 minutes followed by 5 days of reperfusion caused, on average, 49% and 78% reductions in the a- and b-wave amplitudes, respectively, in



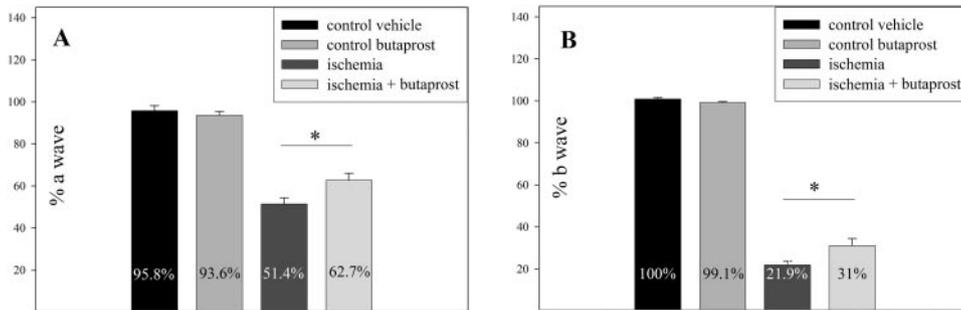
**FIGURE 4.** (A) Localization of EP4 receptor immunoreactivity in rat retinas of different ages. Retinal sections from the P4 stage show intense staining in the ganglion cell layer (GCL) and the inner part of the neuroblastic layer (NBL). Diffuse staining is also associated with other parts of the NBL. Retinal sections from P8 to adult show EP4 receptor immunoreactivity to be associated primarily with the ganglion cell layer (GCL) and to a lesser extent with the inner nuclear layer (INL). IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PR, photoreceptors. (B) Western blot studies show EP4 receptors to exist as a dimer and a monomer in retinas from all aged rats, with the ratios varying and where the dimer always predominates when related to actin. Results are mean  $\pm$  SEM of three independent experiments. Scale bars, 20  $\mu$ m.



**FIGURE 5.** These sections show the localization of different types of EP receptor immunoreactivities in the adult retina (Con) and after ischemia-induced stress insult (IS). Stress (IS) causes little change in the localization of EP4 receptor immunoreactivity. In the case of EP1 receptor immunoreactivity, stress (IS) causes the outer segments of the photoreceptor (*arrowhead*) to stain positively. Although control (Con) retinas show little EP2 and EP3 receptor immunoreactivities in Müller cells, this is not the case after a stress-induced insult. Stress (IS) clearly causes a significant upregulation of EP2 and EP3 receptor immunoreactivities in Müller cells. Scale bars, 20  $\mu$ m.



**FIGURE 6.** The influence of ischemia-induced stress insult (ischemia [Is]) on the ratio of dimer/monomer proteins for different types of EP receptors. Stress (Is) caused a clear shift between dimer and monomer for all EP receptor types relative to actin. Results are mean  $\pm$  SEM of three independent experiments ( $***P < 0.001$ ;  $n = 6$ ; one-way ANOVA and Bonferroni test).



49% and 78%, respectively. However, when eyes were treated with butaprost, reductions in a- and b-wave amplitudes were significantly lower ( $P < 0.05$  by one-way ANOVA and Bonferroni test)—37% and 69%, respectively. Error bars represent  $\pm$  SEM, where  $n = 10$ .

vehicle-treated rats ( $n = 10$ ). In animals treated with butaprost ( $n = 10$ ), reductions in the a- and b-wave amplitudes were approximately 37% and 69%, respectively. The differences in amplitudes of the a- and b-waves between the two groups of animals were modest but statistically significant. Neither butaprost nor its vehicle affected the ERG recordings of untreated (nonischemic) eyes (Fig. 7).

Normal ChAT immunoreactivity associated with amacrine cells appeared as two clearly defined bands in the inner plexiform layer, with cell bodies on either of the sides (Fig. 8). However, ChAT immunoreactivity was almost eliminated in vehicle-treated retina 7 days after ischemia but was only partially affected in rats treated in this way but injected with butaprost. Similarly, nNOS-immunoreactivity in control retinas appeared as three large diffuse bands in the inner plexiform layer and as occasional cell bodies, mainly in the inner nuclear layer. This pattern of nNOS-staining was less affected in rats given ischemia and butaprost rather than vehicle (Fig. 8).

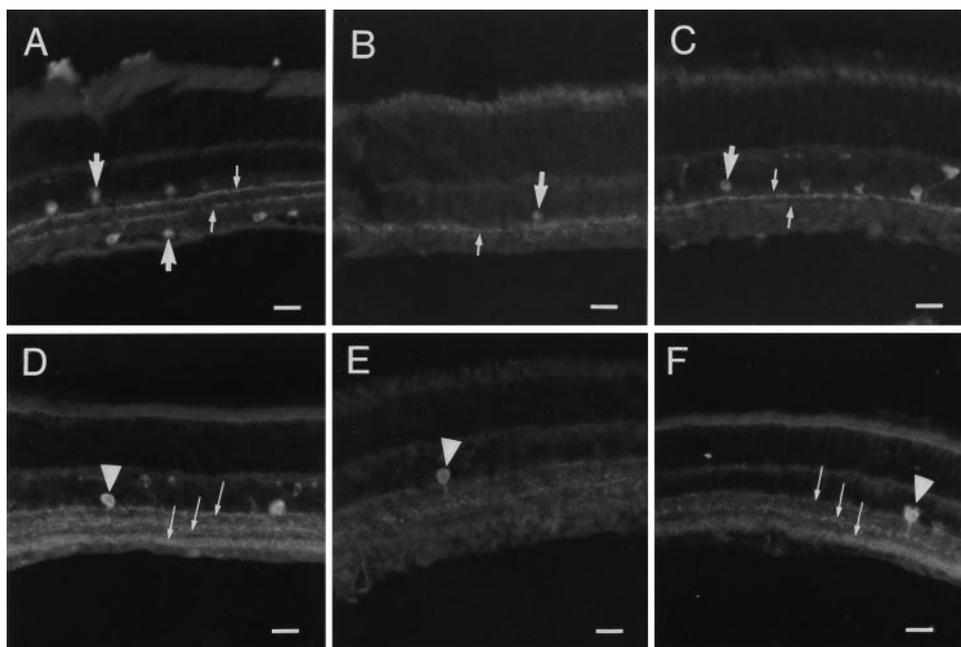
Ischemia for 50 minutes followed by 7 days of reperfusion caused a change in the content of selected retinal and optic nerve proteins, as shown in Figures 9 and 10. These data showed that ischemia/reperfusion statistically decreased the protein level of NF-L but increased the level of PARP and GFAP proteins in the retina (Fig. 9). Importantly, the effects of ischemia/reperfusion on the protein level of NF-L, PARP, and GFAP

were all significantly blunted by butaprost treatment. Because ganglion cell axons in the optic nerve contain NF-L and tubulin proteins, their levels were also determined and were found to decrease by approximately 58% and 50%, respectively, after ischemia/reperfusion (Fig. 10). This level of decrease in optic nerve NF-L and tubulin proteins induced by ischemia/reperfusion was significantly nullified in rats treated with butaprost (Fig. 10).

Ischemia for 50 minutes followed by 7 days of reperfusion also caused changes in the content of selected mRNAs. mRNA levels (normalized to GAPDH) of Thy-1 and NF-L in vehicle-treated rat retinas were significantly reduced, whereas opsin mRNA was unaffected (Fig. 11). In contrast, the mRNA levels of caspase-3, caspase-8, and GFAP were clearly elevated. When butaprost rather than vehicle was injected into the eye after ischemia, the reductions of retinal Thy-1 and NF-L were significantly attenuated. Butaprost treatment did not, however, affect the increased mRNA levels of caspase-8, caspase-3, or GFAP caused by ischemia.

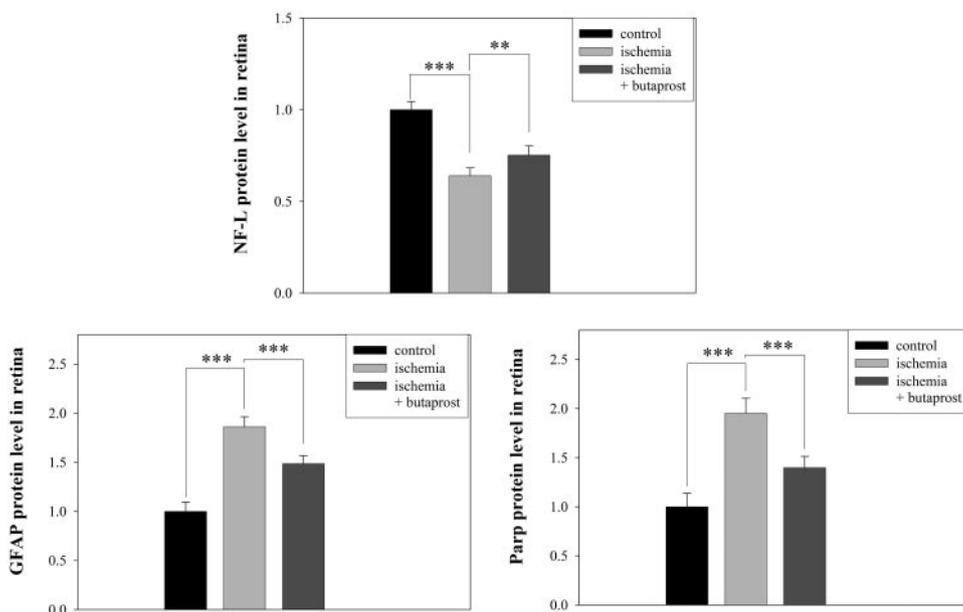
## DISCUSSION

Good evidence exists to suggest that in glaucoma retinal microglia and astrocytes are activated and release substances that can induce the apoptotic death of ganglion cells.<sup>44-46</sup> Acti-



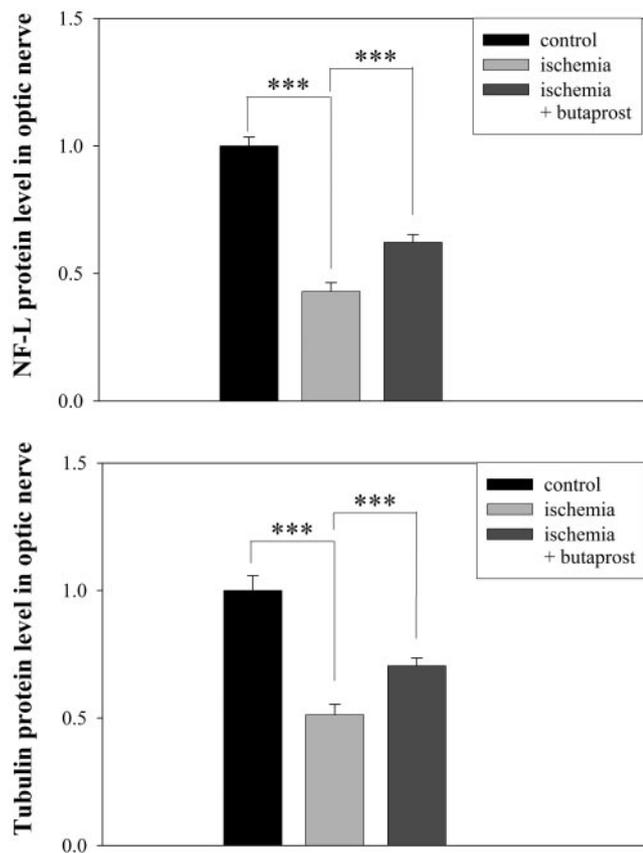
**FIGURE 8.** In the control retina (A) ChAT immunoreactivity is located as a tram line of immunoreactivity in the inner plexiform layer (*small arrows*) and cell bodies on either side (*large arrows*). In contrast, nNOS is located to three distinct bands of immunoreactivity in the inner plexiform layer (*small arrows*) and some cell bodies in the inner nuclear layer (*arrowhead*) in the control retina (D). Ischemia/reperfusion causes a significant reduction of ChAT (B) and nNOS (E) immunoreactivities that are reduced in rats treated with butaprost (C, F, respectively). Scale bars, 45  $\mu$ m

**FIGURE 9.** NF-L (A), GFAP (B), and PARP (C) proteins in retina relative to actin in eyes given ischemia and injected with vehicle (ischemia) or butaprost (ischemia + butaprost) in comparison with control eyes. Data are mean, with error bars indicating  $\pm$  SEM;  $n = 19$  in each case. \*\*\* $P < 0.01$ ; \*\* $P < 0.05$ .



vated microglia can potentially release cytotoxic substances such as reactive oxygen radicals, proteases, and cytokines and proinflammatory prostanoids such as PGE<sub>2</sub>.<sup>11,47</sup> PGE<sub>2</sub> is the natural agonist for EP1, EP2, EP3, and EP4 receptors. Laboratory studies suggest that activation of individual EP receptors

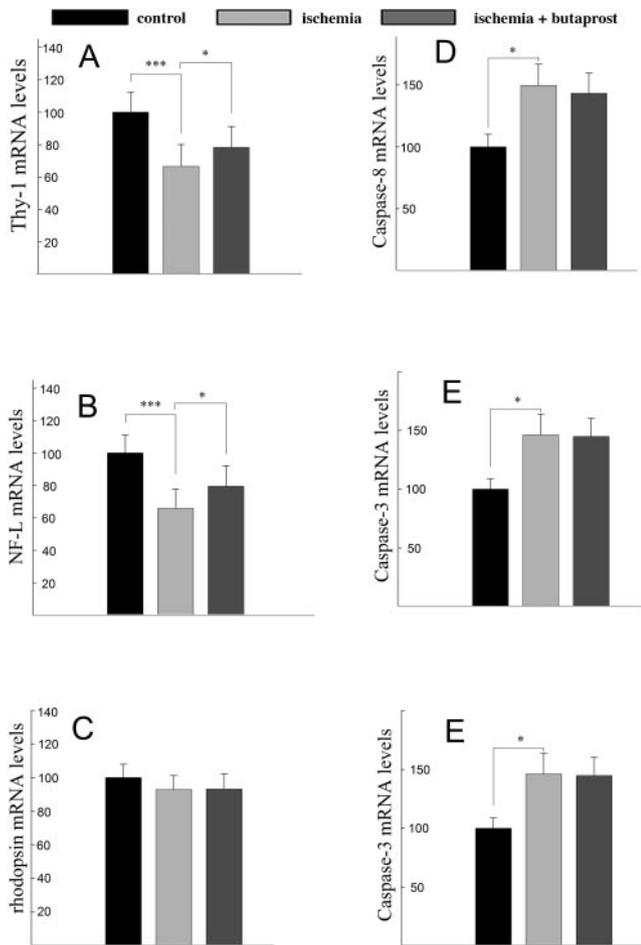
can counteract or enhance a detrimental stimulus, depending on the cell type. It appears that EP2 and EP4 agonists generally protect neurons from defined insults,<sup>3,4,34,48,49</sup> whereas EP1 receptor agonists generally enhance a detrimental effect to neurons.<sup>11,50-53</sup>



**FIGURE 10.** NF-L (A) and tubulin (B) proteins in optic nerves relative to actin in eyes given ischemia and injected with vehicle (ischemia) or butaprost (ischemia + butaprost) in comparison with control eyes. Data are means with error bars indicating  $\pm$  SEM;  $n = 19$  in each case. \*\*\* $P < 0.01$ .

Because the localization of individual EP receptor types in the rat retina was unknown, we determined their localization particularly in relation to their association with ganglion cells. We did not restrict our studies to the adult rat retina because certain EP receptors may have age-related functions in the retina, especially before the stage of eye opening. The studies reported by Najarian et al.<sup>26</sup> may be relevant because the EP2 agonist butaprost was found to have a positive effect on the electroretinogram in newborn pigs subjected to hypoxia. Moreover, they showed that PGE1 antagonists, diclofenac, and ibuprofen intensified hypoxia-induced changes in VEPs and ERGs that were attenuated with a PGE analogue (dimethyl-PGE2) and the EP2 agonist butaprost. Major differences in the localization of the different EP receptor immunoreactivities were, however, not apparent in P8 to adult rat retinas (Figs. 1-4). At P4, before eye opening, the retina was poorly developed and generally did not exhibit a distinctive localization for a specific type of EP receptor. Our only clear conclusion is that EP1, EP2, EP3, and EP4 immunoreactivities are particularly associated with neurons in the ganglion cell layer of retinas at all ages of the rat (P4-adult), as reported to occur for mouse, human, and porcine retinas.<sup>21,22,24</sup> Our Western blot studies suggested that EP receptor type expression varies in rat retinas of different ages because the ratio of dimer/monomer was not constant. It is likely that subtle differences in the localization and functional states of individual EP receptor types probably occurred in the retinas of rats of different ages, but for this to be revealed, a more detailed analysis is necessary. Moreover, G-protein-coupled receptors often exist as homodimers or heterodimers and interact physically to affect their receptor function, and this could vary in retinas at different ages.

The clear expression of EP2 and EP3 (but not EP1 and EP4) receptor immunoreactivity to Müller cells after stress-induced ischemia may be of significance. EP3 receptors have been shown in porcine and human retinal Müller cells,<sup>24</sup> but to our knowledge this is the first report to show these cells to clearly express EP2 receptors. Retinal Müller cells are implicated in a variety of functions that support neuronal survival,<sup>54</sup> and up-



**FIGURE 11.** Thy-1 (A), NF-L (B), rhodopsin (C), caspase-8 (D), caspase-3 (E), and GFAP (F) mRNAs in retina relative to GAPDH mRNA in eyes given ischemia and injected with vehicle (ischemia) or butaprost (ischemia + butaprost) in comparison with control eyes. Data are means with error bars indicating  $\pm$  SEM;  $n = 12$  in each case. \*\*\* $P < 0.01$ ; \* $P < 0.05$ .

regulation of their EP2 and EP3 receptors caused by stress suggests their involvement in the maintenance of neuronal/glial interactions. Our ischemia/reperfusion data indicated that this was indeed the case because direct activation of Müller cell EP2 receptors caused by intravitreal injection of butaprost resulted in an enhancement of neuronal function.

The influence of stress-induced ischemia to the retina also revealed modifications in the expression of monomeric and dimeric forms of the individual EP receptor types. All EP receptors, therefore, appear to be affected by stress-induced ischemia, indicated by changes in their monomer/dimer forms. This is not necessarily revealed by changes in their localization, as documented for EP4 receptors. The changes seen in the monomer/dimer ratios of individual EP receptors caused by stress-induced ischemia suggest that the functional state of adult EP receptors might revert to what occurs in the nonadult retina. Another possibility is that stress-induced ischemia results in oxidative stress that affects plasma membranes, thus altering the ratio of monomer/dimer forms of individual EP receptors.

The ganglion cell layer of the retina contains predominantly ganglion cells in which EP2 receptor immunoreactivity is strongly located (Figs. 2, 5). Moreover, EP2 receptor immunoreactivity is associated with Müller cells, as demonstrated clearly after ischemia-induced stress to the retina (Fig. 5).

Injection of the EP2 specific agonist butaprost into the rat eye is, therefore, likely to affect EP2 receptors located to retinal ganglion and Müller cells. Such an effect might be the reason for the attenuation of an insult of ischemia/reperfusion to the retina. Ischemia, induced by the elevation of intraocular pressure above systolic blood pressure for 50 minutes, reduces the blood supply to the retina, indicated by whitening of the fundus. This insult results in partial damage to the retina. For example, a- and b-wave amplitudes of electroretinograms are significantly reduced 5 days after ischemia. The a-wave provides information about the photoreceptors, whereas the b-wave provides information on the physiology of the ON-bipolar and Müller cells.<sup>55</sup> In our study, reductions in the a- and b-wave amplitudes caused by ischemia/reperfusion were significantly lower in animals treated with butaprost.

The inner retina in particular is affected by ischemia/reperfusion, as can be demonstrated by morphologic and immunohistochemical studies<sup>40</sup> (Fig. 8). It must be emphasized that for sections of different retinas to be compared, they must be of the same eccentricities, which is almost impossible to achieve. By comparing a number of sections from approximately the same retinal areas in a masked fashion, however, it is possible to make an informed judgment. In this study, we concluded that nNOS and ChAT immunoreactivities are greatly reduced after ischemia/reperfusion and are clearly less affected when butaprost is intravitreally administered. Significantly, EP2 immunoreactivity is associated with putative amacrine cells of the rat retina (Fig. 2), although we have no evidence to suggest that nNOS and/or ChAT amacrine cells express EP2 receptors.

Analysis of the levels of retinal mRNA and proteins associated with the retina and optic nerve (Figs. 9–11) agrees with immunohistochemistry and electroretinogram data. The degree of ganglion cell death after ischemia/reperfusion was determined by relating the protein (NF-L) and mRNA (Thy-1 and NF-L) levels of ganglion cell-specific markers with total retinal actin protein and GAPDH mRNA levels, respectively. The validity of this method for obtaining information on ganglion cell death is documented in various publications.<sup>41,42,56</sup> Results show that ischemia/reperfusion causes a dramatic effect on ganglion cells, leading in a large decrease in NF-L mRNA/protein and Thy-1 mRNA levels. This effect is significantly blunted by the injection of butaprost.

NF-L and tubulin proteins are present in ganglion cells. Analysis of these proteins in the optic nerve provides a measure of ganglion cell viability.<sup>57,58</sup> Ischemia/reperfusion clearly reduces the levels of tubulin and NF-L proteins in the optic nerve that are significantly blunted by butaprost (Fig. 10). Thus ischemia/reperfusion causes a reduction in retinal mRNA/proteins and optic nerve proteins associated with ganglion cells that is nullified by the intravitreal injection of butaprost. Because ganglion cells appear to contain EP2 receptors, the possibility exists that their direct activation by butaprost attenuates the detrimental influence of ischemia/reperfusion.

A number of studies have shown that retinal neuronal death after ischemia/reperfusion occurs by apoptosis.<sup>40,59</sup> This was confirmed in the present study, where it was shown that caspase-3 mRNA, caspase-8 mRNA, and PARP protein are upregulated in the retina after ischemia/reperfusion. It is generally thought that during apoptosis, caspase-3, caspase-8, and PARP are upregulated.<sup>60–63</sup> The finding that butaprost significantly attenuates the upregulation of PARP protein caused by ischemia/reperfusion provides evidence that butaprost attenuates the process of apoptosis. It is difficult to explain the negative effect of butaprost on the upregulation of caspase-3 and caspase-8 mRNA caused by ischemia/reperfusion. One possible explanation is that ischemia/reperfusion caused too great an elevation of caspase3/8 mRNA and that the positive affect of butaprost was insufficient to be detected.

GFAP is associated with retinal glia (astrocytes and Müller cells), and when these cells are activated, protein expression is upregulated.<sup>54</sup> It remains unknown exactly why this occurs. In the present study, GFAP protein and mRNA were upregulated after ischemia/reperfusion, consistent with other studies that used immunohistochemistry to show that GFAP immunoreactivity in retinal Müller cells is enhanced after ischemia/reperfusion.<sup>54,64,65</sup> Significantly, butaprost attenuates ischemia/reperfusion-induced upregulation of Müller cell GFAP protein, and EP2 receptors are located to these cells.

This study provides clear evidence that stimulation of EP2 receptors in the retina attenuates the influence of an insult caused by ischemia/reperfusion and that EP2 receptors are particularly located to retinal ganglion and Müller cells. We therefore conclude that agonists affecting EP2 receptors might be beneficial for treating retinal degenerating diseases<sup>9,66</sup> in which ganglion or Müller cell functions are affected. Given that retinal cells also contain other types of PGE<sub>2</sub> receptors, future studies should investigate whether specific EP1, EP3, and EP4 agonists or antagonists counteract the influence of ischemia/reperfusion as effectively as the EP2 agonist butaprost. A combination of specific PGE<sub>2</sub> receptor agonists/antagonists may counteract the negative influence of released PGE<sub>2</sub> from activated microglia, as is thought to occur in glaucoma,<sup>9,66</sup> and may provide a way forward in the treatment of this disease.

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