Increased Prostaglandin E2 (PGE2) Levels in Proliferative Diabetic Retinopathy, and Correlation with VEGF and Inflammatory Cytokines

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PURPOSE. We determined vitreous levels of prostaglandin E2 (PGE2), VEGF, and 15 other cytokines in diabetic and nondiabetic patients undergoing vitrectomy.

METHODS. Of 26 eyes of 26 patients enrolled consecutively, 13 eyes underwent vitrectomy for complications related to proliferative diabetic retinopathy, and the other 13 for epiretinal membrane, macular hole, vitreous opacities, or dislocated intraocular lens. Undiluted vitreous samples were taken at the time of surgery and frozen immediately at –80°C, and later analyzed for PGE2, VEGF, and 15 other cytokines.

RESULTS. PGE2 levels were 53% higher in diabetic eyes. Mean ± standard deviation PGE2 levels were 25.11 ± 11 pg/mL and 16.40 ± 7 pg/mL in diabetic and nondiabetic eyes, respectively (P < 0.03). Mean ± standard deviation VEGF levels were 2225 ± 3798 pg/mL and 66 ± 185 pg/mL in diabetic and nondiabetic eyes, respectively (P < 0.001). Other cytokines, including eotaxin-1, growth related oncogene (GRO), interleukin (IL)-6, IL-8, interferon-γ-inducible protein of 10 kDa (IP-10), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α), and platelet-derived growth factor-AA, also were elevated significantly in diabetic eyes. A significant correlation was seen between PGE2 levels and IP-10 and VEGF (P = 0.04).

CONCLUSIONS. PGE2 levels are significantly higher in the vitreous of patients with complications from proliferative diabetic retinopathy, and correlate with IP-10 and VEGF. The results of our study suggest that PGE2 may have a pathogenic role in diabetic retinopathy and implicates a potential therapeutic role for nonsteroidal anti-inflammatory drugs. (ClinicalTrials.gov number, NCT01609881.) (Invest Ophthalmol Vis Sci. 2012; 53:3906–3911) DOI:10.1167/iovs.12-10410

Diabetic retinopathy (DR) is the most frequent cause of legal blindness among working-age individuals in developed countries.1 Preventable measures include lowering of high blood pressure and strict control of blood glucose,2,3 but a growing body of scientific evidence supports a pathogenic role of inflammation.4 In support of this, a number of pro-inflammatory cytokines are elevated consistently in the vitreous of patients with advanced stages of DR.5–7 Prostaglandins (PGs) are an important class of inflammatory mediators that are biosynthesized from arachidonic acid by cyclooxygenase enzyme. Within the eye, PGs disrupt the blood–ocular barrier, increase vasodilation, and facilitate leukocyte migration.8 Consequently, their inhibition has favorable effects on intraocular inflammation.9 In experimental and animal models, PGs induce VEGF production,10,11 with subsequent development of vascular leakage and retinal neovascularization.12 In addition, PG levels are elevated significantly in animal models of DR13 and progression of retinopathy can be prevented or delayed with PG inhibitors.14–16

To our knowledge, there are no published studies demonstrating elevated vitreous PG levels in patients with DR. Therefore, the primary aim of our study was to analyze and compare PG levels in the vitreous of patients with and without diabetes. Our secondary aim was to determine the relationship of PG with VEGF and 15 other inflammatory cytokines that have been implicated in the pathogenesis of DR.

METHODS

Study Population

The Vanderbilt University Institutional Review Board approved this study, and all patients gave informed consent before enrollment. The study complied with all aspects of the Health Insurance Portability and Accountability Act, and was conducted in accordance with the tenets of the Declaration of Helsinki. The trial is registered in the public domain at clinicaltrials.gov (Identifier NCT01609881).

All adult patients, aged 18 years or more, undergoing pars plana vitrectomy (PPV) were eligible for inclusion. Exclusion criteria consisted of previous PPV in the study eye, prior intravitreal injection (anti-VEGF, corticosteroid, and so forth) within 3 months, co-existent macular, retinovascular, or ocular inflammatory disease (including age-related macular degeneration, retinal venous or arterial occlusive disease, uveitis, and so forth), history of ocular trauma, aphakia, presence of an anterior chamber intraocular lens, and previous enrollment of the fellow eye. Any use of topical and/or systemic anti-inflammatory medications, including corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs), was documented carefully. Only diabetic eyes with active neovascularization at the optic nerve or elsewhere at the time of surgery were enrolled.

Sample Collection

The recruitment objective was 26 eyes of 26 patients, because this was the maximum number of samples that could be tested in triplicates on
Table 1. Baseline Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>PDR Group</th>
<th>Control Group</th>
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</thead>
<tbody>
<tr>
<td>Mean age ± SD (y)</td>
<td>56 ± 10</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>Lens status</td>
<td>( \text{Phakic} )</td>
<td>( \text{Pseudophakic} )</td>
</tr>
<tr>
<td>Prior treatments</td>
<td>Anti-VEGF</td>
<td>PRP</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Systemic use of</td>
<td>Aspirin</td>
<td>NSAIDs</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Corticosteroids</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Immunosuppression</td>
<td>0</td>
</tr>
<tr>
<td>Topical use of</td>
<td>PG analog</td>
<td>Prednisolone acetate</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

A single 96-well Multiplex testing plate after accounting for standards and controls. Approximately 0.5 to 1.0 mL of undiluted vitreous was obtained at the beginning of surgery using the vitreous cutter inserted in the mid-vitreous cavity before active infusion was started. The vitreous sample then was placed immediately on ice and aliquoted into smaller tubes, and stored at \(-80^\circ\text{C}\). There were no observed complications.

Measurement of PG Levels

Vitreous samples were thawed and prostaglandin E2 (PGE2) levels were analyzed using the Prostaglandin E2 Monoclonal EIA Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions. In brief, serial dilutions of standards (7.8–1000 pg/mL) were prepared. Standards, controls, and vitreous samples were added to individual wells, followed by PGE2 acetylcholinesterase tracer and PGE2 monoclonal antibody. The plates were incubated overnight at \(4^\circ\text{C}\). The plates were washed, and Ellman’s Reagent and tracer were added to each well. The plates were covered and incubated at room temperature with gentle shaking for 60 minutes. The plates were read, and the data were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA) and the Cayman Chemical Company EIA triple workbook. Cross-reactivity between PGE2 and prostaglandin F2\(\alpha\) is \(<0.01\%\).

Millipore Multiplex Kit

A microparticle bead-based multiplex assay was used to measure inflammatory cytokines in accordance with manufacturer’s instructions and as described previously. In brief, vitreous samples were thawed and analyzed using the Millipore Multiplex Human Cytokine/Chemokine Panel (Millipore Corporation, Billerica MA). Samples were analyzed for the following 16 cytokines: Eotaxin-1, FL-3 ligand (FL-3L), growth related oncogene (GRO), interferon-gamma (IFN-\(\gamma\)), interleukin (IL)-1\(\beta\), IL-6, IL-8, IL-10, IL-12 (p40), interferon-\(\gamma\)-inducible protein of 10 kDa (IP-10), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor alpha (TNF-\(\alpha\)), VEGF, regulated on activation normal T expressed and secreted (RANTES), platelet-derived growth factor (PDGF)-AA, and PDGF-AB/BB. Serial dilutions of standards (3.2–10,000 pg/mL) were prepared. Standards (duplicates), controls (duplicates), and vitreous samples (triplicates) were added to individual wells on a 96-well plate containing the above 16 beads and incubated at \(4^\circ\text{C}\) overnight on a shaker. The plate was washed, detection antibody was added to each well, and the plates were incubated for 60 minutes on a shaker at room temperature. Streptavidin-phycocerythrin was added to each well and the plates were incubated for 30 minutes on a shaker at room temperature. The plate was washed, sheath fluid was added, and the samples were analyzed using a flow cytometry-based instrument (Bio-Plex Array Reader; Bio-Rad, Hercules, CA). Analysis software (Bio-Plex Manager 4.1; Bio-Rad) converted fluorescence readings to concentration (pg/mL) using a calibration curve generated from the standards. The quality of the fit of the standard curve to the data was good, as judged by graphical representation, and the criteria that all standards between the upper and lower limits of detection had back-calculated concentrations within 70% of the expected values.

Statistical Analysis

Descriptive statistics, including mean and SD, were calculated for case characteristics. Group comparisons were performed with the Wilcoxon rank-sum test using 2-sided analysis. Categorical characteristics were analyzed using a Fisher’s exact test. Spearman’s rank order correlation was used to determine correlations between cytokines among all 26 patients and also to assess the association of age with vitreous PGE2 levels. A \( P < 0.05 \) was considered statistically significant. Cytokines were analyzed if at least half of the eyes had detectable values and those with values below limits of detection were assigned a numerical value of 0 pg/mL for statistical analysis. All vitreous samples were tested in triplicates and the mean value was used for statistical analysis.

RESULTS

Study Population

The study population consisted of 26 eyes of 26 patients divided evenly into patients with and without diabetes. Of the 26 eyes, 13 underwent PPV for complications due to proliferative diabetic retinopathy (PDR), including vitreous hemorrhage (VH) and/or tractional retinal detachment (TRD). The remaining 13 eyes without diabetes (control) underwent PPV for epiretinal membrane (ERM; 9 eyes), macular hole, (MH; 2 eyes), vitreous opacities (1 eye), and dislocated intraocular lens (1 eye). In both populations, patients were enrolled consecutively from January 2012 to May 2012.

Baseline characteristics of both groups are shown in Table 1. Mean age in the PDR and control groups was 56.08 ± 10.20 years and 68.54 ± 5.70 years, respectively (\(P < 0.001\)). All 13 PDR eyes had VH and 9 of 13 had a TRD. Two eyes had a remote history (>3 months) of intravitreal anti-VEGF injection and seven (53.8%) had prior panretinal photocoagulation (PRP). Eleven of 13 (85%) PDR eyes were phakic, but only 5 of 13 (39%) control eyes were phakic (\(P = 0.04\)).

There were no significant differences between the PDR and control groups in regard to systemic use of aspirin, NSAIDs, or other immunosuppressive agents (Table 1). One patient in the control group was on hydroxychloroquine for rheumatoid arthritis. Topical prostaglandin F2\(\alpha\) analogs were used in three patients in the PDR group and one in the control group. Two eyes in the PDR group were on topical prednisolone acetate for neovascular glaucoma.

Prostaglandin Levels

In the PDR and control groups, the concentration of PGE2 was \(25.11 \pm 6.90 \text{ pg/mL} \) and \(10.20 \pm 5.20 \text{ pg/mL} \), respectively (\(P < 0.03\), Fig. 1). Overall, PGE2 levels were 53% higher in the PDR group.

Inflammatory Cytokines

Results of cytokine testing are shown in Table 2. Among the 16 cytokines tested, eotaxin-1, GRO, IL-6, IL-8, IP-10, MCP-1, TNF-\(\alpha\), VEGF, and PDGF-AA all were significantly higher in PDR
Impact of PRP

PGE$_2$ levels were modestly higher in PDR eyes without prior PRP (27.80 pg/mL) versus eyes with prior PRP (24.09 pg/mL), but the results did not reach the level of statistical significance (Table 3). Of the nine detectable cytokines, all were higher in eyes that did not have prior PRP, and IL-8 ($r = 0.04$) and PDGF-AA ($r = 0.04$) reached statistical significance.

Relationship of PGE$_2$ with Other Cytokines

PGE$_2$ levels among all 26 eyes correlated significantly with two cytokines: IP-10 ($r = 0.40$, $P = 0.04$, Fig. 2) and VEGF ($r = 0.41$, $P = 0.04$, Fig. 3). PGE$_2$ levels did not correlate significantly with eotaxin-1, GRO, IL-6, IL-8, MCP-1, TNF-alpha, and PDGF-AA. In contrast, VEGF correlated significantly with all detected cytokines: eotaxin-1 ($r = 0.50$, $P = 0.01$), GRO ($r = 0.42$, $P = 0.03$), IL-6 ($r = 0.60$, $P = 0.001$), IL-8 ($r = 0.73$, $P < 0.001$), IP-10 ($r = 0.55$, $P = 0.004$), MCP-1 ($r = 0.63$, $P < 0.001$), TNF-$\alpha$ ($r = 0.45$, $P = 0.02$), and PDGF-AA ($r = 0.73$, $P < 0.001$).

DISCUSSION

Accumulating scientific evidence suggests that inflammation has a pathogenic role in DR. The results of our pilot study demonstrated that PGE$_2$ is elevated significantly in the vitreous of diabetic patients, and correlates with VEGF and IP-10. To our knowledge, we are the first to report these findings, which may have considerable clinical importance, since therapeutic strategies to prevent DR are needed, and PG inhibitors (NSAIDs) are widely available and used frequently in medical therapy for their anti-inflammatory effects.

Cyclooxygenase (COX) is an important enzyme in the inflammatory process and catalyzes the biosynthesis of 5 classes (PGE$_2$, PGD$_2$, PGF$_2\alpha$, PGI$_2$, Thromboxane A$_2$) of PGs from arachidonic acid. NSAIDs are potent inhibitors of COX enzymes and thereby the synthesis of PGs. Two isoforms, COX-1 and COX-2, are established firmly, and are among the most thoroughly studied and best understood mammalian enzymes. In the human retina, COX enzyme can be detected in retinal endothelial cells, astrocytes, microglia, ganglion cells, amacrine cells, Müller, and RPE cells, and is upregulated in response to inflammatory cytokines. Within the eye, PGs promote vasodilation, disrupt the blood-ocular barrier, facili-

### Table 2. VEGF and Cytokine Levels

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>PDR Group Mean ± SD (pg/mL)</th>
<th>Control Group Mean ± SD (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>14.35 ± 6.3</td>
<td>9.05 ± 4.7</td>
</tr>
<tr>
<td>GRO</td>
<td>26.79 ± 18.5</td>
<td>11.06 ± 7.4</td>
</tr>
<tr>
<td>IL-6</td>
<td>22.75 ± 24.5</td>
<td>4.25 ± 8.6</td>
</tr>
<tr>
<td>IL-8</td>
<td>114.1 ± 174</td>
<td>8.04 ± 6.6</td>
</tr>
<tr>
<td>IP-10</td>
<td>1223 ± 962</td>
<td>1135 ± 3282</td>
</tr>
<tr>
<td>MCP-1</td>
<td>7904 ± 2727</td>
<td>2342 ± 2342</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>0.283 ± 0.19</td>
<td>0.128 ± 0.25</td>
</tr>
<tr>
<td>VEGF</td>
<td>2225 ± 3798</td>
<td>66 ± 185</td>
</tr>
<tr>
<td>PDGF-AA</td>
<td>960.5 ± 672</td>
<td>103.7 ± 60</td>
</tr>
</tbody>
</table>

### Table 3. Cytokine Levels in PDR Group with and without Prior PRP

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>No PRP Mean ± SD (pg/mL)</th>
<th>Prior PRP Mean ± SD (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandin E2</td>
<td>27.80 ± 15.8</td>
<td>24.09 ± 5.1</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>17.36 ± 5.0</td>
<td>11.76 ± 6.4</td>
</tr>
<tr>
<td>GRO</td>
<td>27.36 ± 13.8</td>
<td>26.31 ± 23</td>
</tr>
<tr>
<td>IL-6</td>
<td>23.47 ± 51.43</td>
<td>22.13 ± 19.4</td>
</tr>
<tr>
<td>IL-8</td>
<td>195.38 ± 238.4</td>
<td>44.43 ± 24.0</td>
</tr>
<tr>
<td>IP-10</td>
<td>1536 ± 1106</td>
<td>954 ± 805</td>
</tr>
<tr>
<td>MCP-1</td>
<td>8785 ± 2663</td>
<td>7149 ± 2742</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>0.351 ± 0.21</td>
<td>0.224 ± 0.16</td>
</tr>
<tr>
<td>VEGF</td>
<td>3280 ± 5458</td>
<td>1321 ± 1399</td>
</tr>
<tr>
<td>PDGF-AA</td>
<td>1401 ± 711</td>
<td>583 ± 349</td>
</tr>
</tbody>
</table>

($P < 0.05$)
tate leukocyte migration, and interact with and amplify many other soluble mediators.\textsuperscript{5,20}

There is considerable experimental evidence demonstrating that PGs have a role in the pathogenesis of DR. In animal models of diabetic retinopathy, retinal cells consistently upregulate COX enzyme and PGs.\textsuperscript{13,21} It now is well established that VEGF is a principle mediator of neovascularization in DR.\textsuperscript{12} In vitro studies demonstrate that PGE\textsubscript{2} increases VEGF expression in cultured Müller cells\textsuperscript{10} and agonism or antagonism of the PGE\textsubscript{2} receptor EP\textsubscript{4} increases or decreases VEGF production, respectively, in a dose-dependent manner.\textsuperscript{22} In a variety of experimental systems, PG inhibition prevents or slows progression of DR. PGE\textsubscript{2} is increased by 40% in the retinal vasculature of diabetic rats and is reduced significantly by treatment with insulin.\textsuperscript{13} The NSAID celecoxib significantly inhibits PGE\textsubscript{2} secretion, and retinal VEGF expression and vascular leakage in streptozotocin-induced diabetic rats.\textsuperscript{16,21} and administration of NSAIDs (nepafenac, aspirin, meloxicam) inhibits diabetes-induced retinal microvascular disease and prevents early DR.\textsuperscript{14,15}

In addition to experimental evidence, there is growing clinical evidence supporting a pathogenic role of PGs. It was observed first a half century ago that rheumatoid arthritis patients taking salicylates had a reduced incidence of DR.\textsuperscript{23}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Scatter plot of PGE\textsubscript{2} and IP-10 levels of control and PDR eyes. The horizontal axis is on a logarithmic scale. \textit{Best fit line} was created from logarithmic regression.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Scatter plot of PGE\textsubscript{2} and VEGF. Eyes with no detectable VEGF (11 control eyes) and one PDR patient with VEGF over 14,000 pg/mL were analyzed statistically, but not graphed for presentation purposes only. \textit{Best fit line} represents linear regression.}
\end{figure}
This observation was examined later in two large multicenter clinical trials, the Early Treatment Diabetic Retinopathy Study (ETDRS), which examined the effect of 650 mg aspirin on advanced DR, and the Dipyridamole Aspirin Microangiopathy of Diabetes (DAMAD) Study, which tested the impact of 990 mg aspirin in patients with early DR. While no benefit was found in patients with more advanced DR in ETDRS, a significant effect was seen in the DAMAD study, where higher doses of aspirin were found to slow the development of retinal microaneurysms. This latter observation is supported by a randomized 3-year pilot study where the NSAID sulindac prevented development and progression of DR. Similarly, a recent prospective, controlled trial conducted by the National Eye Institute demonstrated that oral celecoxib significantly reduced vascular leakage in patients with DR despite premature stoppage of treatment due to concerns regarding cardiovascular toxicity. Finally, a recent randomized clinical trial by the Diabetic Retinopathy Clinical Research (DRCR) Network reported that intravitreal injection of corticosteroid (triamcinolone acetonide) significantly reduced progression of DR, which provides further support for anti-inflammatory based therapies.

Only 2 previous studies in the literature, to our knowledge, have analyzed PG levels in the vitreous of humans with DR and, in contrast to our results, reported lower levels. However, both studies were limited by their small sample size, prior treatment history in diabetic eyes, and different indications for vitrectomy (rhegmatogenous retinal detachment). Furthermore, it is unclear to what extent, if any, diabetic eyes had active neovascularization at the time of vitrectomy, which was required of all diabetic eyes in our study and may account for our findings.

Vitreous PGE2 levels have been reported in several studies, but there is considerable variation in measured levels, due in part to differences in indication, sampling method, and immunoassay technique used. Therefore, neither comparing PGE2 levels to normative data nor between different studies is possible. However, animal models have shown a greater than 50% rise in PGE2 levels in the vitreous and retina after laser-induced breaks in Bruch’s membrane, along with an increase in vitreous protein levels and neovascularization, suggesting that relatively small percent increases in PGE2 levels can promote angiogenesis and inflammation.

In addition to PGE2 and VEGF, 8 other inflammatory cytokines were elevated in the vitreous of patients with PDR. Eotaxin-1 levels were higher in the PDR group, which has not been reported consistently. Eotaxin-1 is an eosinophil-specific chemoattractant that has been found to be elevated in allergic conditions and other systemic inflammatory disorders. Similarly, GRO is a chemokine that recruits neutrophils and basophils, is produced in several systemic inflammatory disorders, and has been found to be elevated in the vitreous of PDR patients in only one prior study. Equally important, our findings confirmed other recent studies that have reported elevated vitreous levels of IL-6, IL-8, MCP-1, PDGF-AA, TNF-α, and in patients with PDR.

While a relationship between PGE2 and VEGF is established, we are not aware of a previously reported association of PGE2 with IP-10. IP-10 is a leukocyte chemoattractant chemokine that belongs to the CXC family, and has been found to be elevated in vitreous samples in patients with PDR and proliferative vitreoretinopathy (PVR). Similarly, PGE2 has been reported previously to be elevated in patients with rhegmatogenous retinal detachment and to have a potential role in PVR. The role of IP-10 in retinal disease remains unclear but its association with PGE2 is interesting and merits further investigation. Therefore, future studies of IP-10 levels in retinal disease in the presence and absence of PGE2 inhibition may be informative.

As with all pilot studies, our results should be interpreted with caution. While the difference between PGE2 levels in the vitreous of diabetic and nondiabetic eyes was significant, this is not a direct measure of retinal concentration. Nonetheless, it generally is accepted that vitreous levels correlate with retinal levels and this method of analysis is widely accepted given the unacceptable risks to patients with direct sampling of retinal tissue. We also cannot rule out the fact that PGE2 levels in some of our eyes may have been affected by conditions other than diabetes. For example, a few patients were on systemic aspirin or NSAIDs, topical corticosteroids, and topical prostaglandin analogs before surgery. However, the cross-reactivity of PGF2α (prostaglandin analog) and PGE2 is less than 0.01%, and the number of patients on these medications was small overall and similar between groups. Not surprisingly there was a greater than 10-year difference between our diabetic and nondiabetic patients. However, inflammation and inflammatory markers generally increase with age and our correlation analysis demonstrated a trend of increasing PGE2 vitreous levels with increasing age in our control group that almost reached statistical significance (P = 0.08). Thus the older age of our nondiabetic patients more likely would have resulted in underestimating rather than overestimating our findings.

In conclusion, our results demonstrated that PGE2 levels are increased in the vitreous of patients with PDR, and correlated significantly with VEGF and IP-10. These findings, if substantiated by other larger studies, provided further support of an inflammatory basis of DR and provided rationale for anti-inflammatory based therapies.

References


31. Heier JS, Awh CC, Busbee BG, et al. Vitreous nonsteroidal anti-inflammatory drug concentrations and prostaglandin E2 levels in vitrectomy patients treated with ketorolac 0.4%, bromfenac 0.09% and nepafenac 0.1%. *Retina.* 2009;29:1310–1313.


