Effect of Unoprostone on Topographic and Blood Flow Changes in the Ischemic Optic Nerve Head of Rabbits

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Objective: To determine whether subconjunctival injection of unoprostone isopropyl alters changes in the topography and blood flow of the optic disc induced by endothelin 1 (ET-1) in rabbits.

Methods: From April 1, 2005, to April 28, 2006, we injected ET-1 (20 pmol) intravitreally into rabbits twice per week for 4 weeks. The observation period was 8 weeks. The first group received an intravitreal injection of ET-1 followed by a subconjunctival injection of unoprostone (0.12%, 50 µL). The second group received the same amount of ET-1 followed by a subconjunctival injection of the vehicle of unoprostone. The third group received the intravitreal vehicle of ET-1. The blood flow and topography of the optic nerve head (ONH) were measured by laser speckle flowgraphy and confocal scanning ophthalmoscopy, respectively. The number of cells in the retinal ganglion cell layer and inner nuclear layer was determined histologically.

Results: We found that ET-1 decreased the ONH blood flow, decreased the cells in the ganglion cell layer and inner nuclear layer, enlarged the cup area of the ONH, and reduced the rim area of the ONH. When unoprostone was given with ET-1, no such changes occurred.

Conclusion: Unoprostone can suppress the effects of ET-1 on the circulation and topography of the ONH.

Clinical Relevance: Unoprostone could be a candidate for treating eyes with ischemic ONH.

ity, 45% [10%]; and 12-hour lighting cycle) with free access to food and water. The experimental procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research.

**CHEMICALS**

Human ET-1 was purchased from the Peptide Institute, Inc. (Osaka, Japan). After ET-1 was dissolved in 0.1% aqueous acetic acid to obtain a 10⁻⁶M solution, the concentration was adjusted to 10⁻⁴M by dilution with balanced saline solution. Unoprostone (Rescula eyedrops; 0.12%) and its metabolites, M₁ and M₂, were provided by R-Tech Ueno, Ltd (Tokyo, Japan). M₁ and M₂ were used as authentic standards for quantification. As an internal standard for the quantification, 13,14-dihydro-15-keto-prostaglandin F₂α (Cayman Chemical Company, Ann Arbor, Michigan) was used.

**DRUG ADMINISTRATION AND EXPERIMENTAL PROTOCOL**

Only 1 of the eyes was randomly selected for the experiments. We injected ET-1 (10⁻⁴M, 20 µL) into the posterior vitreous of the selected eye through the pars plana with a Hamilton syringe using a 30-gauge needle with the rabbit under local anesthesia with 0.4% oxybuprocaine hydrochloride (Benoxiv; Santen Pharmaceutical Co, Ltd, Osaka, Japan). This dose of ET-1 was determined by an earlier study. For the injections, each rabbit was placed in a holding box, its pupil was dilated with 1 drop of 0.4% tropicamide (Mydriat M; Santen Pharmaceutical Co, Ltd), and its eye was held open with a Barraquer wire speculum (Inami & Co, Ltd, Tokyo, Japan). The injections were given with the rabbit under local anesthesia with oxybuprocaine on Tuesday and Friday for 4 weeks, and the overall observation period was 8 weeks beginning from the first injection of ET-1.

Twelve rabbits were divided into 3 groups. Two groups received intravitreal ET-1: 1 group was given a subconjunctival injection of 50 µL of 0.12% unoprostone after the ET-1 (ET-1 plus unoprostone group, n = 4), and the second group was given a subconjunctival injection of the vehicle for unoprostone (ET-1 plus vehicle group, n = 4). A third group was given an intravitreal injection of only the vehicle for ET-1 (sham group, n = 4). Measurements of the ONH blood flow, IOP, and ONH topography were performed before and 4 and 8 weeks after the first injection of ET-1 or vehicle.

**BLOOD FLOW AND IOP ASSESSMENT**

To evaluate the changes in ocular circulation, the capillary blood flow in the ONH was measured using laser speckle flowgraphy, which permits a noninvasive, 2-dimensional measurement of circulation within tissue. The details of this instrument have been described. Briefly, when the ocular fundus is illuminated with a diode laser (wavelength, 808 nm), a speckle pattern appears, and the frequency of the speckles varies with blood velocity. The normalized blur (NB) obtained with laser speckle flowgraphy is equivalent to a quantitative index of the blurring of speckle patterns and was originally considered to be an indicator of blood velocity within tissue. Alterations in the NB were shown to represent changes in capillary blood flow in the ONH of rabbits because NB changes were well correlated with blood flow changes simultaneously measured by the hydrogen clearance method.

Rabbits were placed in holding boxes, and measurements were performed with the rabbits under local anesthesia with a drop of oxybuprocaine. For the measurements of the ONH capillary blood flow, the average NB over an area of 0.72 × 0.72 mm of the ONH that was free of surface vessels was measured after mydriasis with a drop of tropicamide. We always recorded the measurement area, including characteristic vessels, to ensure that the same area was analyzed at all times of measurement of the same rabbit. It required 0.18 second to record 98 scans to obtain 1 NB value. The NB at each experimental time was calculated as the average of 5 successive measurements. The IOP was also measured with a pneumotonometer (Medtronic Solan, Jacksonville, Florida) immediately after the blood flow assessments.

**HEIDELBERG RETINA TOMOGRAPHY ANALYSES**

To quantify the changes in the topography of the ONH, Heidelberg retina tomography (HRT2; Heidelberg Engineering, Heidelberg, Germany) was used. Previous experiments using HRT showed that the reproducibility of the topographic data in rabbits was comparable to that in humans. To verify the reproducibility of each parameter, the coefficient of variation, (standard deviation/mean) × 100, was calculated from 6 images of each randomly selected eye before the first injection of ET-1 or vehicle in 12 rabbits. These measurements were taken on the same day as the first measurements of blood flow and IOP.

Rabbits were placed in holding boxes, and measurement was performed after mydriasis was induced with 1 drop of tropicamide. To enhance the imaging quality, the cornea was kept moistened with artificial tears between imaging acquisitions. At each session, a series of 6 topographic images was obtained from each selected eye of the same rabbit, and then the NB data was averaged to obtain the topographic parameters for statistical analyses.

**HISTOLOGIC EVALUATION**

After completion of the 8-week experimental period, the animals were humanely killed with an overdose of intravenous pentobarbital sodium (Nembutal; Abbott, North Chicago, Illinois). The eyes were immediately enucleated, fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 10mM phosphate-buffered saline, rinsed with 10mM phosphate-buffered saline, and embedded in paraffin. A transverse section of each retina (3 µm) was cut parallel to the medullary rays 2 mm directly inferior to the center of the ONH and stained with hematoxylin-eosin. To evaluate the damage to the retina, cells in the ganglion cell layer (GCL) and the inner nuclear layer (INL) of the retina were counted by an examiner (Y.Y.) who was masked to the experimental procedures performed on the rabbit. For the analysis, 9 light photomicrographs taken at ×260 magnification around the center of each retinal section at a distance of approximately 5 mm were obtained in a masked fashion. The examiner counted all of the cells in the GCL and INL in these photographs, with displaced amacrine cells not excluded from the counts, as described. The numbers of cells in the retinal ganglion cell and INL were averaged for each eye to obtain data for the statistical analyses.

**QUANTIFICATION OF UNOPROSTONE METABOLITE CONCENTRATION**

Three rabbits were used for this experiment. Rabbits were anesthetized by intramuscular injection of ketamine hydrochloride (Ketalar; Daiichi Sankyo Pharma Co, Ltd; Tokyo, Japan) and xylazine hydrochloride (Celactal; Bayer Japan Co, Ltd, Osaka, Japan) at doses of 35 and 10 mg/kg. Then 50 µL of 0.12% unoprostone was injected subconjunctivally into both eyes of 2 rabbits. At 0.5 and 1 hour after the injection, the eyes were collected after exsanguinations. Another rabbit received no treatment and served as a control.
The eyes were dissected, and retinal samples were homogenized with distilled water using Ultratarax (IKA Co., Ltd; Stauffen, Germany) to prepare 10% or 20% (wt/vol) retina homogenates. The concentrations of metabolites M1 and M2 in the retinal homogenates were determined by liquid chromatography–tandem mass spectrometry. The internal standards and acetonitrile were added to the retinal homogenate samples, and then the samples were centrifuged at 10,000g for 2 minutes at room temperature. The supernatants were then evaporated to dryness by a centrifugal evaporator. The dried residues were dissolved with methanol and water (2:8, vol/vol), and then the samples were filtrated by centrifugation at 5000g for 1 minute at room temperature (Centricut Ultra-mini, 0.45 µm; Kurabo, Osaka, Japan). The filtrates were injected into a liquid chromatography–tandem mass spectrometry system.

Liquid chromatography–tandem mass spectrometry analysis was performed with a high-performance liquid chromatography system (Alliance 2795 system; Waters Corporation, Milford, Mass.) coupled to a guard column (Inertsil ODS-3; GL Sciences Inc, Tokyo, Japan) and an analytical column (Delovosil ODS-UG-3; 2.0 × 50 mm; Nomura Chemical, Seto, Japan). The mobile phases were composed of acetonitrile, water, and acetic acid (20:80:0.1) and acetonitrile and acetic acid (100:0.1). The gradient was as follows: 0 minutes, 100% acetonitrile, water, and acetic acid; 2 minutes, 40% acetonitrile, water, and acetic acid and 60% acetonitrile and acetic acid; 2 through 5 minutes, 40% acetonitrile, water, and acetic acid and 60% acetonitrile and acetic acid; and 5.01 through 7 minutes, 100% acetonitrile and acetic acid. The flow rate was 0.25 mL/min. The column compartment was kept at 35°C. The high-performance liquid chromatography eluate was introduced via electrospray ionization directly into a mass spectrometer (API 3000 triple quadrupole mass spectrometer; MDS Sciex, Concord, Ontario, Canada) using a Turbo Ion Spray interface. Multiple-reaction monitoring analysis of M1 and M2 was performed using the following transitions: mass/charge of 381 to 183 (M1) and mass charge of 327 to 141 (M2).

The tandem mass spectrometry data analyses were performed by Analyst (version 1.1; MDS Sciex). The concentrations of M1 and M2 were determined by the peak area ratios of M1 and M2 using an internal standard method. Calibration ranges were 0.25 to 250 ng/g for M1 and 0.5 to 250 ng/g for M2.

**STATISTICAL ANALYSES**

The data are expressed as mean(SEM). Statistical comparisons among groups were performed using analysis of variance followed by unpaired t tests. Statistical comparisons of data at different time points in each group were performed using repeated-measures analysis of variance followed by paired t tests. Differences were accepted as statistically significant at P < .05.

**ONH BLOOD FLOW AND IOP**

The mean NB values determined in the same part of the ONH are shown in Figure 1. In the ET-1 plus vehicle eyes, the NB values were significantly reduced at 4 and 8 weeks, whereas in the ET-1 plus unoprostone eyes, the reduction was not significant. The mean NB values in the sham control eyes did not change significantly during the study. The changes in the mean IOPs are shown in Figure 2. In the ET-1 plus unoprostone eyes and the ET-1 plus vehicle eyes, the IOPs were reduced significantly at 4 and 8 weeks, whereas the sham control eyes did not change significantly during the study.

**ONH TOPOGRAPHY**

The mean (SEM) (n = 12) of the coefficients of variations for each HRT2 parameter are as follows: 0.00% (0.00%) for disc area, 16.63% (2.88%) for rim area, 9.44% (6.97%) for cup area, 35.34% (4.26%) for rim volume, 23.66% (2.88%) for cup volume, 9.41% (2.32%) for cup-disc area ratio, 8.20% (2.16%) for linear cup-disc ratio, 53.46% (12.23%) for cup shape measure, 13.28% (1.22%) for mean cup depth, 9.22% (2.5%) for maximum cup depth, 21.90% (5.82%) for height variation contour, 118.76% (39.30%) for mean retinal nerve fiber layer (RNFL) thickness, and 124.76% (38.58%) for RNFL cross-sectional area. Most of the parameters, except for the RNFL cross-sectional area, mean RNFL thickness, cup shape, and
rim volume, showed good reproducibility with the coefficients of variation below 25%.

Many of the HRT2 parameters decreased after the injection of ET-1, but unoprostone inhibited the changes. In fact, the differences between sham control and ET-1 plus vehicle eyes were significant for rim area, cup area, rim volume, cup volume, cup-disc area ratio, linear cup-disc ratio, mean RNFL thickness, and RNFL cross-sectional area, although no significant difference was detected between sham control and ET-1 plus unoprostone eyes (Table 1).

**HISTOLOGIC CHANGES**

The histologic changes in the retina are shown in Figure 3. The numbers of cells in the GCL and INL were decreased in the ET-1 plus vehicle eyes compared with the sham control eyes but not in the ET-1 plus unoprostone eyes. A quantitative assessment of the protective effect of unoprostone is shown in Figure 4 and Figure 5. The number of cells in the GCL and INL in the retinas of the ET-1 plus vehicle eyes was significantly fewer than in the sham control eyes and ET-1 plus unoprostone eyes.

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**Table 1. Mean (SEM) Values (Percentage of Baseline) of HRT2 Parameters at 8 Weeks After the First Injection of ET-1 or Vehicle**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham Control</th>
<th>ET-1 Plus Vehicle</th>
<th>ET-1 Plus Unoprostone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disc area, mm²</td>
<td>1.00 (0.00)</td>
<td>1.00 (0.00)</td>
<td>1.00 (0.00)</td>
</tr>
<tr>
<td>Rim area, mm²</td>
<td>0.99 (0.07)</td>
<td>0.45 (0.11)</td>
<td>1.01 (0.13)</td>
</tr>
<tr>
<td>Cup area, mm²</td>
<td>1.02 (0.02)</td>
<td>1.35 (0.09)</td>
<td>1.00 (0.05)</td>
</tr>
<tr>
<td>Rim volume, mm³</td>
<td>0.88 (0.06)</td>
<td>0.28 (0.08)</td>
<td>0.99 (0.19)</td>
</tr>
<tr>
<td>Cup volume, mm³</td>
<td>1.03 (0.04)</td>
<td>1.80 (0.16)</td>
<td>0.96 (0.06)</td>
</tr>
<tr>
<td>Cup-disc area ratio</td>
<td>1.00 (0.02)</td>
<td>1.35 (0.09)</td>
<td>0.97 (0.05)</td>
</tr>
<tr>
<td>Linear cup-disc ratio</td>
<td>1.01 (0.01)</td>
<td>1.16 (0.04)</td>
<td>0.98 (0.03)</td>
</tr>
<tr>
<td>Cup shape measure</td>
<td>0.74 (0.38)</td>
<td>0.66 (0.06)</td>
<td>0.57 (0.06)</td>
</tr>
<tr>
<td>Mean cup depth, mm</td>
<td>1.11 (0.08)</td>
<td>1.07 (0.09)</td>
<td>1.03 (0.08)</td>
</tr>
<tr>
<td>Maximum cup depth, mm</td>
<td>1.10 (0.09)</td>
<td>1.06 (0.05)</td>
<td>0.93 (0.06)</td>
</tr>
<tr>
<td>Height variation contour, mm</td>
<td>0.87 (0.13)</td>
<td>1.04 (0.05)</td>
<td>0.92 (0.13)</td>
</tr>
<tr>
<td>Mean RNFL thickness, mm</td>
<td>1.67 (0.78)</td>
<td>−1.46 (0.48)</td>
<td>0.72 (0.32)</td>
</tr>
<tr>
<td>RNFL cross-sectional area, mm²</td>
<td>0.85 (0.42)</td>
<td>−1.39 (0.47)</td>
<td>0.72 (0.32)</td>
</tr>
</tbody>
</table>

Abbreviations: ET-1, endothelin 1; HRT, Heidelberg retina tomography; RNFL, retinal nerve fiber layer.

*P < .01, †P < .05 show statistically significant differences among the 3 groups by analysis of variance and also statistically significant differences from sham control by unpaired t test.

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**Figure 3.** Photomicrographs of transverse sections of the posterior retina. Sections were obtained from the sham control (A), endothelin 1 (ET-1) plus vehicle (B), and ET-1 plus unoprostone (C) eyes. The number of cells in the ganglion cell layer (GCL) and inner nuclear layer (INL) is reduced by repeated applications of ET-1 (B), whereas it is not changed significantly in the ET-1 plus unoprostone eyes (C). Bar=50 µm. ONL indicates outer nuclear layer.

**Figure 4.** Mean number of cells in the ganglion cell layer (GCL) (n=4). Analysis of variance revealed a statistically significant difference among the 3 groups (P=0.004) (unpaired t tests, *P < .01, †P < .05). When unoprostone was given, there was no reduction in the number of GCL cells. Sham indicates sham control; vehicle, endothelin 1 plus vehicle; unoprostone, endothelin 1 plus unoprostone. Error bars indicate SEM.

**Figure 5.** Mean number of cells in the inner nuclear layer (INL) (n=4). Analysis of variance revealed a statistically significant difference among the 3 groups (P=0.009) (unpaired t tests, *P < .01, †P < .05). When unoprostone was given, there was no reduction in the number of INL cells. Sham indicates sham control; vehicle, endothelin 1 plus vehicle; unoprostone, endothelin 1 plus unoprostone. Error bars indicate SEM.
The concentrations of M₁ and M₂ in the retina after a subconjunctival injection of unoprostone are given in Table 2. The average concentration of M₁ was 1.43 ng/g at 0.5 hour after the injection. At 1 hour, the concentration decreased below the lower limit of quantification. On the other hand, the average concentration of M₂ was 1.125 ng/g at 1 hour after the injection.

### Table 2. Concentrations of M₁ and M₂ in the Retina After Subconjunctival Injection of Unoprostone Eyedrops in Rabbits

<table>
<thead>
<tr>
<th>Time point, h</th>
<th>M₁, ng/g</th>
<th>M₂, ng/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right side</td>
<td>1.73</td>
<td>0.674</td>
</tr>
<tr>
<td>Left side</td>
<td>1.13</td>
<td>BLQ²</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right side</td>
<td>BLQ¹</td>
<td>1.35</td>
</tr>
<tr>
<td>Left side</td>
<td>BLQ¹</td>
<td>0.899</td>
</tr>
</tbody>
</table>

Abbreviations: BLQ¹, below the lower limit of quantification (<0.250 ng/g); BLQ², below the lower limit of quantification (<0.500 ng/g).

In this study, the concentrations of M₁ and M₂ might have affected the basal (Ca²⁺) channels in ET-1–treated bovine trabecular meshwork and ciliary muscle cells. A similar action might have occurred in the ONH ischemia induced by ET-1. However, another study reported that the mechanism of the relaxation of vascular smooth muscle by unoprostone differed from that of IOP reduction and did not depend on the maxi-K potassium channels. Instead, the relaxation might have been mediated by inhibition of Ca²⁺ entry possibly through capacitative Ca²⁺ channels. Moreover, M₂ was shown to suppress the Ca²⁺ influx through Ca²⁺ release–activated Ca²⁺–current channels in trabecular meshwork and ciliary muscle cells.

In conclusion, we have demonstrated that subconjunctival injection of unoprostone might produce M₁ and M₂ at pharmacologically effective levels against ET-1 action in the eye.
graphical changes and blood flow reduction in the ONH of rabbits. These findings show the possibility of using unoprostone to treat optic neuropathic disease caused by ischemia, including glaucoma. However, because our experiments were performed in rabbits, the transfer of these results to human optic neuropathy should be considered cautiously.

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Author Contributions: Dr Sugiyama had full access to all of the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Additional Contributions: Ryosuke Ono, MSc, Akiko Morikawa, MSc, and Yosuke Kawai, MSc, of R-Tech Ueno Ltd, and Asako Komori, BA, provided technical support.

REFERENCES