Effect of P2X<sub>7</sub> Receptor Activation on the Retinal Blood Velocity of Diabetic Rabbits

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Objective: To test the effect of activation of P2X<sub>7</sub> receptors on retinal blood velocity in diabetic rabbits.

Methods: Immunohistochemical analysis was performed on healthy and diabetic rabbit eyes using P2X<sub>7</sub> receptor antibodies. Diabetes was induced using alloxan. Retinal blood velocity was measured with the laser speckle circulation analyzer. Visual function was assessed using electroretinography.

Results: Cells in inner retinal layers were positive for anti-P2X<sub>7</sub> receptor antibodies in healthy rabbits. The distribution of positive cells extended to outer layers and some small vessels stained in diabetic rabbits. When assayed 24 hours after an intravitreal injection of 150 nmol of benzoylbenzoyl adenosine triphosphate (BzATP), a P2X<sub>7</sub> agonist, the retinal blood velocity in healthy rabbits was reduced by approximately 30%; this reduction continued for at least 4 weeks. Only in diabetic rabbits did an injection of 50 nmol of BzATP reduce retinal blood velocity by approximately 30% and the amplitudes of electroretinography a waves, b waves, and oscillatory potentials for at least 4 weeks.

Conclusions: Soon after the onset of alloxan-induced diabetes, retinal blood velocity and function become more vulnerable to reduction initiated through P2X<sub>7</sub> receptors.

Clinical Relevance: Our findings support the hypothesis that the retinal circulation disorder accelerated by activation of P2X<sub>7</sub> receptors may be involved in the early changes of diabetic retinopathy.

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The pathophysiologic mechanism of diabetic retinopathy is not yet fully elucidated; however, the apoptotic death of microvascular pericytes and endothelial cells is one of the hallmarks of diabetic retinopathy. The loss of pericytes seems to play an important role in the progression of diabetic retinopathy. Many studies have examined retinal blood flow in diabetes. As the disease progresses before vasoproliferation, retinal blood flow was found to be above normal. However, some researchers have reported reduced retinal blood flow in patients with diabetes mellitus without diabetic retinopathy or with background diabetic retinopathy. These changes in retinal blood flow might be related to the dysfunction or loss of microvascular pericytes.

Our previous immunocytochemical and electrophysiologic studies have shown that microvessels of the retina express functional purinergic P2X<sub>7</sub> receptors. We also demonstrated that retinal microvascular-
METHODS

CHEMICALS
Adenosine triphosphate, the P2X<sub>7</sub> agonist benzoylbenzoyl-ATP (BzATP), the P2X<sub>7</sub> antagonist oxidized ATP, and alloxan monohydrate were purchased from Sigma Chemical Co (St Louis, Mo).

ANIMALS
Male albino rabbits that weighed 2.5 to 3.4 kg were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). They were housed in an air-conditioned room at a mean ± SD temperature of 22°C±1°C and a mean ± SD level of humidity of 66% ± 3% humidity with a 24-hour light-dark cycle. They were handled in accordance with the Annual Meeting of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

IMMUNOHISTOCHEMICAL ANALYSIS
Two healthy rabbits and 3 rabbits made diabetic by alloxan for approximately 6 weeks were killed by injecting a lethal dose of pentobarbital sodium, and the eyes were removed. The excised eyes were fixed in 4% paraformaldehyde for approximately 24 hours, dehydrated, and embedded in paraffin. Continuous 5-µm sections were then prepared using a microtome. After deparaffinization, sections were treated with a 3% hydrogen peroxide solution for 5 minutes and incubated with primary antibodies (anti–human P2X<sub>7</sub> IgG at a 1:20 ratio for 60 minutes [Santa Cruz Biotechnology Ltd, Santa Cruz, Calif]) and secondary antibodies (biotin-conjugated goat anti–rabbit IgG, at a 1:200 ratio for 30 minutes [Vector Laboratories, Burlingame, Calif]) and the avidin-biotin-peroxidase complex for 30 minutes [Vector Laboratories]). The resulting sections were stained with diaminobenzidine, and nuclear staining was performed using 3% methyl green.

MEASUREMENT OF RETINAL BLOOD VELOCITY
Retinal blood velocity was measured with the laser speckle circulation analyzer, which permits noninvasive and 2-dimensional measurements of tissue circulation in the retina. The apparatus consists of a fundus camera equipped with an argon laser (wavelength, 488 nm) and an image sensor (100 x 100 pixels). The principle of this method has been described previously. The normalized blur (NB) value, a quantitative index of blood velocity in the retinal microcirculation, was recorded 5 times at each time point, and then the mean value was calculated. The image speckles from a retinal field free of visible surface vessels and approximately 1 papillary diameter from the optic nerve head along the medullary rays were recorded. The subsequent measurements of tissue circulation in the retina. The apparatus consists of a fundus camera equipped with an argon laser (wavelength, 488 nm) and an image sensor (100 x 100 pixels). The principle of this method has been described previously. The normalized blur (NB) value, a quantitative index of blood velocity in the retinal microcirculation, was recorded 5 times at each time point, and then the mean value was calculated. The image speckles from a retinal field free of visible surface vessels and approximately 1 papillary diameter from the optic nerve head along the medullary rays were recorded.

MEASUREMENT OF INTRAOCULAR PRESSURE AND MEAN BLOOD PRESSURE
Intraocular pressure (IOP) was measured with a calibrated pneumotonometer (model 30 Classic, Medtronic Solan, Jacksonville, Fla) with the rabbit under local anesthesia with benoxinate hydrochloride. The mean blood pressure (MBP) was measured at the front leg by an automatic sphygmomanometer (BP-98E, Softron, Tokyo). A close correspondence between the pressure determined by this sphygmomanometer and that obtained through a pressure transducer cannula placed in the femoral artery had been previously confirmed. Ocular perfusion pressure (OPP) was determined as MBP minus IOP.

ASSESSMENT OF RETINAL FUNCTION
To assess the changes of retinal function, electroretinography (ERG) was performed. For recording, a photic stimulator (SLS 4100), a biophysical amplifier (AVM-10), and an averager (DAT-1100) (all from Nihon-Kohden, Tokyo) were used. Before the ERG recordings, the animals were adapted to the dark for 60 minutes. The ERGs was performed with a 4.7-cal light stimulus set 20 cm in front of the eye, and recordings were made with a gold ring active electrode on the cornea by averaging 4 responses to the light stimuli at 0.1 Hz. A diffuser was placed before the stimulated eye to ensure a more full-field stimulation, and the mean lumiance on the corneal surface was 43.8 lux/s. Bandpass filters were set at 0.5 to 100 Hz for a waves and b waves and at 50 to 300 Hz for oscillatory potentials (OPs). The OPs that appeared first and second were referred to as OP<sub>1</sub> and OP<sub>2</sub>, respectively. The amplitudes and the implicit times of the a waves and b waves, OP<sub>1</sub>, and OP<sub>2</sub>, of the ERGs were measured. Analog data were recorded by a rectilinear pen system and simultaneously stored and digitized using a microcomputer (MacLab 2e, AD Instruments, Castle Hill, Australia). Analysis of the various stored parameters was performed using the microcomputer. We performed ERG before and 2 and 4 weeks after intravitreal injection of 10 µL of BzATP (50 nmol) or physiologic saline solution, as a control, in 5 nondiabetic rabbits.

MODEL OF DIABETES AND EXPERIMENT PROTOCOL
After an overnight fast, rabbits received an intravenous injection of alloxan (80 mg/kg) diluted in physiologic saline solution. Blood glucose, IOP, MBP, and retinal blood flow were measured at 3 and 6 hours, 3 days, and 1 and 2 weeks after the alloxan application in 7 rabbits. If the blood glucose level was less than 30 mg/dL (<2.78 mmol/L), sufficient glucose (10%) was provided. Two weeks after the indication of diabetes, BzATP (15, 50, or 150 nmol) or physiologic saline solution was intravitreally injected into the eyes of diabetic rabbits. The retinal blood flow and ERG were examined according to the same protocol as the nondiabetic rabbits. Five rabbits were used for each dose of BzATP.

STATISTICAL ANALYSIS
Statistical analysis was performed using 1-way analysis of variance. If a statistically significant change was detected, further assessment was made with the Dunnett test. Statistical significance was set at P<.05.
RESULTS

IMMUNOHISTOCHEMICAL EVIDENCE FOR P2X7 RECEPTORS IN THE RETINAS OF HEALTHY AND DIABETIC RABBITS

The results of immunohistochemical analysis in the retinas of healthy rabbits showed cells positive for an anti-P2X7 receptor antibody in the inner plexiform and ganglion cell layers (Figure 1B). In the retinas of diabetic rabbits, the distribution of positive cells was extended to the outer nuclear layer. Some small vessels also stained densely. Bar represents 30 µm.

Figure 1. Immunohistochemical evidence of P2X7 receptors in the retina of healthy and diabetic rabbits. A, Minimal immunoreactivity was found in the retina of a healthy rabbit when the primary antibody was omitted from the protocol. B, Cells in the inner plexiform and ganglion cell layers of a normal retina stained with an anti-P2X7 receptor antibody. C, The distribution of positive cells extended to the outer nuclear layer in the diabetic retina. Some small vessels also stained densely.

Figure 2. Changes in retinal blood velocity after intravitreal injection of adenosine triphosphate (ATP) and benzoylbenzoyl-ATP (BzATP) in nondiabetic rabbits. NB indicates normalized blur. A, injection of ATP; B, injection of BzATP. Data are expressed as mean ± SEM (error bars) for 5 rabbits. Significant differences were found between the control and treated groups. *P<.05 by repeated-measures analysis of variance. †P<.05, ‡P<.01 by the Dunnett test.

EFFECTS OF INTRAVITREAL INJECTION OF ATP OR A P2X7 AGONIST ON RETINAL BLOOD VELOCITY IN NONDIABETIC RABBITS

Intravitreal injection of 1500 nmol of ATP decreased the retinal blood velocity significantly with a maximum reduction of more than 30% at 2 weeks; 150 nmol of ATP had no significant effect (Figure 2A). Although 150 nmol of the P2X7 agonist BzATP reduced the blood velocity to almost the same degree as 1500 nmol of ATP, a significant reduction was detected 1 day after the injection and lasted for at least 4 weeks. These changes were inhibited by the P2X7 antagonist oxidized ATP (Figure 2B).
CHANGES IN BLOOD GLUCOSE LEVEL, IOP, MBP, OPP, AND RBV AFTER INJECTION OF ALLOXAN

Blood glucose levels increased in 3 hours, decreased temporarily in 6 hours, and increased to 400 mg/dL (22.2 mmol/L) or more in 3 days. That level was maintained for at least 2 weeks (Table 1). The IOP, MBP, and OPP did not show any significant changes for at least 2 weeks.

The retinal blood velocity was reduced at 3 and 6 hours, but it recovered to the initial level within 3 days and then became stable at 2 weeks (Table 1).

EFFECTS OF INTRAVITREAL INJECTION OF A P2X7 AGONIST ON IOP, MBP, OPP, AND RBV AFTER INJECTION OF ALLOXAN

A total of 50 nmol of BzATP produced no significant changes in IOP, MBP, or OPP (Table 2). It decreased the retinal blood velocity with a maximum reduction of more than 30% in 4 weeks in diabetic rabbits (Figure 3A), although the same dose had no significant effect in healthy rabbits (Figure 2B). A total of 150 nmol of BzATP increased the blood velocity temporarily in a day but reduced it to the same level as 50 nmol in 4 weeks (Figure 3A). The dose-response curves show the enhancement of BzATP-induced reduction of the retinal blood velocity in diabetic rabbits compared with non diabetic rabbits (Figure 3B).

EFFECTS OF INTRAVITREAL INJECTION OF A P2X7 AGONIST ON ERG IN NONDIABETIC AND DIABETIC RABBITS

As shown in Figure 4, ERG a and b waves did not notably change in the 2 weeks after administration of alloxan; however, 2 weeks after the injection of 50 nmol of BzATP, the amplitudes of the a and b waves and OPs were more reduced than in the controls. A total of 50 nmol of BzATP significantly reduced the amplitudes of a waves, b waves, OP1, and OP2 in diabetic rabbits for at least 4 weeks, although it had no significant effect on these parameters in nondiabetic rabbits (Table 3). The implicit times of the a waves, b waves, OP1, and OP2 were not significantly changed after BzATP injections, although they have the tendency to increase (data not shown).

COMMENT

To the best of our knowledge, this is the first study that demonstrates that the activation of P2X7 receptors can cause the persistent reduction of retinal blood velocity and function in vivo. Although a high concentration of the P2X7 agonist was needed to decrease retinal blood velocity in healthy rabbits, a lower concentration was effective soon after the onset in an alloxan-induced diabetic rabbit model. Therefore, diabetes-induced vulnerability of retinal blood velocity affected by P2X7 receptor
activation may be involved in the mechanism of early changes in diabetic retinopathy.

To measure retinal blood velocity, we used a laser speckle circulation analyzer developed in Japan. Since Tamaki et al15 have already shown a significant correlation between the relative change in NB and that in the retinal blood flow rate determined using the microsphere technique, we estimated blood flow by measuring blood velocity.

Our results show that vessels and other types of cells in the retina were immunoreactive to P2X7 receptor antibodies. Other researchers have reported the expression of the P2X7 receptor in the neurons and Muller glia cells of the retina17,18; however, they did not direct their attention to the retinal vessels. We confirmed the immunoreactivity of retinal vessels to P2X7 receptor antibodies, so it is natural that the retinal vasculature responds to P2X7 agonists. We have already reported that a P2X7 agonist, BzATP, as well as ATP caused the microvascular lumen to narrow.10 In the current study, we did not measure the short-term effect of ATP and BzATP on the retinal blood velocity. The BzATP decreased the blood velocity and probably blood flow within 24 hours; therefore, the effect might be partly due to retinal vessel constriction, as shown in the previous study.10 Since the change lasted 4 weeks, other mechanisms, including apoptosis of the microvascular cells (which we had shown in the previous study),11 might be involved in the circulation reduction. In addition, ATP had no effect on blood velocity at 1 day after injection, suggesting the existence of a different mechanism of ATP and BzATP action. This finding is consistent with the ability of ATP to activate receptors other than P2X7 receptors. On the other hand, the 50% effective concentration of BzATP to the P2X7 receptor is much smaller than that of ATP (3:100), meaning that BzATP is the most sensitive P2X7 agonist.19 In addition, BzATP is sensitive to the P2X7 receptor.

Some reports have described retinal blood velocity or flow changes in diabetic animal models. In streptozotocin-induced diabetic rats and spontaneous type 2 diabetic rats, reduced retinal blood flow was indicated using retinal angiography.20,21 In this investigation, we used alloxan to
create diabetic rabbits. Alloxan inhibits insulin release from beta cells for 2 to 4 hours, followed by destruction of beta cells and a transient increase of serum insulin in 6 to 12 hours.\(^2\) The blood glucose level temporarily increased, followed by a decrease to a fatal level unless glucose was administered to the rabbits. After 3 days the rabbits became hyperglycemic (glucose level \(> 300 \text{ mg/dL} \)) and maintained this condition for at least 2 weeks. Retinal blood velocity decreased transiently but returned to the previous level in 3 days and became stable in 2 weeks. The transient reduction of the blood velocity might be due to the decrease in OPP, which was not statistically significant. The BzATP at 50 nmol, which had no significant effect on the blood velocity in healthy rabbits, reduced the blood velocity in diabetic rabbits significantly within 3 days after the intravitreal injection. Since the systemic condition (ie, MBP) and IOP of the animals did not change significantly, the blood velocity change seems to be induced by the response of microvessels to the agonist. Consistent with our previous study\(^1\) that used microvascular cells of rats, the retinal blood velocity seemed to react more to a P2X\(_7\) agonist in diabetic retinas than in healthy ones. The reason that BzATP at 150 nmol increased the retinal blood velocity transiently is not exactly known; however, it might be a result of the inflammation induced by the agonist.\(^3\)

The ERG recordings indicated that 50 nmol of BzATP reduced the amplitudes of a and b waves and OPs significantly in diabetic rabbits but not significantly in nondiabetic ones. The ERG b waves and OPs are well-known indicators of inner retinal ischemia in humans and diabetic animals.\(^2\)\(^,\)\(^2\)\(^5\) The retinal vascular system in rabbits is extremely underdeveloped, and most parts of the retina are nourished by choroidal circulation. The simultaneous reduction of ERG a and b waves and OPs may indicate that the stimulation of the P2X\(_7\) receptor accelerates functional impairment of the inner and outer retinal segments in diabetic retinas, including photoreceptors and bipolar, amacrine, and Muller cells, probably owing to a reduction in retinal circulation as well as choroidal circulation. Another possibility is that BzATP may directly affect cellular function in the retinas of diabetic animals, since Muller cells express P2X\(_7\) receptors.\(^1\) To fully understand this subject, more research needs to be conducted.

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### Table 3. Changes in Amplitudes of ERG Waves After Intravitreal Injection of BzATP (50 nmol) in Diabetic and Nondiabetic Rabbits*

<table>
<thead>
<tr>
<th>Change in ERG Wave Amplitude, µV</th>
<th>Initial Value</th>
<th>2 Weeks After Alloxan</th>
<th>2 Weeks After BzATP</th>
<th>4 Weeks After BzATP</th>
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<tr>
<td><strong>a Wave</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Diabetic</td>
<td></td>
<td></td>
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<tr>
<td>BzATP†</td>
<td>77.8 ± 8.2</td>
<td>66.7 ± 11.4</td>
<td>50.8 ± 20.0†</td>
<td>48.0 ± 13.4</td>
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<tr>
<td>Control</td>
<td>65.5 ± 6.8</td>
<td>67.7 ± 26.9</td>
<td>67.5 ± 28.5</td>
<td>55.0 ± 17.9</td>
</tr>
<tr>
<td>Nondiabetic</td>
<td></td>
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<tr>
<td>BzATP</td>
<td>76.7 ± 21.0</td>
<td></td>
<td>71.3 ± 34.4</td>
<td>65.0 ± 8.2</td>
</tr>
<tr>
<td>Control</td>
<td>69.3 ± 28.1</td>
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<td>61.0 ± 4.6</td>
<td>59.0 ± 10.8</td>
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<tr>
<td><strong>b Wave</strong></td>
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<tr>
<td>Diabetic</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BzATP†</td>
<td>340 ± 64</td>
<td>330 ± 54</td>
<td>244 ± 62†</td>
<td>197 ± 67§</td>
</tr>
<tr>
<td>Control</td>
<td>310 ± 15</td>
<td>285 ± 67</td>
<td>287 ± 107</td>
<td>239 ± 55</td>
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<td></td>
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<tr>
<td>BzATP</td>
<td>376 ± 77</td>
<td>328 ± 67</td>
<td>332 ± 57</td>
<td>360 ± 42</td>
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<tr>
<td>Control</td>
<td>357 ± 48</td>
<td>373 ± 60</td>
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<tr>
<td><strong>OP(_1) and OP(_2)</strong></td>
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<tr>
<td>Diabetic</td>
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</tr>
<tr>
<td>BzATP†</td>
<td>53.2 ± 8.1</td>
<td>46.4 ± 13.2</td>
<td>30.6 ± 12.0†</td>
<td>30.5 ± 11.7†</td>
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<tr>
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<tr>
<td>BzATP</td>
<td>43.0 ± 12.0</td>
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<td>43.5 ± 11.3</td>
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<td>35.8 ± 11.3</td>
<td>44.0 ± 11.2</td>
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</table>

Abbreviations: BzATP, benzoylbenzoyl adenosine triphosphate; ERG, electroretinography; OP, oscillatory potential.

*Data are expressed as mean ± SD for 5 rabbits. There were significant changes from the initial values.

†\(P<.05\) by analysis of variance.

‡\(P<.05\) by the Dunnett test.

§\(P<.01\) by the Dunnett test.
REFERENCES


Cheng and colleagues also noted that prior to final transplantation, 5% of these patients have experienced vision loss to a visual acuity of 20/70 or worse. Therefore, the lifetime risk of vision loss to a visual acuity of 20/70 is 1 in 2000. Studies by Lam et al and Dart found essentially the same rate of infection.

The risk of vision loss from *Acanthamoeba* keratitis may also be calculated using published data. The incidence varies by country from an average of 0.3 to 1.5 cases per 10,000 persons per year, with the highest rate in Scotland. The low number yields a 30-year risk of 1 in 1000 or higher. If half of these patients, a conservative estimate, sustain vision loss from the infection, the lifetime risk of vision loss is also 1 in 2000. This may seem high, but it is only 1 case per 60,000 persons per year.

The risk of vision loss from refractive surgery can be calculated more directly. Chang et al reported an average infection rate of 1 case per 800 persons, with 25% of infected eyes experiencing moderate vision loss (1 case per 3200 persons). Covering 32,000 procedures, Hammond et al reported an incidence greater than 1 line, the minimum detectable, was 1 case per 1250 persons. A loss of 2 or more lines, which would be more significant but much less frequent, was not specified. Our own data from the Casey Eye Institute, Portland, Ore, on 18,000 procedures over 10 years found no eyes with vision loss greater than 2 lines. We propose that the incidence of vision loss greater than 2 lines may be 1 case per 10,000 persons.

These calculated risks are obviously approximate and subject to change. Highly oxygen-permeable contact lenses should lessen the risks of wearing contact lenses; however, laser surgery will also become safer. The data sets described earlier cannot be compared directly, and it is difficult psychologically to equate long- vs short-term risks. Nevertheless, data from large, peer-reviewed studies strongly suggest that our intuition regarding these risks needs to be reassessed. We look forward to further investigations of these risks.

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