Involvement of P2X<sub>7</sub> Receptors in the Hypoxia-Induced Death of Rat Retinal Neurons

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PURPOSE. To investigate the hypoxia-induced death of rat retinal neurons and to determine whether P2X<sub>7</sub> activation is involved in this type of neuronal death.

METHODS. Cultured retinal neurons from fetal rats were used. The effects and time course of various degrees of hypoxia (1–5% O<sub>2</sub>) in the death of retinal neurons, were examined. The effects of P2X<sub>7</sub> antagonists, oxidized adenosine triphosphate (oxidized ATP; 30–100 μM), and brilliant blue G (BBG; 100 nM–10 μM) on hypoxia-induced neuronal death, including apoptosis, were assessed by using trypan blue exclusion, TUNEL assays, and cleaved caspase-3 immunoreactivity. Immunocytochemical analysis was performed to determine whether these neurons express P2X<sub>7</sub> receptors. The effects of P2X<sub>7</sub> receptor stimulation, induced by the P2X<sub>7</sub> agonist benzoylbenzoyl-ATP (BzATP), on neuronal viability and intracellular Ca<sup>2+</sup> levels ([Ca<sup>2+</sup>]<sub>i</sub>) were examined.

RESULTS. Retinal neuronal death increased according to the degree of hypoxia and became more severe after 12 hours. Both oxidized ATP and BBG significantly decreased hypoxia-induced neuronal death. Immunocytochemistry demonstrated that P2X<sub>7</sub> receptors were expressed by the cultured retinal neurons. ATP and BzATP caused P2X<sub>7</sub>-receptor-dependent neuronal death in a dose-dependent manner and led to a sustained increase in [Ca<sup>2+</sup>]<sub>i</sub>, with BzATP being more effective than ATP. These effects were hypoxia-induced factor-1<sub>i</sub>-independent and were prevented by oxidized ATP.

CONCLUSIONS. The results suggest that the death of retinal neurons can be triggered by hypoxia and that P2X<sub>7</sub> activation is involved in the hypoxia-induced death of retinal neurons. P2X<sub>7</sub> antagonists can prevent hypoxia-induced damage in retinal neurons. (Invest Ophthalmol Vis Sci. 2010;51:3236–3245) DOI:10.1167/iovs.09-4192

The ischemic injury of retinal neurons is thought to be triggered by pathologic increases in the intracellular Ca<sup>2+</sup> levels ([Ca<sup>2+</sup>]<sub>i</sub>), which are mediated by Ca<sup>2+</sup> influx through voltage- and ligand-gated Ca<sup>2+</sup> channels. In fact, a study reported that calcium channel blockers have direct retinal neuroprotective effects against hypoxic damage in an experimental model.

Recently, it was clearly established that purine nucleosides and nucleotide plays an important regulatory role in the function of many organ systems. Notably, ATP receptors are involved in synaptic signal transfer, immunity, inflammation, cell death, and several trophic actions on neuronal and glial cells after brain injury. Immunolabeling for P2X<sub>7</sub> was detected in amacrine and ganglion cells, suggesting that extracellular ATP provides both neuromodulatory and trophic influences on visual processing and the neural retina. There is also a report that P2X<sub>7</sub> receptors may be involved in neuronal transmission, rather than in cytolysis, because they are expressed exclusively in neurons in the primate retina. Others have suggested that activation of P2X<sub>7</sub> receptors modulates the uptake of neurotransmitters from the extracellular space by Müller cells and also the photoreceptor and rod bipolar cell responses in the retina. We have reported that extracellular ATP regulates the physiology of retinal pericytes. By activating P2X<sub>7</sub> receptors, ATP alters ionic currents, increases [Ca<sup>2+</sup>]<sub>i</sub>, and induces contractions in freshly isolated pericytes from the rat retina. We have also reported that purinergic vasotoxicity is normally prevented in the retina by several mechanisms, whereas the dysfunction of these protective mechanisms may be a cause of cell death within the retinal microvasculature. An upregulation of purinergic receptors has been suggested to be part of the reactive changes in Müller cells during proliferative vitreoretinopathy. It has also been reported that short-term stimulation of the P2X<sub>7</sub> receptor can raise [Ca<sup>2+</sup>]<sub>i</sub> in rat retinal ganglion cells, whereas sustained stimulation of the receptor can kill the cells. Another research study has suggested that ATP released during hypoxic stress from carotid body chemoreceptors (and/or red blood cells) causes glossoopharyngeal nerve neuronal depolarization mediated by multiple P2X receptors, including P2X<sub>7</sub> receptors, and that activation of this pathway leads to calcium influx.

In the present study, we investigated the hypoxia-induced death of retinal neurons and tested the hypothesis that activation of P2X<sub>7</sub> receptors may be involved in the mechanism of this type of neuronal cell death.

MATERIALS AND METHODS

Animals

Pregnant Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The rats were housed in an air-conditioned room, at a temperature of approximately 23°C, 60% humidity, and a 12:12 hour light–dark cycle. All experimental procedures conducted on the animals were carried out in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Chemicals

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

Primary cultures obtained from the retinas of fetal rats were used for the experiments, as previously described. After the mother Wistar rat...
was euthanatized, retinas were removed from her fetal rats (gestational age, 18 to 19 days old) under microscope magnification. The tissue was minced, mechanically dissociated using scalpel blades, filtered using nylon mesh (No. 305; NBC Meshtec, Tokyo, Japan) and plated as single-cell suspensions on plastic coverslips (22 mm × 22 mm, 100–1305; ScienceLab.com, Houston, TX). The coverslips were placed in 60-mm dishes (1.0 × 10^6 cells/mL; Falcon; BD Biosciences, Bedford, MA), in Earle’s minimum essential medium (MEM) containing 2 mM glutamate, penicillin-streptomycin (100 U/mL and 50 μg/mL, respectively), and 25 mM HEPES. Plastic coverslips were precoated by soaking in 0.1% polyethyleneimine solution in 0.15 M borate buffer for more than 12 hours. The medium was supplemented with 10% heat-inactivated fetal bovine serum. Media and supplements were purchased from Invitrogen-Gibco (Rockville, MD). Retinal cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and air. Supplemented medium for the culture was exchanged every 2 days except for day 5. To eliminate non-neuronal cells, 10 μM cytosine arabinoside (ara-C) was added to the cultures on day 5. Previous immunohistochemical studies have shown that neuronal cells cultured with this protocol consist mainly of amacrine cells and less than 1% glial cells.16 Primary cultures of retinal neurons that were maintained for 10 days were used.

Assessment of Neuronal Death

The degree of neuronal death was assessed quantitatively with the trypan blue exclusion method. All experiments were performed in Earle’s MEM at 37°C. After completion of the drug exposure, the cell cultures were stained with 1.5% trypan blue solution at room temperature for 10 minutes and then fixed in isotonic formalin (pH 7.0; 2–4°C). The fixed cultures were rinsed with PBS and examined with a Hoffman modulation microscope at ×200 magnification (Hoffman Modulation Contrast System/IX-70; Olympus, Tokyo, Japan).

The number of dead cells was determined by counting all isolated cells in a microscopic field until the total number of cells on each coverslip reached more than 200. Cells in clusters were not counted. The experimental treatment of the cells was masked to the examiner. Cells stained with trypan blue were considered dead, and unstained cells were deemed to be alive. Data are expressed as the percentage of dead cells (stained cells) in the total number of cells counted.

TUNEL Assay and Immunocytochemical Evidence for P2X7 Receptors

Dual staining was performed to examine whether TUNEL (TdT-mediated dUTP nick-end labeling) apoptotic staining is related to P2X7 receptors. Neurons were fixed with 4% paraformaldehyde in PBS for 25 minutes at room temperature and exposed to 0.3% hydrogen peroxide and 0.2% Triton X-100 for 15 minutes. After the reaction was blocked in 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA) and 1% bovine serum albumin in 10 mM PBS for 1 hour at room temperature, the neurons were washed with PBS and stained with the primary antibody rabbit anti-P2X7 (anti-human P2X7 IgG; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 for 16 hours at 4°C. In negative control experiments, the primary antibody was omitted. A commercial kit (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI) was used to incorporate biotinylated dUTP at 3’-OH DNA ends using terminal deoxynucleotidyl transferase. The neurons were then reacted with Cy3-conjugated goat anti-rabbit IgG (affinity-purified IgGs; Jackson ImmunoResearch Laboratories), at 1:150, for 45 minutes at room temperature. Images were acquired with a fluorescence mi-
Hypoxic stimuli were induced by using a gas oxygen controller (Proox the Effects of P2X7 Antagonism and Hypoxia-Neuronal Death under Hypoxic Conditions and

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Results

Neuronal Death under Hypoxic Conditions, the Effects of P2X7 Antagonism and HIF-1α Inhibition, and Immunocytochemical Evidence for P2X7 Receptors

Figure 1 shows representative photomicrographs of retinal neurons demonstrating hypoxic toxicity, as detected by the trypan blue exclusion method, and the inhibition of this toxicity by a P2X7 antagonist. Dead retinal neurons were noted as trypan blue-positive cells under hypoxic conditions, and no positively stained neurons were found under control conditions (Figs. 1A, 1B). Exposure to a P2X7 antagonist, oxidized ATP (100 μM), reduced the number of trypan blue-positive cells after hypoxia (Fig. 1C). Exposure to 3% O2 induced a significantly greater extent of neuronal death than 5% O2, whereas 1% O2 induced significantly more death than 5% O2 (Fig. 2A). The time-course of neuronal death induced by 1% O2 is shown in Figure 2B. Although there was no significant difference in the levels of neuronal death between hypoxic and control conditions at 6 hours, hypoxia-induced neuronal death

hypoxia, neuronal death was assessed as mentioned earlier. During hypoxia (5% O2), the neurons were exposed to the P2X7 antagonists oxidized ATP (30 or 100 μM) or brilliant blue G (BBG; 100 nM, 1 or 10 μM) and the HIF-1α inhibitor YC-1 (30 or 100 μM; A.G. Scientific, San Diego, CA). As YC-1 dissolves only in DMSO (0.1%), we set the control for YC-1-exposed neurons to 0.1% DMSO, in contrast to our other control condition. Neuronal death was then evaluated.

Neuronal Death Induced by P2X7 Receptor Activation

To test the hypothesis that activation of P2X7 receptors induces the death of retinal neurons, we incubated the neurons with the purinergic agonists ATP (30-300 μM) or BzATP (10-100 μM) for 24 hours under the control conditions mentioned earlier. Subsequently, we measured cell death using the trypan blue exclusion method. In addition, we tested the effect on neuronal death of a 60-minute pretreatment with the P2X7 antagonist oxidized ATP or the HIF-1α inhibitor YC-1.

Measurement of [Ca2++]i

After 10 days in culture, the retinal neurons were incubated in Earle’s MEM containing 2 μM acetoxyethyl ester (AM) of fura 2 (Dojindo, Kumamoto, Japan) for 30 minutes and then washed three times with Earle’s MEM. Fura 2-AM-loaded retinal neurons were placed on a coverslip precoated with neutralized cell adhesive (Cell-Tak; BD Biosciences, Franklin Lakes, NJ). The coverslip with neurons was set in a perfusion chamber and mounted on the stage of an inverted microscope (IX70; Olympus, Tokyo, Japan) connected to an image-analysis system (Argus/HiSCa; Hamamatsu Photonics, Hamamatsu, Japan).

All the experiments were performed at 37°C. The volume of the perfusion chamber was approximately 80 μL and the rate of perfusion was 500 μL min−1. Fura 2-AM was excited at 340 and 380 nm, and emission was measured at 510 nm. The fluorescence ratio (F340/F380) was calculated and stored in an image-analysis system (Argus/HiSCa). Changes in F340/F380 values in the retinal neurons were examined when ATP or P2X7 antagonists or agonists were added to the perfusion. Each experiment was performed on four to seven cells, and the F340/F380 values were expressed as the mean ± SD.

Statistical Analyses

The data are expressed as the mean ± SEM, unless otherwise noted. Statistical comparisons between groups were performed with the Bonferroni test, Dunnett’s test, or the paired t-test. Fisher’s exact test was used for statistical comparisons of positive cells between two groups. Differences were deemed significant at P < 0.05.

RESULTS

Neuronal Death under Hypoxic Conditions and the Effects of P2X7 Antagonism and Hypoxia-Induced Factor-1α (HIF-1α) Inhibition

Hypoxic stimuli were induced by using a gas oxygen controller (Proox model 110; BioSpherix, Redfield, NY). Retinal neurons were incubated under control conditions (20% O2, 5% CO2, 37°C) or hypoxic conditions (1%, 3%, or 5% O2, 5% CO2, 37°C) for 6, 12, or 24 hours. After

Assessment of Cleaved Caspase-3 Immunoreactivity

Neurons were fixed by 4% paraformaldehyde in PBS for 25 minutes at room temperature and then exposed to 0.3% hydrogen peroxide and 0.2% Triton X-100 for 15 minutes. After specimens were blocked by 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA) and 1% bovine serum albumin in 10 mM PBS for 1 hour at room temperature, the neurons were washed with PBS and stained with primary antibody, rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) at 1:500 for 16 hours at 4°C. Subsequently, the neurons were reacted with Cy3-conjugated goat anti-rabbit IgG (affinity-purified IgGs; Jackson ImmunoResearch Laboratories) at 1:150 for 45 minutes at room temperature. Neuronal nuclei were counterstained with diamino-2-phenylindole (DAPI), and images were acquired by fluorescence microscope (VZ-8000; Keyence, Osaka, Japan). An observer who was masked to the treatment of the neurons examined at least 150 neurons on each coverslip.
increased significantly after 12 hours. Exposure to the P2X7 antagonists, oxidized ATP (30, 100 μM), and BBG (100 nM and 1, 10 μM), reduced the hypoxia-induced cell death in a dose-dependent manner (Fig. 3A). In comparison, exposure to the HIF-1α inhibitor YC-1 also significantly reduced hypoxia-induced cell death (Fig. 3B). The expression of P2X7 receptors in cultured retinal neurons was confirmed under both control and hypoxic conditions (Figs. 4A, 4B), although P2X7 antagonist treatment reduced P2X7 receptor immunoreactivity (Figs. 4C, 4D). No TUNEL-positive neurons were detected under control conditions (Fig. 4A), but there were several TUNEL-positive neurons expressing P2X7 receptors during hypoxia (Fig. 4B). Moreover, exposure to P2X7 antagonists reduced the number of TUNEL-positive neurons (Figs. 4C, 4D). In addition, although cleaved caspase-3 immunoreactivity was not detected under control conditions (Fig. 5A), it was enhanced during hypoxia (Fig. 5B), and a P2X7 antagonist also significantly decreased hypoxia-induced caspase-3 immunoreactivity (Fig. 5C). In summary, there was a significantly greater number of damaged neurons (according to both TUNEL assays and caspase-3 immunoreactivity) during hypoxia in the absence of P2X7 antagonists (Table 1).

Effects of P2X7 Activation on Neuronal Death

ATP (30–300 μM) increased neuronal death in a dose-dependent manner, whereas the P2X7 antagonist oxidized ATP reduced cell death significantly, but only partly (Fig. 6). On the other hand, the P2X7 agonist BzATP increased neuronal death in a dose-dependent manner, whereas oxidized ATP reduced death significantly and almost completely (Fig. 6). The HIF-1α inhibitor YC-1 had no significant effects on the neuronal death induced by the P2X7 agonist (Fig. 6).

Changes in [Ca2+]i Induced by P2X7 Activation

Treatment with ATP (300 μM) increased [Ca2+]i, significantly (Fig. 7A), but the increase was completely prevented when the cells were pretreated with oxidized ATP (100 μM) before the addition of ATP (Fig. 7B). Furthermore, BzATP (100 μM) also increased [Ca2+]i, significantly, after a transient decrease;
which was probably due to a mechanical effect (Fig. 7C). The increase in $[\text{Ca}^{2+}]_{\text{i}}$ induced by BzATP appeared larger than that induced by ATP.

**DISCUSSION**

The present study revealed the effects of various degrees of hypoxia on the death of retinal neurons and the time course for the neuronal death. It demonstrated for the first time the neuroprotective effects of P2X7 antagonists on retinal neurons from hypoxia-induced death. In addition, it showed that P2X7 activation causes neuronal death in a dose-dependent manner and leads to sustained increases in $[\text{Ca}^{2+}]_{\text{i}}$. Taken together, these data show that P2X7, activation is most likely involved in mediating the hypoxia-induced death of retinal neurons.

The finding that the death of retinal neurons increased in proportion to the degree of hypoxia (at least between 1%–5% O2), demonstrated that hypoxia alone can induce death in this type of cell. In addition, since neuronal death increased after 12 hours, it is possible that hypoxia triggers apoptosis in retinal neurons. In fact, the present study revealed that approximately 60% of dead cells were TUNEL-positive.

Our immunocytochemical study demonstrated that our cultured retinal neurons, mainly amacrine cells, express P2X7 receptors. Amacrine cells have neural connections with amacrines, bipolar, and ganglion cells in the inner plexiform layer of the retina, and sometimes send processes to the outer plexiform layer. They regulate the activities of these neurons by releasing several neurotransmitters. In addition to these physiological properties, amacrine cells can be displaced to the...
Effects of P2X7 Antagonists

P2X7 receptors and HIF-1α/H9251 hypoxia-induced neuronal death. The relationship between P2X7 receptors and HIF-1α remains to be further investigated.

Tissue hypoxia has been postulated to occur in eyes with various diseases, including glaucoma and diabetic retinopathy. Yamada et al. have demonstrated the direct neuroprotective effects of calcium channel blockers on the hypoxia-induced cell death of retinal ganglion cells in culture. Furthermore, the hypoxia-induced release of glutamate from the isolated retina or from cultured retinal cells has been reported. In contrast, a report suggested that hypoxia-induced damage of retinal ganglion cells is mostly independent of glutamate receptor-mediated excitotoxicity, but occurs via a Bax-dependent apoptotic pathway. The contribution of glutamate to the hypoxia-induced death of retinal ganglion cells in vivo needs more investigation.

A previous study has shown that stimulation of P2X7 receptors raise Ca2⁺ in rat retinal ganglion cells, whereas sustained stimulation of the receptors kills them. In addition, there is a report suggesting that the Ca2⁺ entry mediated by P2X7 receptors induces glutamate release in cerebellar granule neurons. Furthermore, activation of P2X7 receptors has been reported to enhance the induction of type 2 nitric oxide synthase in microglial cells. An in vitro experiment indicated that elevated concentrations of nitric oxide exhibit neurotoxic effects and mediate glutamate-induced neurotoxicity in cultured retinal neurons.

A postischemic, time-dependent upregulation of P2X7 receptor expression by neurons and glial cells has been reported, suggesting a role for these receptors in the pathophysiology of cerebral ischemia in vivo. It has also been reported that P2X7 receptors may become hypersensitive in primary cerebrocortical cultures after in vitro ischemia, because of the increased efficiency of their transduction pathways. In contrast, another study revealed that the treatment of mice with P2X7 antagonists did not affect ischemic cell death, although the animal species used for the study was different from the strain used in the present study. Furthermore, it has been reported that P2X7 receptors modulate microglial cells and that antagonists of these receptors reduce brain infarct in rat. It has also been reported that these receptors modulate β-amyloid-induced cytokine secretion from human microglia and thus may play a role in the neuroimmunopathology of Alzheimer’s disease. The relationship between these receptors and glial cells in the retina remains to be investigated. We previously reported a diabetes-induced increase in the vulnerability of retinal microvessels to the lethal effects of P2X7 receptor activation. In addition, our experiments using rabbits suggest that retinal circulation disorder accelerated by the activation of P2X7 receptors may be involved in the early changes in diabetic retinopathy.

Our current findings suggest that modulation of P2X7 receptors could protect retinal neurons from hypoxia-induced cell death. Others also reported that the inhibition of P2X receptors had a neuroprotective effect similar to that of the inhibition of N-methyl-D-aspartate receptors in rat hippocampal slice cultures. The limitation of our study is that our results were obtained from cultured retinal neurons consisting mainly of amacrine cells, and so the results cannot be directly extended to other types of retinal neurons, including retinal

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**Table 1.** Hypoxia-Induced Apoptosis in Retinal Neurons and the Effects of P2X7 Antagonists

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<th>Positive Neurons</th>
<th>Negative Neurons</th>
<th>Percent Positive</th>
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<td>Control</td>
<td>13</td>
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<td>5% O2</td>
<td>97</td>
<td>396</td>
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<td>5% O2+BBG</td>
<td>12</td>
<td>156</td>
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<tr>
<td>Cleaved caspase-3</td>
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<tr>
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<td>37</td>
<td>475</td>
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A TUNEL assay was performed after 6 hours of hypoxia; active caspase-3 was assayed after 4 hours of hypoxia. The P2X7 antagonists Ox-ATP and BBG prevented apoptosis in retinal neurons.

* P < 0.001 (Fisher’s exact test).

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**Figure 6.** ATP- or BzATP-induced neuronal death detected by the trypan blue exclusion method and the effects of a P2X7 antagonist or an HIF-1α inhibitor. ATP increased cell death in a dose-dependent manner, whereas oxidized ATP significantly, but only partly, reduced it. A P2X7 antagonist, BzATP, increased cells death in a dose-dependent manner, whereas oxidized ATP significantly and almost completely reduced it, although YC-1 had no significant effects. Data are expressed as the mean ± SEM (n = 4-13). *Significant differences from the control group (Dunnett’s test, P < 0.05).
ganglion cells. Further studies of other types of retinal neurons and in vivo studies under hypoxic conditions are necessary for developing new treatments for eye diseases caused by hypoxia.

In conclusion, our results show that P2X activation may be involved in the hypoxia-induced cell death of retinal neurons and that P2X antagonists can prevent hypoxic damage in retinal neurons.

Acknowledgments

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References


