Enhancement of P2X7-Induced Pore Formation and Apoptosis: An Early Effect of Diabetes on the Retinal Microvasculature

Tetsuya Sugiyama, Masato Kobayashi, Hajime Kawamura, Qing Li, and Donald G. Puro

PURPOSE. A sight-threatening complication of diabetes is cell death in retinal capillaries. Currently, the mechanisms responsible for this classic manifestation of diabetic retinopathy remain uncertain. The hypothesis for the current study is that diabetes increases the vulnerability of retinal microvessels to the potentially lethal consequences of having their P2X7 purinoceptors activated. A pathophysiological role is suspected for these receptor-operated channels because, in addition to transducing retinovascular responses to extracellular adenosine triphosphate (ATP), the sustained opening of these channels can induce the formation of large transmembrane pores.

METHODS. In pericyte-containing retinal microvessels that were freshly isolated from nondiabetic and streptozotocin-injected rats, cells with pores were identified by the uptake of YO-PRO-1. Cell viability was assayed by trypan blue dye exclusion, and cleaved caspase-3 immunoreactivity, TUNEL positivity, and nuclear morphology were used to detect apoptotic cells. Patch-clamp recordings assessed electrophysiological parameters.

RESULTS. Activation of P2X7 receptors caused large pores to form and apoptosis to occur in retinal capillaries of nondiabetic and diabetic rats. Of importance to diabetes, the agonist concentration needed to open pores and trigger apoptosis decreased markedly soon after the onset of streptozotocin-induced hyperglycemia. However, despite this increased sensitivity, diabetes minimally affected the P2X7-induced ionic currents. Thus, rather than upregulate the number of functional P2X7 receptor/channels, diabetes appears to facilitate the channel-to-pore transition that occurs during activation of these purinoceptors. In this way, normally nonlethal concentrations of P2X7 ligands may trigger apoptosis in microvessels of the diabetic retina.

CONCLUSIONS. A diabetes-induced increase in the vulnerability of retinal microvessels to the lethal effect of P2X7 receptor activation may be a previously unrecognized mechanism by which diabetic retinopathy progresses.

A hallmark of diabetic retinopathy is the apoptotic death of microvascular pericytes and endothelial cells. The loss of pericytes, which are contractile cells located on the abluminal wall of capillaries, appears to play a critical role in the formation of microaneurysms and neovascular tufts. Damage to the endothelium can result in a breakdown of the blood-retinal barrier and a loss of vision due to edema within the retina.

At present, the mechanisms by which diabetes induces apoptosis in the retinal microvasculature remain uncertain, although oxidative stress, formation of advanced glycation end products, upregulation of protein kinase C, increased polyol pathway flux, and focal leukostasis may be important. In fact, multiple lethal pathways may be activated during chronic hyperglycemia.

In this study, we tested the hypothesis that diabetes adversely affects retinal microvessels by increasing their vulnerability to potentially lethal consequences of having their P2X7 purinoceptors activated. In capillaries of nondiabetic retinas, we have recently found that these ligand-gated cation channels mediate the vasoconstrictive action of extracellular ATP, which is likely to be a glial-to-vascular signal. However, in addition to this putative physiological role, our earlier study also suggested a potentially pathological effect of activating microvascular P2X7 receptors. Namely, at high agonist concentrations, ligand binding not only opens cation channels, but also results in the formation of large transmembrane pores.

The opening of similar pores in other cell types is known to cause cell death by disrupting ionic gradients and/or by providing pathways for an efflux of vital intracellular molecules of 900 Da or less.

Based on experiments using pericyte-containing microvessels freshly isolated from the adult rat retina, we now report that soon after the onset of streptozotocin (STZ)-induced diabetes, markedly lower P2X7 agonist concentrations effectively opened pores and triggered apoptosis in the retinal microvasculature. Our observations point to the previously unappreciated possibility that microvascular damage in the diabetic retina may be mediated by receptors for vasoactive molecules.

METHODS

Microvessel Isolation

Animal use conformed to the guidelines of the Association for Research in Vision and Ophthalmology for the use of Animals in Ophthalmic and Vision Research and the University of Michigan Committee on the Use and Care of Animals. As detailed previously, 6- to 8-week-old Long-Evans rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN; and Charles River, Cambridge, MA) were killed with carbon dioxide, and their retinas were rapidly removed and incubated in 2.5 mL Earle's balanced salt solution (Invitrogen-Life Technologies, Carlsbad, CA), which was supplemented with 0.5 mM EDTA, 20 mM glucose, 15 U papain ( Worthington Biochemicals, Freehold, NJ), and 2 mM cysteine for 30 minutes at 30°C and bubbled with 95% oxygen-5% carbon dioxide to maintain pH and oxygenation. After transfer to solution A...
(140 mM NaCl, 3 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 10 mM Na-HEPES, 15 mM mannitol, and 5 mM glucose at pH 7.4 with osmolarity adjusted to 310 mOsmol · L⁻¹), each retina was then gently sandwiched between two glass coverslips (15 mm diameter; Warner Instrument Corp., Hamden, CT). Vessels adhered to the coverslip that was in contact with the vitreous side of the retina. By repeating this tissue print step, we were able to obtain several coverslips containing microvessels from a retina.

**Cell Viability Assay**

As detailed previously, microvessels were exposed to 0.04% trypan blue. Cells that did not exclude trypan blue were classified as dead. The viability of this often-used viability assay did not appear to be affected by the formation of P2X₇ pores. Specifically, 2% of microvascular cells (the results section, Fig. 1C) even though P2X₇ pores are induced in more than half of the cells after one hour of BzATP exposure. The percentage of cells stained with this dye was determined by examining isolated microvessels at >100 magnification with an inverted microscope equipped with bright-field optics. Because we could not establish with certainty whether a cell stained with trypan blue was an endothelial cell or a pericyte, subclassification of microvascular cells into these two types was not feasible. At least 200 microvascular cells per coverslip were counted. Except where noted, cell viability assays were performed on microvessels maintained under control or experimental conditions for 24 hours.

**Detection of Apoptosis**

Microvessels were assessed for cleaved caspase-3 immunoreactivity, TUNEL positivity or nuclear morphology after incubation for 4, 8, or 14 hours, respectively, in solution A, without or with BzATP (Fig. 1). For each assay, microvessels were then exposed for 30 minutes with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature. Nuclear morphology was assessed by their characteristic “bump-on-a-log” appearance on the abluminal wall of microvessels. Pipettes, which had resistances of approximately 5 Mohm and contained a solution consisting of 0.1 M KCl, 64 mM K₅SO₄, 6 mM MgCl₂, 10 mM K-HEPES, 240 μg · mL⁻¹ amphotericin, and 240 μg · mL⁻¹ nystatin at pH 7.4 with the osmolarity adjusted to 280 mOsmol · L⁻¹, were mounted in the holder of a patch-clamp amplifier (Dagan Corp., Minneapolis, MN) and then sealed to the cell bodies of

**Experimental Model of Diabetes**

After an overnight fast, 4- to 5-week-old Long-Evans rats received an intraperitoneal injection of STZ (75 mg · kg⁻¹) diluted in 0.8 mL citrate buffer. Animals were maintained on a 12-hour alternating light–dark cycle and received food and water ad libitum. Immediately before the harvesting of the retinal microvessels, the blood glucose level was 329 ± 18 mg · dL⁻¹ (n = 20).

**Electrophysiology**

As detailed elsewhere, the perforated-patch configuration of the patch-clamp technique was used to monitor ionic currents from pericytes located on microvessels that had been isolated from a retina within 3 hours. A coverslip with microvessels was placed in a recording chamber, which was perfused (~2 mL · min⁻¹) with solution A, without or with various concentrations of the P2X₇ agonist BzATP. Vessels were examined at ×300 magnification with an inverted microscope equipped with phase-contrast optics. Pericytes were identified by their characteristic "bump-on-a-log" location on the abluminal wall of microvessels. Pipettes, which had resistances of approximately 5 Mohm and contained a solution consisting of 0.1 M KCl, 64 mM K₅SO₄, 6 mM MgCl₂, 10 mM K-HEPES, 240 μg · mL⁻¹ amphotericin, and 240 μg · mL⁻¹ nystatin at pH 7.4 with the osmolarity adjusted to 280 mOsmol · L⁻¹, were mounted in the holder of a patch-clamp amplifier (Dagan Corp., Minneapolis, MN) and then sealed to the cell bodies of

![Figure 1](https://iovs.arvojournals.org)
amplitude of the inward current induced during continuous exposure estimate cell membrane capacitance. As detailed recently,8 the peak point, 8/H11006/H11002, membrane resistance was calculated by measuring steady state currents at 6.1; OriginLab, Northampton, MA) for data analysis and graphics dis-
every 250 ms using a data-acquisition system (DigiData 1200B; Axon

Unless noted otherwise, chemicals were obtained from Sigma/RBI (St.

Chemicals

microscope (Eclipse TE300; Nikon) equipped for
differential interference contrast (DIC) optics with 20

Initially, microvessels were exposed to solution A supplemented with

5-
methylene-ATP; BzATP, benzoylbenzoyl-ATP. (B) Effect of oxidized-ATP (oxoATP) on BzATP-induced cell death. Isolated microvessels were initially incubated at 37°C for 2 hours in solution A supplemented with 300 μM oxidized ATP, then maintained for 1 hour at room temperature in this solution with 100 μM BzATP (oxoATP+BzATP) or without this P2X7 agonist (oxoATP). Other vessels (BzATP) were exposed to 100 μM BzATP for 1 hour after a 2-hour incubation at 37°C in solution A. For each group, microvessels were subsequently maintained in solution A at room temperature for 15 h prior to quantification of cell death. *P < 0.001 when compared with the control group. (C) Time course for cell death during exposure to solution A without or with 100 μM BzATP. For each point, 8 ≤ 1 vessel-containing coverslips were assessed. After 8 hours, data points in the BzATP group were significantly (P < 0.05) greater than the 24-hour value in the absence of BzATP. Activation of P2X7 receptors resulted in delayed cell death in pericyte-containing retinal microvessels isolated from the rat retina.

Statistics

Data are expressed as the mean ± SEM. Probability was evaluated by
Student’s t-test or the Fisher exact test (Tables 1, 2).

RESULTS

P2X7-Induced Cell Death in Retinal Microvessels

To test the hypothesis that activation of P2X7 receptors induces cell death in the retinal microvasculature, we incubated pericyte-containing microvessels with various purinergic agonists (Fig. 2A). We found that exposure to ATP and the P2X7 receptor agonist BzATP significantly (P < 0.001) increased microvascular cell death. Consistent with studies showing that cloned P2X7 receptors are more potently activated by BzATP than ATP,18 1 mM BzATP was significantly (P < 0.001) more effective than 1 mM ATP in causing cell death in isolated retinal microvessels. Because BzATP can also activate P2X1 receptors,18 we exposed some microvessels to α,β-methylene-ATP, which is a relatively specific agonist for the P2X7 receptors.18 Our finding that 1 mM α,β-methylene-ATP did not increase cell death above the control level (Fig. 2A) supported the conclusion that the lethal effect of BzATP was mediated by P2X7, not P2X1, receptors.

In other experiments, we used the irreversible blocker of P2X7 receptors, 2′,3′-dialdehyde-ATP (oxidized-ATP).18 Isolated microvessels were preincubated with 300 μM oxidized-ATP for 2 hours at 37°C before exposure to 100 μM BzATP. Consistent with P2X7 receptors mediating microvascular cell death, oxidized-ATP markedly (P < 0.001) inhibited BzATP-induced cell death (Fig. 2B). Taken together, our pharmacologic experiments indicate that the activation of P2X7 receptors causes cell death in the pericyte-containing microvasculature of the retina. Determination of the time course for cell death during a sustained exposure of microvessels to BzATP (100 μM) revealed that approximately 80% of the cell death detected by the trypan blue assay occurred after 8 hours (Fig. 2C). Even with a relatively brief exposure to this P2X7 agonist, most of the BzATP-induced cell death occurred after a delay of many hours. For example, between 8 and 24 hours after a 15-second

YO-PRO-1 Uptake

A coverslip with freshly isolated pericyte-containing microvessels was

positioned in a chamber located on the stage of a transmission electron

microscope (Eclipse TE300; Nikon) equipped for

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(A) Cell death in isolated retinal microvessels after 24 hours of exposure to various purinergic agonists; 6 ≥ 1 vessel-containing coverslips were assessed for each value. *P < 0.001 when compared with the control group. α,β-methylenoATP, α,β-methylene-ATP; BzATP, benzoylbenzoyl-ATP. (B) Effect of oxidized-ATP (oxoATP) on BzATP-induced cell death. Isolated microvessels were initially incubated at 37°C for 2 hours in solution A supplemented with 300 μM oxidized ATP, then maintained for 1 hour at room temperature in this solution with 100 μM BzATP (oxoATP+BzATP) or without this P2X7 agonist (oxoATP). Other vessels (BzATP) were exposed to 100 μM BzATP for 1 hour after a 2-hour incubation at 37°C in solution A. For each group, microvessels were subsequently maintained in solution A at room temperature for 15 h prior to quantification of cell death. *P < 0.001 when compared with the oxoATP+BzATP group. ns, P = 0.33, when compared with the oxoATP+BzATP group. (C) Time course for cell death during exposure to solution A without or with 100 μM BzATP. For each point, 8 ≤ 1 vessel-containing coverslips were assessed. After 8 hours, data points in the BzATP group were significantly (P < 0.05) greater than the 24-hour value in the absence of BzATP. Activation of P2X7 receptors resulted in delayed cell death in pericyte-containing retinal microvessels isolated from the rat retina.

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YO-PRO-1 Uptake

A coverslip with freshly isolated pericyte-containing microvessels was

positioned in a chamber located on the stage of a transmission electron

microscope (Eclipse TE300; Nikon) equipped for fluorescence and
differential interference contrast (DIC) optics with 20X objectives. Initially, microvessels were exposed to solution A supplemented with 5 μM YO-PRO-1 (Molecular Probes, Eugene, OR), which is a 629 Da propidium diiodide dye that on entry into a cell, binds to nucleic acids and thereby causes detectable fluorescence. After 15 minutes, microvessels were then exposed for 60 minutes at room temperature to the YO-PRO-containing solution without or with the addition of a purinergic agonist. Fluorescence was detected with excitation and emission wavelengths of 475 and 510 nm, respectively; the light source was a high-intensity mercury lamp coupled to a monochromator (Cairn Research, Faversham, UK). DIC optics facilitated detection of cells without fluorescence. At least 80 (mean ± SEM) microvascular cells per coverslip (n = 37) were examined.

Chemicals

Unless noted otherwise, chemicals were obtained from Sigma/RBI (St.

Louis, MO).
P2X7 agonist. The P2X7 agonist BzATP induced apoptosis in retinal microvessels. To test this possibility, we used cleaved caspase-3 without (control) or with 100 μM BzATP. Active caspase-3 was assayed 4 hours after the onset of BzATP exposure; the TUNEL assay was performed after 8 hours of BzATP exposure, and microvessels were stained with Hoechst 33258 after 14 hours of exposure to the P2X7 agonist. The P2X7 agonist BzATP induced apoptosis in retinal microvessels.

**Table 1.** Apoptosis in Retinal Microvascular Cells

<table>
<thead>
<tr>
<th>Apoptosis Assays</th>
<th>Positive Cells</th>
<th>Negative Cells</th>
<th>Percent Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved caspase-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>27</td>
<td>902</td>
<td>2.9</td>
</tr>
<tr>
<td>BzATP (100 μM)</td>
<td>216</td>
<td>91</td>
<td>18.1*</td>
</tr>
<tr>
<td>TUNEL Control</td>
<td>227</td>
<td>1298</td>
<td>6.6</td>
</tr>
<tr>
<td>BzATP (100 μM)</td>
<td>1343</td>
<td>28</td>
<td>14.5*</td>
</tr>
<tr>
<td>Spheroid nuclei</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>76</td>
<td>786</td>
<td>3.5</td>
</tr>
<tr>
<td>BzATP (100 μM)</td>
<td></td>
<td></td>
<td>8.8*</td>
</tr>
</tbody>
</table>

Cleaved caspase-3 reactivity, TUNEL positivity and Hoechst stained nuclei were assessed in microvessels after exposure to solution A without (control) or with 100 μM BzATP. Active caspase-3 was assayed 4 hours after the onset of BzATP exposure; the TUNEL assay was performed after 8 hours of BzATP exposure, and microvessels were stained with Hoechst 33258 after 14 hours of exposure to the P2X7 agonist. The P2X7 agonist BzATP induced apoptosis in retinal microvessels.

**P2X7-Induced Cell Death in Diabetic Microvessels**

Because apoptotic cell death in the pericyte-containing microvasculature is a well-known feature of diabetic retinopathy,1 we also assessed the effect of BzATP on retinal microvessels isolated from rats made diabetic by STZ injection. As shown in Figure 3A, there was a marked shift to the left in the dose-response relationship for cell death induced in diabetic, compared with nondiabetic, microvessels. Specifically, the concentration of BzATP that was half-maximally effective in causing microvascular cell death was 45 μM for the nondiabetic microvessels, but only 0.6 μM for those of the diabetic retina. In agreement with diabetes increasing the vulnerability of retinal microvessels to apoptotic cell death triggered by BzATP, exposure to a 1-μM concentration of this P2X7 agonist induced a significant (P < 0.001) increase in apoptosis in diabetic, but not (P > 0.25) in nondiabetic, microvessels (Table 2).

How soon after the onset of STZ-induced diabetes was the vulnerability to BzATP increased? To address this question, we compared BzATP-induced death in retinal microvessels isolated from nondiabetic rats and from rats that were diabetic for various periods (Fig. 3B). One week after the onset of experimental diabetes, cell death induced by 1 μM BzATP was not significantly (P = 0.38) affected. However, by the second week of diabetes, this P2X7 agonist induced markedly (P < 0.001) more cell death. These findings demonstrate that the vulnerability of retinal microvessels to BzATP-induced cell death increases early in the course of diabetes.

We considered the possibility that the increased lethality of P2X7 receptor activation was a manifestation of a nonspecific vulnerability of diabetic microvessels. However, contrary to this possibility, the procedure to isolate microvessels did not result in significantly (P = 0.24) more cell death in the diabetic group; immediately after isolation, 96% ± 1% and 94% ± 1% of the microvascular cells were viable in the nondiabetic (n = 53 coverslips) and diabetic groups (n = 22, 23 ± 2 days after STZ injection), respectively. Nor did maintenance for 24 hours in solution A (without P2X7 agonists) result in more (P = 0.55) cell death in diabetic microvessels; 88% ± 2% (n = 14) and 90% ± 2% (n = 10) of the cells were viable in the nondiabetic and diabetic microvessels, respectively. Also consistent with a lack of effect of diabetes on cell death in the absence of P2X7 agonists, apoptosis was not significantly (P > 0.5) different in the nondiabetic and diabetic microvascular cells incubated in solution A (without BzATP; Table 2). Additional evidence suggesting that the membrane integrity of diabetic microvascular cells was not different from that in the control cells was the lack of a significant (P = 0.51) difference in the percentage of cells that were permeable to the propidium diiodide dye YO-PRO-1. Specifically, after a 60-minute incubation in 5 μM YO-PRO-1 (without BzATP), 11% ± 1% (n = 3 coverslips) and 15% ± 5% (n = 3) of the cells were fluorescent in the nondiabetic and diabetic (26 ± 2 days after STZ injection) microvessels, respectively. Furthermore, indicative of diabetes not causing generalized damage, pericytes in nondiabetic (n = 35) and

**Table 2.** Apoptosis Induced by 1 μM BzATP in Nondiabetic and Diabetic Microvessels

<table>
<thead>
<tr>
<th>Apoptosis Assays</th>
<th>Percent Apoptotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nondiabetic Cells</td>
</tr>
<tr>
<td></td>
<td>Diabetic Cells</td>
</tr>
<tr>
<td>Cleaved caspase-3</td>
<td></td>
</tr>
<tr>
<td>No BzATP</td>
<td>2.8</td>
</tr>
<tr>
<td>BzATP (1 μM)</td>
<td>1.8</td>
</tr>
<tr>
<td>BzATP-induced</td>
<td>−0.5**</td>
</tr>
<tr>
<td>Spheroid nuclei</td>
<td></td>
</tr>
<tr>
<td>No BzATP</td>
<td>3.4</td>
</tr>
<tr>
<td>BzATP (1 μM)</td>
<td>2.5</td>
</tr>
<tr>
<td>BzATP-induced</td>
<td>−0.9**</td>
</tr>
</tbody>
</table>

Differences in basal (no BzATP) apoptosis in nondiabetic and diabetic groups were not significant (P > 0.05). Differences between the values in Tables 1 and 2 for the incidence of apoptotic cells in nondiabetic microvessels maintained in solution A (without BzATP) were not significant (P > 0.6). Diabetic rats were injected with streptozotocin 20 ± 2 days (n = 4) prior to microvessel isolation. BzATP at 1 μM induced apoptosis in diabetic, but not in nondiabetic, retinal microvessels.

* Significant (P ≤ 0.001) BzATP-induced apoptosis; ns, no significant (P > 0.25, Fisher's exact test) BzATP-induced apoptosis.

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diabetic (n = 11; 27 ± 2 days after STZ injection) microvessels had similar (P ≥ 0.14) membrane potentials (~46 ± 2 mV vs. ~45 ± 2 mV), membrane resistances (557 ± 24 MΩ vs. 595 ± 34 MΩ), and membrane capacitances (16 ± 1 pF vs. 13 ± 2 pF). Taken together, these observations indicate that the increased P2X7-induced cell death in diabetic microvessels was not secondary to nonspecific cell injury.

**Effect of Diabetes on P2X7-Induced Pore Formation**

Recently, we demonstrated that activation of P2X7 receptors in retinal microvessels is associated with the formation on large transmembrane pores.8 Because the opening of pores may have lethal consequences due to the leakage of vital intracellular molecules and the disruption of ionic gradients, we assessed the link between P2X7-induced pore formation and microvascular cell death.

If P2X7-induced pores play an essential role in microvascular cell death, then the increased lethality of BzATP should be associated with increased pore formation. To test this prediction, we assessed the effect of this P2X7 agonist on the permeability of microvascular cells to YO-PRO-1 (629 Da), which we and other investigators have used to detect P2X7-induced pores.8,12,19,20 Consistent with a casual relationship between pores and death, exposure to 10 μM BzATP induced YO-PRO permeability in substantially more (P < 0.001) diabetic than nondiabetic microvascular cells (Fig. 4A). This increase in BzATP-induced YO-PRO-1 uptake in diabetic microvessels correlated with the increase in BzATP-induced cell death in these vessels (Fig. 3A).

To obtain additional evidence consistent with pore formation being linked with cell death, YO-PRO uptake into microvascular cells was assessed before and after STZ-induced diabetes (Fig. 4B). Similar to the time course for the development of increased P2X7 lethality in diabetic microvessels (Fig. 3B), the BzATP-induced uptake of YO-PRO-1 increased markedly (P < 0.001) 2 weeks after STZ injection. This 2-week interval between STZ injection and increased pore formation strongly suggests that this diabetogenic drug did not directly affect P2X7 receptors, but by causing chronic hyperglycemia, indirectly altered the response of retinal microvascular cells to P2X7 ligands. Taken together, our observations show that relatively soon after the onset of experimental diabetes, exposure of retinal microvessels to BzATP resulted in enhanced pore formation and apoptotic cell death.
Effect of Diabetes on P2X<sub>7</sub>-Induced Currents

In a series of experiments, we compared the amplitudes of the BzATP-induced current in nondiabetic and diabetic microvessels. If the number of functional P2X<sub>7</sub>-receptor/channels was significantly greater in the diabetic microvessels, then the current induced by this P2X<sub>7</sub> agonist should be larger. However, diabetes did not significantly increase the amplitudes of the currents induced by 1 to 100 μM BzATP (Fig. 4C). This lack of an effect of diabetes on the current generated by the activation of P2X<sub>7</sub>-receptors contrasts with the diabetes-induced increase in P2X<sub>7</sub>-mediated apoptotic cell death (Fig. 3, Table 2) and pore formation (Figs. 4A, 4B). Thus, it appears that the number of functional P2X<sub>7</sub>-receptor/channels was not substantially increased in diabetic microvascular cells, even though P2X<sub>7</sub>-induced YO-PRO-1 uptake and cell death were markedly greater.

A parsimonious explanation for these observations is that, rather than upregulating the number of P2X<sub>7</sub>-receptor/channels, diabetes facilitates the process by which the activation of these receptor/channels results in the formation of lethal pores. In addition, because pores would be expected to increase the membrane conductance, our inability to detect an effect of diabetes on the BzATP-induced current also suggests that the opening of a relatively small population of pores in the microvascular cells of the retina results in a detectable uptake of YO-PRO-1 and the triggering of apoptosis.

Discussion

This study demonstrates that the activation of P2X<sub>7</sub>-receptors can result in the formation of large transmembrane pores and the initiation of apoptosis in retinal microvascular cells. Although relatively high P2X<sub>7</sub>-agonist concentrations were necessary to open pores and trigger cell death in microvessels of the nondiabetic rat retina, far lower ligand concentrations were effective soon after the onset of STZ-induced hyperglycemia. This diabetes-induced increase in sensitivity suggests that extracellular nucleotide concentrations that are nonlethal in the normal retina may cause pores to open and, consequently, microvascular cells to die in the diabetic retina. Thus, an increase in the vulnerability of retinal microvessels to the lethal effect of P2X<sub>7</sub>-receptor activation is a candidate mechanism by which diabetes may cause microvascular cell death in the diabetic retina.

Our working hypothesis is that chronic hyperglycemia due to diabetes enhances the opening of lethal pores during exposure of retinal microvessels to P2X<sub>7</sub>-ligands. Consistent with one aspect of this model, Solini et al. 21 observed that maintenance of cultured fibroblasts in high glucose increased P2X<sub>7</sub>-induced apoptosis. Unfortunately, these investigators did not assay for the presence of pores in the fibroblasts, although they did observe that elevated glucose levels caused the P2X<sub>7</sub>-receptors to aggregate into ringlike structures within the plasma membrane. However, a causal relationship between receptor aggregation, pore formation, and cell death remains to be established. 22

At present, the mechanism by which hyperglycemia enhances P2X<sub>7</sub>-induced pore formation in retinal microvessels remains uncertain. One possibility is that an increased number of functional P2X<sub>7</sub>-receptor/channels results in the formation of more pores. Although this is likely to be the mechanism by which P2X<sub>7</sub>-induced pore formation and cell death are enhanced when monocytes become macrophages, it does not appear to account for our observations. Inconsistent with an increase in functional receptor/channels in diabetic microvascular cells, we observed that the ionic current induced by the P2X<sub>7</sub>-agonist BzATP was not significantly affected by diabetes.

Similarly, Brimium et al. 23 also noted a lack of correlation between the density of functional P2X<sub>7</sub>-receptor/channels and the opening of transmembrane pores in Müller (glial) cells.

An alternative mechanism by which diabetes may increase the lethal effect of P2X<sub>7</sub>-agonists without increasing the number of receptor/channels is to facilitate the transition from activated P2X<sub>7</sub>-receptor/channels to open transmembrane pores. Although we think that this scenario is likely, it is difficult at present to test this hypothesis directly. A major challenge is that the steps linking the activation of P2X<sub>7</sub>-receptors with the opening of pores are not well understood. 24

Elucidation of this process in nondiabetic cells should set the stage for the subsequent characterization of the mechanism by which diabetes facilitates the formation of pores during the activation of P2X<sub>7</sub>-receptor/channels.

Our experiments led us to conclude that only a relatively small number of transmembrane pores are opened in diabetic microvessels during activation of their P2X<sub>7</sub>-receptor/channels. Evidence supporting this conclusion is that diabetes did not cause a detectable increase in the BzATP-induced current, even though pores provide high-conductance pathways for ionic fluxes. 25 Thus, the diabetes-mediated increase in P2X<sub>7</sub>-induced YO-PRO uptake and apoptotic cell death must not require the opening of a large number of transmembrane pores. This is in agreement with the suggestion of others 26 that the number of P2X<sub>7</sub>-induced pores is likely to determine the mechanism of cell death. If many pores are opened, then death is induced rapidly (within minutes), due to osmotic imbalance. 25,27 Conversely, more subtle changes in the intracellular milieu caused by the opening of a few pores causes delayed apoptotic death, as we observed in the retinal microvasculature.

The enhanced formation of lethal transmembrane pores during the activation of P2X<sub>7</sub>-receptor/channels may be a mechanism by which apoptosis is triggered in the microvasculature of the diabetic retina. Potentially exacerbating cell death by this mechanism, the concentration of extracellular ATP may be increased due to the release of this nucleotide by platelets whose aggregation is enhanced in the diabetic retina. 28 However, because extracellular ATP levels have not been measured at sites adjacent to microvascular cells in either the normal or the diabetic retina, the possibility remains that the concentration of this nucleotide may be reduced in the diabetic retina. If ATP levels are markedly decreased, then ATP-induced death in the diabetic retinal microvasculature may be minimal in vivo.

Clearly, although probably not feasible at present, measurement of the luminal and abluminal concentration of ATP in retinal capillaries will help to test our hypothesis that an enhancement of the P2X<sub>7</sub>-channel-to-pore transition plays a role in diabetic retinopathy.

Our ideas concerning P2X<sub>7</sub>-mediated apoptosis in the retinal microvasculature are based on experiments using freshly isolated microvessels. One benefit of studying microvessels in isolation is that the concentration of purinergic agonists and antagonists could be precisely controlled. In addition, the duration of exposure to these chemicals could be closely regulated. Furthermore, our use of isolated microvessels eliminated confounding effects mediated through the P2X<sub>7</sub>-receptors that are located in nonvascular retinal cells. 29,30 However, it remains to be demonstrated that the apoptotic response triggered in isolated microvessels during P2X<sub>7</sub>-receptor activation also occurs in vivo. In addition, caution must be used when extrapolating results of our experiments using rat microvessels to the human condition. For example, the P2X<sub>7</sub>-receptors expressed by rats and humans appear to have different ligand affinities, 31 and the STZ model in rodents does not fully mimic diabetes in humans. Consequently, the results of future studies...
assembling P2X-mediated transmembrane pore formation and microvascular apoptosis in vivo will be of keen interest.

Despite the noted caveats, the use of freshly isolated microvessels provides a useful experimental preparation to help generate and test new hypotheses concerning mechanisms by which diabetes affects the retinal microvasculature. From our experiments, we propose that diabetes increases the sensitivity of the retinal microvasculature to the lethal effect of P2X7 receptor activation. This may be a previously unrecognized mechanism that plays a role in the progression of diabetic retinopathy. If so, then pharmacologic interference with P2X7-mediated apoptosis may be of some benefit in the prevention of sight-threatening complications of diabetes.

Acknowledgments

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References