Endothelin-1 Enhances Glutamate-Induced Retinal Cell Death, Possibly through ET$_A$ Receptors

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**PURPOSE.** To determine the modification of the glutamate-induced death of retinal neurons by endothelin (ET)-1.

**METHODS.** Cultured retinal neurons from fetal rats were exposed to glutamate (1.0 mM) alone or glutamate with ET-1 (10$^{-10}$–10$^{-7}$ M) for 10 minutes. Neuronal death was assessed by the trypan blue exclusion or TUNEL assays at 2, 6, and 24 hours after the exposure. The effects of adding BQ-123 or BQ-788, ET$_A$, and ET$_B$ receptor antagonists, respectively, in combination with ET-1 was also assessed.

**RESULTS.** Immunohistochemical analyses showed that the ETs as well as ET$_A$ and ET$_B$ receptors were expressed on cultured retinal neurons consisting mainly of amacrine cells. A brief exposure of the cultured retinal neurons to glutamate alone significantly increased the number of dead cells, and the addition of ET-1 with glutamate caused a further significant increase in retinal neuronal death compared with the cells exposed to glutamate alone. A significant increase in neuronal death was detected at doses of 10 nM of ET-1 and higher after a 24-hour exposure (P < 0.05, Dunnett), whereas brief exposure of neurons to up to 1 µM ET-1 alone did not cause delayed cell death of neurons. BQ-123 (10 nM) suppressed the enhancement of retinal toxicity caused by ET-1 (10 nM), whereas BQ-788 had no significant effect.

**CONCLUSIONS.** These results indicate that ET-1 enhances glutamate-induced retinal cell death, possibly through ET$_A$ receptors. ET-1 may act synergistically with glutamate to damage retinal neurons under hypoxic conditions. (Invest OphthalmoV Ital Sci. 2005;46:4684 – 4690) DOI:10.1167/iovs.05-0785

The endothelins (ETs) are a family of 21-amino-acid peptides with three isomers, ET-1, ET-2, and ET-3. ET-1 is the most potent and long-acting vasoconstricting peptide presently known. ET-1 and its G-protein-coupled receptors, ET$_A$ and ET$_B$, are abundantly expressed and widely distributed in ocular tissues, including the sensory retina. Activation of ET$_A$ receptors on the ocular vascular systems induces strong vasoconstriction, whereas stimulation of ET$_B$ receptors leads to dilation through the formation of nitric oxide (NO). Despite extensive studies on the effects induced by ET-1 on the vascular system including retinal and optic nerve head circulation, the precise roles of ET-1 on neurotransmission and neuronal damage in the retina remain to be clarified.

In the brain, it has been determined that the endothelins modulate astroglial activation, gial proliferation, and communication between glial and neuronal cells at gap junctions. In addition, endothelins have been found to be involved in neuronal apoptosis in the central nervous system. Recently, the roles of ET-1 in the pathogenesis of glaucoma have been clarified from different aspects. ET-1 causes loss of optic nerve fibers, reduces the number of retinal ganglion cells, impairs axonal transport in the optic nerve fibers, and induces astroglial proliferation in the human optic nerve head. These findings strongly suggest that ET-1 plays a pivotal role in neuronal survival and glial proliferation in addition to its vascular effects.

Excitotoxicity, caused by glutamate, acting mainly through N-methyl-D-aspartate (NMDA) receptors, has been implicated in the neuronal damage caused by hypoxia and glaucoma. Although little is known about the direct effects of ET-1 on neurons, it has been demonstrated that ET-1 increases neuronal activities and augments neuronal responses to glutamate. From these observations in other systems, we hypothesized that ET-1 would be expressed in retinal neurons and would have several synergistic effects on the glutamate-induced neurotoxicity in the retina. Because of the potent and long-lasting effects of ET-1 on the vascular system, in vitro studies using cultured neurons are needed to determine the direct actions of ET-1 on retinal neurons. Thus, we evaluated the modification of the glutamate-induced death of retinal neurons by ET-1, using cultured retinal neurons from fetal rats.

**MATERIALS AND METHODS**

**Animals**

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

**Chemicals**

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

**Cell Cultures**

Cells for the primary cultures were obtained from the retinas of fetal Wistar rats (gestational age, 19 days), and prepared as described in detail. In brief, retinal tissues were mechanically dissociated, and single-cell suspensions were plated onto plastic coverslips (1.0 × 10$^6$ cells/mL). Retinal cultures were incubated in Earle’s minimum essential medium (MEM) containing 2 mM glutamine, penicillin-streptomycin-
cin (100 U/mL and 50 μg/mL, respectively), and 25 mM HEPES, in an atmosphere of 5% CO₂ and air. The medium was supplemented with 10% heat-inactivated fetal bovine serum. Media and supplements were purchased from Invitrogen-Gibco (Rockville, MD). 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 retinal cells cultured with this protocol consist mainly of amacrine cells, and the population of glial cells is less than 0.1%. Primary cultures of retinal neurons that were maintained for 10 days were used.

**Immunohistochemistry**

To identify the types of retinal neurons in our culture system, immunohistochemical analyses were performed with an avidin-biotin complex (ABC) kit (Vectastain Elite; Vector Laboratories, Burlingame, CA). After 10 days in culture, retinal neurons were fixed with 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS) at room temperature for 30 minutes. After the cells were washed in PBS, endogenous peroxidase activity was blocked by exposing them to 0.3% H₂O₂ for 30 minutes. After a 5-minute exposure to 0.1% Triton X-100, neurons were exposed to rabbit polyclonal anti-neuron-specific enolase (1: 100), mouse monoclonal anti-HPC-1 (1:100), mouse monoclonal anti-Thy-1 (1:100; Chemicon, Temecula, CA), or mouse monoclonal anti-vimentin (1:100) antibodies for 16 hours at 4°C. In control experiments, no primary antibody was used. The neurons were then incubated with biotin-conjugated goat anti-rabbit or anti-mouse IgG (Vector Laboratories) at 1:200 for 1 hour, followed by development with diaminobenzidine.

To determine the percentage of each cell type (all retinal neurons, amacrine cells, retinal ganglion cells, and glial cells) in our cultures, at least 200 isolated cells on each coverslip were identified and counted. We evaluated five coverslips, and more than 1000 cells were analyzed to determine the ratio of each cell type. Data are expressed as the percentage of cells immunolabeled with the different specific antibodies in the total number of cells counted.

To determine whether amacrine cells express ET₄ receptors, neurons were double labeled. For this, cultured cells were fixed with 2% paraformaldehyde in 10 mM PBS at room temperature for 30 minutes. After they were washed with PBS, neurons were treated with PBS containing 0.1% Triton X-100 for 10 minutes at room temperature. After they were blocked with PBS containing 1% BSA and 5% donkey serum, they were exposed to mouse monoclonal anti-HPC-1 (1:100), rabbit polyclonal anti-ET₄, or ET₄ receptor (1:100) antibodies as well as rabbit polyclonal anti-ET antibodies (1:100; Bachem, King of Prussia, PA) diluted in blocking buffer for 1 hour. After the cells were washed several times with PBS, they were reacted with donkey anti-mouse Cy3- (1:500) and anti-rabbit FITC (1:200)-conjugated secondary antibodies (affinity-purified IgGs; Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for another hour. Images were acquired with a fluorescence microscope (VP 500; Olympus, Tokyo, Japan) and digitally merged on a personal computer to identify cells immunopositive for both antibodies.

**Assessment of Neurotoxicity**

Brief exposure of retinal neuronal cells to glutamate for 10 minutes followed by postincubation in glutamate-free medium for 1 hour or longer causes delayed cell death through necrosis and/or apoptosis. For our study, the cultured retinal neurons were exposed to 1 mM glutamate alone or 1 mM glutamate with 0.1, 1.0, 10, and 100 nM of ET-1 for 10 minutes in serum-free medium followed by postincubation in glutamate- and serum-free medium for different lengths of time to determine whether there is a synergistic effect of ET-1 on the glutamate-induced death of retinal neurons. To determine whether there was a time-dependent effect of ET-1 on the neurotoxicity induced by a brief exposure to glutamate (1 mM) alone or glutamate (1 mM) with ET-1 (10 nM) for 10 minutes, the retinal cells were assessed at 2, 6, and 24 hours after the exposure.

To determine which type of receptors mediate the effects of ET-1 on the glutamate-induced retinal cell death, BQ-123 (10 nM), a selective ET₄ receptor antagonist, or BQ-788 (10 nM), a selective ET₄ receptor antagonist, was added to the medium during the 10-minute exposure to glutamate (1 mM) with ET-1 (10 nM).

The degree of neurotoxicity caused by glutamate in combination with ET-1 was assessed quantitatively by the trypan blue exclusion method on five randomly selected coverslips. 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°C–4°C). The fixed cultures were rinsed with physiological saline and examined with a Hoffman modulation microscope at ×200 (Hoffman Modulation Contrast System/IX-70; Olympus, Tokyo, Japan).

To determine the number of living cells, all isolated cells in a microscopic field on five coverslips were counted until the total number of cells counted on each coverslip was more than 200. Cells in clusters were not counted. The prior treatment of the cells was performed by other colleagues and was masked to the examiner. Cells stained with trypan blue were considered to be dead while unstained cells were alive. Data are expressed as the percentage of living cells (unstained cells) in the total number of cells counted.

**TUNEL Assay**

In addition to the trypan blue exclusion assay, the TUNEL (TdT-mediated dUTP nick-end) assay was performed to determine the effects of ET-1 on glutamate-induced neurotoxicity and its receptor interactions. TUNEL-positive neurons were counted 6 hours after exposure to glutamate (1 mM) alone or glutamate with ET-1 (10 nM) in combination with BQ-123 (10 nM) or BQ-788 (10 nM). For the assay, the cells were fixed in 4% paraformaldehyde in PBS for 25 minutes at room temperature and then exposed to 0.2% Triton X-100 for 5 minutes. A commercial kit (DeadEnd; Promega, Madison, WI) was used to incorporate biotinylated dUTP at 3′-OH DNA ends, by using terminal deoxynucleotidyl transferase. Neurons were then washed in PBS, exposed to 0.3% hydrogen peroxide for 5 minutes to block endogenous peroxidase, and incubated in a horseradish peroxidase-streptavidin solution in PBS (1:500) for 30 minutes at room temperature. After the ABC method and development with diaminobenzidine, at least 200 neurons on each coverslip (n = 5 for each condition) were examined by an observer masked to the treatment of the cells. Data are expressed as the percentage of the TUNEL-negative cells (unstained cells) in the total number of cells counted.

**Effects of ET₄ Receptor Agonist on Glutamate-Induced Neuronal Death**

To determine the role of ET₄ receptor on the glutamate-induced death of neurons, we added a selective agonist of the ET₄ receptor, IRL-1620 (10 nM), to the medium of neurons exposed to glutamate (1 mM) for 10 minutes. Neurons were then incubated in glutamate- and serum-free medium for 6 hours, and neuronal death was assessed by trypan blue exclusion and TUNEL assays.

**Statistical Analyses**

The data are expressed as the mean ± SEM, unless otherwise noted. Statistical analysis was performed by one-way analysis of variance (ANOVA). If a significant change was detected in the one-way ANOVA, then a post hoc test was performed for statistical comparisons among groups. For comparisons between two groups, the Student’s t-test was used. The level of significance was set at P < 0.05.

**RESULTS**

**Characterization of Cultured Retinal Neurons**

Immunohistochemical analyses demonstrated that 95.1% ± 0.2% of the total isolated cells were stained with anti-neuron-
specific enolase antibody suggesting that almost all isolated cells were neurons. In contrast, the percentage of cells stained with anti-vimentin antibody, a marker for glia, was 0.7% ± 0.1%. Thus, 95% of the cells in our cultures were neurons and fewer than 1% were glial cells.

The immunoreactivity of the cultured retinal neurons to HPC-1, a specific marker for amacrine cells,29,30 is shown in Figure 1. The percentage of cells immunopositive to anti-HPC-1 antibody was 83.6% ± 2.9% of the total isolated cells (Fig. 1A, 1B), whereas the percentage of cells immunolabeled with anti-Thy 1 antibody, a retinal ganglion cell marker, was 2.3% ± 0.4%. The immunoreactivity to the cells to anti-HPC-1 antibody was more easily recognized by the immunofluorescence assay (Fig. 1C).

Our cultured retinal neurons stained positively with antibodies to ETs and to their receptors (Fig. 2). In the double-labeling studies, the neurons immunopositive to the antibodies against ETA/ETB receptors and ETs were essentially the same as the neurons immunopositive to anti-HPC-1 antibody (Fig. 2). Thus, our cultured retinal neurons consisted mainly of amacrine cells (>80%), and these neurons expressed ETs and their specific receptors.

Effects of ET-1 on Glutamate-Induced Neuronal Death

As reported,28 a 10-minute exposure to 1 mM glutamate followed by a 24-hour incubation in glutamate-free media decreased the number of live cells significantly (from the control level of 75.3% ± 2.1% to 45.5% ± 2.1%; Fig. 3A, P < 0.05, Dunnett). Addition of 10 μM MK-801, an NMDA receptor antagonist, significantly suppressed the reduction of live cells caused by glutamate alone (Fig. 3A, P < 0.05, Dunnett) and the cell viability was 71.7% ± 1.2%.

To determine the effect of ET-1 on glutamate-induced death, the retinal cells were cultured in a mixture of ET-1 and glutamate for 10 minutes and examined after 24 hours (Fig. 3A). The addition of ET-1 with glutamate caused a further decrease in the number of live cells compared with the cells exposed to glutamate alone. There was a decrease in the percentage of live cells detected at doses of 10 nM and higher (P < 0.05, Dunnett, Fig. 3A), and the degree of decrease was dose dependent.

The effects of an exposure to ET-1 alone for 10 minutes on the viability of neurons 24 hours after the exposure are shown in Figure 3B. Up to 1 μM of ET-1 alone did not decrease the cell viability indicating that a brief exposure to ET-1 alone did not lead to delayed cell death of the retinal neurons.

The time-dependent effects of ET-1 on the glutamate-induced cell death were assessed using 10 nM ET-1 and postincubation times of 2, 6, and 24 hours. ET-1 (10 nM) accelerated the glutamate-induced toxicity and significantly decreased cell viability at 6 and 24 hours after the exposure compared to the cells exposed to glutamate alone (Fig. 3C, P < 0.05, Scheffé).

Antagonistic Effects of BQ-123 and BQ-788

From these findings, the following studies on the role of ET-1 receptors were performed using 10 nM ET-1 and postincubation times of 6 hours. Representative photomicrographs of retinal neurons taken 6 hours after exposure to 1 mM glutamate alone or to glutamate with ET-1 in combination with specific antagonists of the receptor subtypes are shown in Figure 4. The effects of BQ-123 and BQ-788 on the synergistic action of ET-1 (10 nM) in glutamate (1 mM)-induced neuronal death are demonstrated by the changes in the percentage of live cells in Figure 5. The percentage of live cells was significantly decreased (to 55.9% ± 0.6%; Figs. 4B, 5) from the control level of 74.8% ± 2.0% (P < 0.05, Dunnett) at 6 hours after the exposure to glutamate (1 mM; Figs. 4A, 5). Addition of 10 nM ET-1 to the glutamate (1 mM) caused a further significant decrease in cell viability to 43.2% ± 1.9% 6 hours after the exposure (Figs. 4C, 5; P < 0.05, Dunnett).

When BQ-123 (10 nM), a selective ETα receptor antagonist, was coadministered with 10 nM ET-1, the reduction of cell viability was suppressed and the percentage of live cells 6...
hours after the exposure was increased to 56.3% ± 1.0% (Figs. 4D, 5). In contrast, BQ-788 (10 nM), a selective ETB receptor antagonist, applied simultaneously with ET-1 (10 nM), did not suppress the effects of ET-1 (Fig. 4E), and cell viabilities 6 hours after the exposure were 46.2% ± 1.1% (Fig. 5), which was not different from the values of the cells exposed to glutamate (1 mM) with ET-1 (10 nM).

The percentage of live cells 6 hours after exposure to glutamate in combination with IRL-1620 (10 nM), a selective ETB receptor agonist, was 54.7% ± 1.2%, which was not different from the level of cells exposed to glutamate alone.

TUNEL Assay
Representative photomicrographs of retinal neurons stained with the TUNEL method taken 6 hours after exposure to 1 mM glutamate alone or glutamate with ET-1 and specific antagonists of the receptor subtypes are shown in Figure 6. The antagonistic effects of BQ-123 and BQ-788 were quantified by calculating the percentages of TUNEL-negative cells (Fig. 7). TUNEL-negative cells were significantly decreased to 52.2% ± 2.9% from the control level of 72.7% ± 0.7% 6 hours after the exposure to glutamate (1 mM), and the addition of 10 nM ET-1 (10 nM) to the glutamate (1 mM) caused a further significant reduction (to 42.5% ± 2.0%; Figs. 6C, 7). The percentage of TUNEL-negative cells in the neurons exposed to glutamate with ET-1 was significantly increased (to 52.3% ± 2.0%) by the combined treatment with BQ-123 (Figs. 6D, 7; P < 0.05, Fishers). However, BQ-788 (10.0 nM) failed to suppress the effects by ET-1 (Figs. 6E, 7).

The percentage of TUNEL-negative cells exposed to glutamate in combination with IRL-1620, a selective ETB receptor agonist, was 52.9% ± 1.8%, which was not different from the level of cells exposed to glutamate alone.

DISCUSSION
These results with two different cell death assays demonstrated that ET-1 enhanced the glutamate-induced death of retinal neurons consisting mainly of amacrine cells. The synergistic effects of ET-1 on the glutamate-induced death of retinal neurons were observed at ET-1 concentrations of 10 nM and higher, which suggests that ET-1 plays a role in modifying the neuronal death signaling pathway in the retina.
Glutamate-induced neurotoxicity is triggered by Ca\(^{2+}\)/H\(_{11001}\) influx into neurons by NMDA receptors,\(^{31}\) and this pathway is critical because 0.8 mM extracellular Mg\(^{2+}\)/H\(_{11001}\), which is known to block these channel but not the voltage-dependent Ca\(^{2+}\)/H\(_{11001}\) channels, has a neuroprotective effect against glutamate-induced neurotoxicity.\(^{31}\) ET-1 is known to increase the intracellular Ca\(^{2+}\)/H\(_{11001}\) concentration in glial and neuronal cells. Therefore, the ET-1-induced enhancement in glutamate neurotoxicity may be related to the ET-1-induced increase in intracellular Ca\(^{2+}\)/H\(_{11001}\) concentration.

The mechanism for the ET-1-induced changes in the intracellular Ca\(^{2+}\)/H\(_{11001}\) concentration is different for glial and neuronal cells, and the contribution of the ET\(_{A}\) and ET\(_{B}\) receptors to the effects are somewhat complex and still not fully understood. For example, the ET-1-induced Ca\(^{2+}\)/H\(_{11001}\) increase in the ND7/104 sensory neuronal cell line has been shown to be mediated by ET\(_{A}\) receptors and not by ET\(_{B}\) receptors.\(^{32}\) However, the ET-1-induced increase in intracellular Ca\(^{2+}\)/H\(_{11001}\) concentration in astrocytes in the hippocampus and the optic nerve head is through both ET\(_{A}\) and ET\(_{B}\) receptors.\(^{10,22}\) In contrast, activation of ET\(_{B}\) receptors suppresses amyloid β-induced Ca\(^{2+}\)/H\(_{11001}\) uptake into cortical neurons and protects the cells from apoptosis through ET\(_{B}\) receptors.\(^{12}\) Our very different effects of BQ-123 and BQ-788 suggest that ET-1 increases the Ca\(^{2+}\)/H\(_{11001}\) influx to retinal neurons, most likely through ET\(_{A}\) receptors.

The results of our immunohistochemical studies indicate that our cultured retinal neurons, mainly amacrine cells, not only possessed ET\(_{A}\) and ET\(_{B}\) receptors but also expressed ETs. Hypoxia is one of the most potent inducers of the expression of the ET-1 gene in endothelial cells\(^{33}\) and cardiomyocytes,\(^{34}\) and this induction is achieved by the upregulation of hypoxia-inducible factor (HIF)-1\(_{α}\),\(^{34}\) which activates the transcription of target genes including ET-1.\(^{35,36}\) In the eye, hypoxia also augments ET-1 expression in the optic nerve head astrocytes in the presence of TNF-α.\(^{37}\) The mRNA of HIF-1\(_{α}\) is expressed in most human and rodent tissues. If this is also the case in the...
retinal neurons, enhancement of ET-1 expression by hypoxia may make these retinal neurons more vulnerable to glutamate in an autocrine mode of action.

Amacrine cells were the main type of retinal neuron in our cultures, and they have neural connections with bipolar, horizontal, and retinal ganglion cells (RGCs). They regulate the activities of these neurons by releasing several neurotransmitters such as glycine, γ-aminobutyric acid (GABA), acetylcholine, and dopamine, and they are important for maintaining the quality of vision by modulating the visual field size, switching from rod to cone pathways, and regulating ON-OFF signals. In addition to these physiological properties, amacrine cells can be displaced to the retinal ganglion cell layer and are involved in retinal remodeling and survival from several retinal degenerations. If these amacrine cells are able to release ET-1 as they do with other neurotransmitters, the displaced amacrine cells may affect the survival of other neurons including RGCs in a paracrine mode of action. Indeed, in the hypothalamus, it has been shown that ET-1 regulates the neurotransmission of dopaminergic neurons. Because the expression of ET-1 is increased in the diabetic retina, the synergistic effects of ET-1 on glutamate-induced neurotoxicity may have clinical importance in the development of diabetic retinopathy. This may also account in part for the fact that diabetes is a risk factor for the development of glaucomatous optic neuropathy.

The limitation of our study is that our findings were obtained from cultured retinal neurons consisting mainly of amacrine cells and thus cannot necessarily be extended to other types of retinal neurons (e.g., retinal ganglion cells) and also to adult retinal neurons. Recently, however, it has been demonstrated that ET-1 causes apoptotic cell death of RGC-5 cells, a transformed cell line of RGCs, through ET<sub>B</sub> receptors (Krishnamoorthy R, et al. IOVS 2005;46:ARVO E-Abstract 4656). Clearly, future studies should be directed to investigation of the mechanism of the synergistic action of ET-1 on glutamate-induced death of retinal neurons. However, our findings may suggest that ET-1, in both autocrine and paracrine modes of action, is deeply connected with the survival of retinal neurons.

References

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