
Glial Endothelin-1 Regulates Retinal Blood Flow During Hyperoxia in Cats

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**PURPOSE.** To investigate the role of endothelin-1 (ET-1) in retinal glial cells in regulating retinal blood flow (RBF) during hyperoxia in cats.

**METHODS.** We measured the vessel diameter (D), blood velocity (V), and blood flow (F) simultaneously in first-order retinal arterioles using a laser Doppler velocimetry system. The animals were under general anesthesia during hyperoxia (100% oxygen) for 10 minutes 24 hours after intravitreal injection of L-2-aminoacidic acid (LAA), a gliotoxic compound, or dilute hydrochloric acid (0.01 N) used as the vehicle control. We also measured the changes in the RBF after intravitreal injection of BQ-123, a specific ET type A receptor antagonist, in LAA-treated eyes. To examine if endothelin-converting enzyme-1 (ECE-1), as an ET-1–generating enzyme located in retinal glial cells, immunohistochemical examinations with costaining of antiglial fibrillary acidic protein (GFAP) antibody and anti-ECE-1 antibody were performed in whole-mount retinas.

**RESULTS.** During hyperoxia, the decreases in D, V, and F in response to hyperoxia were attenuated significantly (P < 0.01 for all comparisons) in the LAA-treated eyes compared with the vehicle control (LAA, D, −8.5 ± 1.5%; V, −13.8 ± 1.5%; F, −27.8 ± 3.0% versus vehicle control, D, −16.8 ± 1.3%; V, −26.3 ± 2.0%; F, −48.9 ± 2.4%). In LAA-treated eyes, intravitreal injections of BQ-123 did not change the rate of hyperoxia-induced RBF compared to LAA-treated eyes. The anti-ECE-1 antibody was costained with anti-GFAP antibody in the whole-mount retinas.

**CONCLUSIONS.** The current findings suggest that retinal glial ET-1 may play an important role in regulating RBF during hyperoxia in cats.

Keywords: retinal blood flow, retinal glial cells, hyperoxia, endothelin, endothelin converting enzyme

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Glial cells, including Müller cells and astrocytes as the main glial cells in the retina, are vital for maintaining normal retinal function. Recent animal experiments have shown that glial cells play a principal role in regulating retinal blood flow (RBF) in various conditions, such as increased intraocular pressure (IOP) and photic stimulation. We also showed previously that glial cells play a crucial role in regulating RBf in flier-induced hyperemia in cats. Indeed, impaired glial cellular activity might be related to the pathologic mechanisms of ocular disorders such as diabetic retinopathy (DR) and glaucoma. Moreover, some clinical studies have reported that the hyperoxia-induced vasoconstriction in the retina deteriorates in patients with these diseases. However, it remains unclear how glial cells regulate RBF during hyperoxia.

Systemic hyperoxia constricts the retinal arterioles and decreases the RBF in humans and animals through release of endothelin (ET-1). Previously, we also reported that ET-1 plays a major role in hyperoxia-induced vasoconstriction in cats. Endothelin-1 is a 21 amino acid polypeptide, expressed in both endothelial and glial components in the retina, that has a potent constrictor action by binding to the high-affinity ET type A (ETA) receptor in retinal vascular smooth muscle cells and pericytes. Endothelin-1 is generated from proendothelin-1, the precursor of ET-1, via the action of endothelin-converting enzyme-1 (ECE-1).

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**MATERIALS AND METHODS**

**Animal Preparation**

The Animal Care Committee of Asahikawa Medical University approved the protocols for the use of animals, which adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Science and the ARVO Statement for the Use of Animals in Ophthalmic and Visual Science.
Glial ET-1 Regulates RBF During Hyperoxia in Cats

Vision Research. Fifteen adult European shorthair cats provided by Shirai Laboratory Animals Co., Ltd. (3.4–4.5 kg; Koshigaya, Saitama, Japan) of either sex were tracheostomized and ventilated mechanically with room air containing 2.0% sevoflurane. The flow rate of sevoflurane was maintained at 1.5 L/min during the experiment. Catheters were placed in the femoral arteries and vein. The mean arterial blood pressure (MABP) and heart rate (HR) were monitored continuously with a transducer (PowerLab; ADInstruments, Inc., Colorado Springs, CO, USA) and recorder (LabChart; ADInstruments, Inc.) in the proximal thoracic descending aorta. Pancuronium bromide (0.1 mg/kg/h) (Daichi Sankyo Co., Tokyo, Japan) was infused continuously via the femoral vein to maintain skeletal muscle relaxation during the experiment. With the animal prone, the head was fixed in a stereotaxic instrument. Arterial pH, arterial partial carbon dioxide tension (PaCO₂), arterial partial oxygen tension (PaO₂), and bicarbonate ion (HCO₃⁻) were measured intermittently with a blood gas analyzer (model ABL5; Radiometer, Copenhagen, Denmark). The rectal temperature was monitored and maintained between 37°C and 38°C with a heated blanket. The pupils were dilated with 0.5% tropicamide (Santen Pharmaceutical Co., Osaka, Japan). A 0-diopter contact lens (Seed Co. Ltd., Tokyo, Japan) was placed on the cornea, which was protected by instillation of a drop of sodium hyaluronate (Healon; Abbott Medical Optics, Inc., Abbott Park, IL, USA). A 26-gauge butterfly needle was inserted into the anterior chamber and connected to a pressure transducer and a balanced salt solution (Alcon, Fort Worth, TX, USA) reservoir for monitoring and maintaining the IOP at 10 mm Hg, respectively.

RBF Measurements

A laser Doppler velocimetry system (Laser Blood Flowmeter, model 100; Canon, Inc., Tokyo, Japan) customized for felines was used to measure the retinal arteriolar diameter (D) (in micrometers) and velocity (V) (mm/s) as described previously. The RBF in the arterioles (μL/min) was calculated based on the acquired V and D. Laser Doppler measurements of the temporal retinal arterioles were performed in one eye of each animal. We studied the first-order arterioles because they have relatively straight segments and were sufficiently distant from the adjacent vessels for consistent measurements.

The RBF was calculated using the formula $RBF = S \times V_{mean}$, where $S$ is the cross-sectional area of the retinal arteriole at the laser Doppler measurement site, assuming a circular cross section, and $V_{mean}$ is the mean blood V calculated as $V_{mean} = V_{max}/2$. The MABP was determined using the formula $MABP = diastolic BP + (systolic BP – diastolic BP)/3$, which is the index of the systemic BP. Because the cats were prone during the experiments, the ocular perfusion pressure (OPP) was calculated as OPP = MABP – IOP.27,28

Intravitreal Injections and Chemicals

A 30-gauge needle (100-μL syringe; Hamilton, Reno, NV, USA) was used to perform the intravitreal injections 3 mm posterior to the limbus with care taken to not injure the lens and retina. The head of the needle was positioned over the optic disc region. All drugs were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). BQ-123 was dissolved in phosphate-buffered saline (PBS). L-2-aminoadipic acid was dissolved in 0.01 N hydrochloric acid (HCl) because LAA does not dissolve in PBS. The volume of the intravitreal injections was 50 μL, which does not alter any retinal circulation parameters and minimizes the systemic effects of the inhibitors. Because the volume of the cat vitreous is approximately 2.5 mL, the solution (50 μL) injected into the vitreous cavity is diluted by a factor of 50 near the retinal vessels. Hereafter, we refer to drug concentrations as injected concentrations.

The 60 nM concentration of LAA (estimated final concentrations in the vitreous cavity were 1.2 mM) was chosen because 1.25 mM DL-α-aminoadipic acid causes swelling of the Müller cells and astrocytes and the remaining neural cells are intact. As we reported previously, an observation period of 24 hours after intravitreal injection of LAA was used because no pathologic changes or dysfunction on electroretinography (ERG) was observed in the neural retina and the maximal responses in RBF were obtained in our previous experiment.

Induction of Hyperoxia

Hyperoxia was induced by inhalation of 100% oxygen for 10 minutes 24 hours after injection of 0.01 N HCl or LAA into each cat. The RBF was measured every minute during and after hyperoxia. An average of five measurements obtained at 2-minute intervals was defined as the baseline value before initiation of hyperoxia (Fig. 1). Blood gas analysis was performed before, at the end, and 10 minutes after the end of hyperoxia.

Effect of Intravitreal Injection of BQ-123 After Initiation of Hyperoxia in LAA-Treated Eyes

We used BQ-123 to study if the suppressed responses in RBF during hyperoxia in LAA-treated eyes resulted from the action of ET. BQ-123, an ETA receptor antagonist, was injected into the vitreous for an extracellular concentration of 2.0 × 10⁻⁵ M near the retinal vessels. This concentration ensures a maximal retinal hemodynamic response. Hyperoxia was induced 60 minutes after injection of BQ-123 because we confirmed previously that the effects of BQ-123 (1 mM) reached the maximal level 60 minutes after the injection. BQ-123 was injected 23 hours after LAA to confirm the maximal responses of LAA and BQ-123 (Fig. 1). The RBF was measured every minute during and after hyperoxia (Fig. 1).

Immunohistochemistry

For whole-mount assessment, the eyes were enucleated and fixed in 1% paraformaldehyde for 1 hour. The retina was blocked and permeabilized in 5% goat serum with 0.3% Triton (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS for 1 to 2 hours. The retinas were transferred to primary antibodies diluted in block solution and incubated for 1 hour at room temperature. We used the following specific primary antibodies: a mouse anti-glial fibrillary acidic protein (GFAP)-cy3 antibody (1:400; Sigma-Aldrich Corp.) and a goat anti-EC-E1 (1:200; R & D Systems, Minneapolis, MN, USA). The secondary antibody was anti-goat IgG Alexa Fluor 647–conjugated antibody (Abcam, Inc., Cambridge, MA, USA). The slides were mounted (Dako, Tokyo, Japan), observed for green (cy3) and red (Alexa Fluor 647) staining, and analyzed with a fluorescence microscope (FluoView FV1000; Olympus, Tokyo, Japan). The emission wavelengths were 567 nm (575- to 620-nm band-pass filter, cy3) and 647 nm (655- to 755-nm band-pass filter, Alexa Fluor 647).

Ex Vivo Experiment Using Isolated Retinal Arterioles

We used the porcine retinal arterioles to confirm the effects of LAA on the contractility of the retinal arterioles in cats. The eyes were enucleated immediately from pigs of either sex (age, 16–24 weeks; weight, 25–35 kg) after the animals were killed.
in a local abattoir and transported to the laboratory in a moist chamber on ice.

Isolation and Cannulation of Microvessels

The techniques used to identify, isolate, cannulate, pressurize, and visualize the retinal microvessels have been described previously. Briefly, single second-order retinal arterioles (90–110 μm in situ) were dissected with microdissection forceps and the isolated retinal arterioles were cannulated with a pair of glass micropipettes and pressurized to 55-cm H2O intraluminal pressure without flow using two independent pressure reservoir systems. The internal diameter of the isolated vessels was recorded continuously using video microscopic techniques throughout the experiments.

Control Experiment

Cannulated and pressurized arterioles were bathed in physiological saline solution with albumin (0.1%) at 36°C to 37°C to allow development of basal tone. After the vessels developed a stable basal tone (~30–40 minutes), they were incubated with LAA 60 mM or 0.01 N HCl (vehicle control) for an hour. Thereafter, the vessels were exposed to ET-1 at 1 nM (Sigma-Aldrich Corp.), and the ET-1-induced vasoconstriction was evaluated every 5 minutes for 20 minutes. After 20 minutes, we regarded the vessel caliber as the maximal response.

Statistical Analysis

All data are expressed as the mean percentage ± standard error of the mean. The changes in RBF were calculated as percentage decreases from the baseline measurements. For statistical analysis, we used analysis of variance (ANOVA) for repeated measurements, followed by post hoc comparisons with the Dunnett procedure. Group comparisons of the RBF, systemic parameters, and the ET-1-induced vasoconstrictions in isolated retinal arterioles were performed using the Mann-Whitney U test or Wilcoxon signed rank test. P < 0.05 was considered significant.

RESULTS

Systemic Changes in Response to Hyperoxia

During hyperoxia, there were no significant differences in the pH, PaCO2, HCO3-, MABP, HR, or OPP, whereas the PaO2 increased significantly (P < 0.01) in all groups (Table 1). Intravitreal injection of BQ-123 did not alter any systemic parameters (Supplementary Table S1).

Effects of LAA and BQ-123 on RBF at Rest

Twenty-four hours after injection of LAA (60 mM) alone or LAA (60 mM) + BQ-123 (1 mM), there were no significant changes in the retinal circulatory parameters (n = 5) (Table 2). We preliminarily confirmed the absence of significant differences in the ocular and systemic circulatory parameters at rest between PBS- and the 0.01 N HCl-treated eyes (Supplementary Tables S2, S3).

Effects of LAA on Hyperoxia-Induced Changes in Retinal Circulation

The D, V, and F decreased gradually after induction of hyperoxia compared with baseline in the vehicle control group (Fig. 2A). Ten minutes after the onset of hyperoxia in the vehicle control group, the percentage decreases from baseline were ~16.8 ± 1.3% in D, ~26.3 ± 2.0% in V, and ~48.9 ± 2.4% in F (Fig. 2B). However, 10 minutes after the onset of hyperoxia in LAA-treated eyes, the decreases in the RBF during hyperoxia were attenuated significantly compared with the vehicle controls (LAA 60 mM, D, ~8.5 ± 1.5%; V, ~13.8 ± 1.5%; F, ~27.8 ± 3.0%) (Fig. 2B). After hyperoxia, the retinal circulatory parameters began to return to the baseline values. Ten minutes after the end of hyperoxia, the retinal circulatory parameters recovered to the baseline value in the vehicle controls and LAA-treated eyes. In a preliminary study, we confirmed that there were no significant differences in the ocular and systemic circulatory parameters during hyperoxia.
TABLE 1. Changes in Systemic Parameters During Hyperoxia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before Hyperoxia</th>
<th>After Hyperoxia</th>
<th>Difference</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.41 ± 0.03</td>
<td>7.40 ± 0.02</td>
<td>0.01</td>
<td>0.93</td>
</tr>
<tr>
<td>PaCO₂, mm Hg</td>
<td>29.8 ± 0.7</td>
<td>30.9 ± 0.7</td>
<td>1.1</td>
<td>0.32</td>
</tr>
<tr>
<td>PaO₂, mm Hg</td>
<td>106.0 ± 2.5</td>
<td>106.8 ± 2.2</td>
<td>0.8</td>
<td>0.93</td>
</tr>
<tr>
<td>HCO₃⁻, mEq/L</td>
<td>24.0 ± 4.7</td>
<td>24.0 ± 4.4</td>
<td>0.0</td>
<td>1.00</td>
</tr>
<tr>
<td>MABP, mm Hg</td>
<td>101.8 ± 2.0</td>
<td>101.3 ± 2.1</td>
<td>0.5</td>
<td>0.62</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>123.8 ± 4.6</td>
<td>124.6 ± 4.8</td>
<td>0.8</td>
<td>0.49</td>
</tr>
<tr>
<td>OPP, mm Hg</td>
<td>90.2 ± 2.5</td>
<td>89.7 ± 2.6</td>
<td>0.5</td>
<td>0.63</td>
</tr>
</tbody>
</table>

The data are expressed as the means ± standard errors of the mean. For the comparison before with hyperoxia, we used the Wilcoxon signed-rank test. *P < 0.05 is considered significant. Before, before induction of hyperoxia; hyperoxia, at the end of hyperoxia; after, 10 minutes after the end of hyperoxia.

The data were analyzed using the Mann-Whitney U test. No significant differences were observed between PBS- and the 0.01 N HCl-treated eyes (Supplementary Fig. S1; Supplementary Table S2).

TABLE 2. Effect of BQ-123 on Ocular Parameters (n = 5)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.01 N HCl LAA 60 mM</th>
<th>LAA 60 mM + BQ-123 1 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter, µm</td>
<td>118.1 ± 2.6</td>
<td>115.2 ± 3.1</td>
</tr>
<tr>
<td>Velocity, mm/s</td>
<td>35.6 ± 2.3</td>
<td>35.0 ± 2.1</td>
</tr>
<tr>
<td>Flow, µl/min</td>
<td>11.7 ± 0.8</td>
<td>10.9 ± 0.6</td>
</tr>
</tbody>
</table>

The data are expressed as the means ± standard errors of the mean. The Mann-Whitney U test was used to compare the vehicle controls (0.01 N HCl) with LAA or LAA + BQ-123. There are no significant differences in D, V, or F between the groups.

Effects of BQ-123 in LAA-Treated Eyes During Hyperoxia

As reported previously, 60 minutes after injection of BQ-123 in LAA-treated eyes (before induction of hyperoxia), there were no significant differences in D, V, and F compared with the preinjection levels.

In the LAA (60 mM)-treated eyes with injections of BQ-123 (1 mM), the percentage reductions of all retinal circulatory parameters were comparable to those in the LAA-treated eyes 10 minutes after induction of hyperoxia (Fig. 3). After hyperoxia, the D, V, and F immediately returned to the baseline level (data not shown).

Immunohistochemistry

To examine if the retinal glial cells have endothelin converting enzyme-1 (ECE-1), the costaining of GFAP and ECE-1 was assessed in the whole retina (Fig. 4). Glial fibrillary acidic protein immunofluorescence histochemistry (Fig. 4A), binding of ECE-1 (Fig. 4B), and merged (Fig. 4C) were performed on flat-mounted feline retinal preparations. In the feline retina, ECE was expressed in the retinal glial cells and vessels, although the neurons were not stained (Fig. 4C). In a preliminary study, the control sections were incubated with no primary antibody, with only secondary antibody conjugates, and these sections did not stain (data not shown).

Constriction of LAA-Infused Isolated Retinal Arterioles Induced by ET-1

We examined the effects of LAA on the contractility resulting from ET-1 (1 nM) in the ex vivo porcine retinal isolated vessels using isolated porcine retinal arterioles. There was no significant difference (∼65.6 ± 4.7% vs. −71.9 ± 2.5%; n = 4, P = 0.34) in the contractility of the retinal arterioles between the LAA 60 mM and 0.01 N HCL groups (Fig. 5).

**DISCUSSION**

In the current study, we found that decreases in RBF during hyperoxia were suppressed in LAA-treated eyes compared with vehicle control eyes (Fig. 2), suggesting that retinal glial cells might participate in the regulation in RBF during hyperoxia. Previously, Takagi et al.12 reported that ET-1 did not increase significantly in cultured bovine retinal endothelial cells exposed to hyperoxia in their Northern blot analysis, suggesting that it is reasonable to consider that vascular endothelial cells might not be involved with production of ET-1 in response to systemic hyperoxia. In addition, the
inhibition ratio of the hyperoxia-induced decrease in RBF in eyes injected with LAA and BQ-123 was comparable to that in eyes treated only with LAA (Fig. 4), suggesting that ET-1 might originate from the retinal glial cells during hyperoxia. This possibility also was supported by immunohistochemistry findings, in which the glial cells and ECE-1 were costained (Fig. 4).

Another possibility is that hyperoxia might increase ET-1 production not only in the glial cells but also in the retinal neurons and trigger an alternative pathway for retinal vessel constriction. Although we could not exclude this possibility, our immunohistochemistry findings also showed that the neurons were not stained by anti-ECE-1 in the feline retina (Fig. 4), suggesting that retinal neurons might not be involved in regulating RBF during hyperoxia via production of ET-1.

A hyperoxia-induced decrease in RBF was not suppressed completely by LAA and/or BQ-123 in the current study (Figs. 2, 3), which suggested that there are other mechanisms besides the glial cells or ET-1 in the reduction of RBF during hyperoxia. Rubanyi and Vanhoutte\textsuperscript{35} suggested the possible explanation for the residual response in the hyperoxia-induced decrease in RBF that superoxide anions, which were generated from oxygen in hyperoxia, transiently inactivated nitric oxide (NO) as an endothelium-derived relaxing factor and the inactivated NO causes the vasoconstriction in the canine coronary artery.\textsuperscript{35}

We reported previously that L-NG-nitroarginine methyl ester, a NO synthase inhibitor, did not increase the rate by which RBF decreased during hyperoxia,\textsuperscript{15} which supported the theory of Rubanyi and Vanhoutte.\textsuperscript{35} Although we did not examine the effects of superoxide anions on the current results, the transient inactivation of NO by superoxide anions might lead to the residual response besides ET-1 in the hyperoxia-induced decrease in RBF.

In the current study, immunohistochemistry findings indicated that anti-ECE-1 and anti-GFAP were costained in the feline retina (Fig. 4), suggesting that ECE-1 was expressed in the retinal glial cells. In contrast, there was no staining of anti-ET-1 antibody in the glial cells (data not shown), possibly because ET-1 is merely released under normoxic conditions.\textsuperscript{36}

Unfortunately, we could not immediately enucleate eyes under induction of hyperoxia in the retinal circulation in response to hyperoxia in the groups in (A). The data are expressed as the mean percentages ± SEs of the baseline values. *P < 0.05 compared with baseline by 2-way repeated-measures ANOVA followed by the Dunnett procedure.
the hyperoxic condition because of a technical difficulty in the current study. Alternatively, further experiments using cultured Müller glial cells or retinal astrocytes exposed to hyperoxic conditions are needed to elucidate the mechanisms underlying the hyperoxia-induced increase in ET-1 production in retinal glial cells.

Some ocular diseases, such as DR and glaucoma, impair the regulation of ocular blood flow and reduce the response in hyperoxia-induced vasoconstriction in the retina. In addition, impaired glial cell activity might be related to the pathologic mechanisms of those ocular disorders. In the current study, we showed the dysregulation in the RBF during hyperoxia in LAA-treated eyes (Fig. 2). Although we did not use the disease model in this study, the results indicated that glial dysfunction might be associated with the pathogenesis of DR and/or glaucoma via impaired RBF regulation. Further basic studies using the disease model and more clinical investigations are warranted to examine whether improved glial function is a novel target for treating ocular vascular disorders.

The current study had some limitations. First, the current results did not show which retinal glial cells, the Müller glial cells or astrocytes, play a central role in regulating RBF during hyperoxia, because it is difficult to suppress each cellular function separately pharmacologically using a gliotoxic compound, which affects both of them. Second, although the current data did not provide a definitive explanation for the effect of general anesthesia, we found in a preliminary study that sevoflurane per se did not change the vessel D of isolated
porcine retinal arterioles (data not shown), and changes in the concentrations of pancuronium bromide did not alter the RBF in cats anesthetized with sevoflurane (data not shown).\(^4\)\(^5\)

Finally, as we reported previously,\(^5\) we could not quantify the degree of functional damage in the retinal glial cells after intravitreal injection of LAA at a concentration of 60 mM, although LAA at 60 mM selectively damaged the glial cells without hurting the neurons and retinal vasculature in the feline retinas. More advanced techniques and research are needed to resolve these issues. In the current study, we examined the effects of LAA on the contractility caused by ET-1 (1 nM) in the retinal vessels using isolated porcine retinal arterioles (Fig. 5) and found that there was no significant difference in the contractility of the retinal arterioles between the LAA and the vehicle control groups, suggesting that this compound did not directly affect the contractility of the retinal blood vessels.

In summary, we found that the hyperoxia-induced decrease in RBF was suppressed significantly in LAA-treated eyes and the suppressed response was comparable to that in LAA-treated eyes with intravitreal injection of BQ-123 as the ETA receptor antagonist, suggesting that the glial cells might be involved in regulating RBF during hyperoxia by releasing ET-1 in the glial cells. Because previous reports have indicated that glial cells could be involved in regulating RBF during hyperoxia by releasing ET-1 in the glial cells. Because previous reports have indicated that glial cells Participation in the autoregulation of optic nerve head blood flow in rabbits. Invest Ophthalmol Vis Sci. 2012;53:3726–3732.


20. Meyer P, Flammer J, Luscher TF. Endothelin-dependent regulation of the ophthalmic microcirculation in the perfused


