Physiology and Pharmacology

Involvement of Glial Cells in the Autoregulation of Optic Nerve Head Blood Flow in Rabbits

Maho Shibata, Tetsuya Sugiyama, Takuji Kurimoto, Hidehiro Oku, Takashi Okuno, Takatoshi Kobayashi, and Tsunehiko Ikeda

Purpose. To investigate the involvement of glial cells in the autoregulation of optic nerve head (ONH) blood flow in response to elevated intraocular pressure (IOP).

Methods. Rabbit eyes were treated with an intravitreal injection of L-2-aminoadipic acid (LAA), a gliotoxic compound. Twenty-four hours after the injection IOP was artificially elevated from a baseline of 20 to 50 or 70 mm Hg and maintained at each IOP level for 30 minutes. ONH blood flow was measured by laser speckle flowgraphy every 10 minutes. Ocular perfusion pressure (OPP) was calculated to investigate the relationship between ONH blood flow and OPP. To evaluate the effects of LAA on the function and morphology of retinal neurons and glial cells, electroretinogram (ERG) was monitored after injections of LAA (2.0 and 6.0 mM) or saline as a control. Histologic and immunohistochemical examinations were then performed.

Results. In the LAA-treated eyes, histologic changes selectively occurred in the retinal Müller cells and ONH astrocytes. There was not any significant reduction of amplitude or elongation of implicit time of each parameter in the ERG after LAA injection compared with control. ONH blood flow in LAA-treated eyes was significantly decreased with a reduction of OPP during IOP elevation to 50 and 70 mm Hg, whereas blood flow was maintained in control eyes during IOP elevation to 50 mm Hg.

Conclusions. These results indicate the involvement of glial ells in the autoregulation of ONH blood flow during IOP elevation.
(*Invest Ophthalmol Vis Sci.* 2012;53:3726–3732) DOI: 10.1167/iovs.11-9316

Glaucoma is a complex disease with various risk factors including vascular factors, genetics, and other systemic conditions.[1] Decreased ocular perfusion pressure (OPP) is reported to be associated with an increased prevalence of primary open-angle glaucoma (POAG).[2,3] Furthermore, recent studies have also reported that reduced OPP and reduced systolic blood pressure were risk factors, independent of intraocular pressure (IOP), for the progression of glaucoma.[4,5]

The blood flow in the retina and optic nerve head (ONH) is stably maintained despite certain changes in OPP and this ability is recognized as a function of autoregulation. ONH blood flow is adjusted to the metabolic needs through an autoregulatory mechanism, which maintains a constant nutrient supply.[6] Several mechanisms of this autoregulation have been previously demonstrated and include metabolic, myogenic, neurogenic, humoral, and endothelial-mediated factors.[7,8] On the other hand, dysfunction of autoregulation has been suggested to be involved in the etiology of glaucoma.[9–13]

Glial cells are important components of the eye for maintaining neuronal activity and structural stability. Astrocytes are the predominant cell type in the ONH, whereas Müller cells and astrocytes are the main glial cells in the retina.[14] It has been shown that the endfoot processes of retinal astrocytes surround the vascular wall and form a thin layer of sheath.[15] This close association suggests that astrocytes could potentially play a role in the regulation of blood flow in the ONH and retina. Although previous reports showed some evidence that astrocytes are the cells responsible for pathologic changes in the glaucomatous ONH,[14] involvement of glial cells in the autoregulatory mechanism has not been well determined. We hypothesize that the connection between glial cells and vascular tissues plays important roles in the autoregulation.

L-2-Aminoadipic acid (LAA), a six-carbon homolog of glutamate, is known as a gliotoxic compound. Since Olney et al.[16] described its selective effect on Müller cells after subcutaneous administration of DL-α-aminoadipic acid to infant mice, there have been several reports suggesting the specific effect on glial cells by intravitreal injection of LAA.[17,18]

In this study, we measured the time course of changes in ONH blood flow in response to elevated IOP by laser speckle flowgraphy (LSFG) to examine the autoregulatory mechanism and also investigated the effects of LAA on the autoregulation. Furthermore, the effects of LAA on glial cells and neurons were verified using electroretinograms (ERGs) and both histologic and immunohistochemical examinations.

Materials and Methods

Animals

Male albino rabbits weighing 2.7 to 3.2 kg (Shimizu Laboratory Supplies, Kyoto, Japan) were used and handled in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research. The experimental protocol was approved by the Committee of Animal Use and Care of the Osaka Medical College, Japan.

Chemicals

LAA was purchased from Sigma Chemicals (St. Louis, MO). LAA solution was diluted in saline to a final concentration of 2.0 and 6.0 mM.

From the Department of Ophthalmology, Osaka Medical College, Takatsuki, Osaka, Japan.

Supported in part by the Osaka Eye Bank.

Submitted for publication December 14, 2011; revised March 18 and April 23, 2012; accepted May 5, 2012.

Disclosure: **M. Shibata**, None; **T. Sugiyama**, None; **T. Kurimoto**, None; **H. Oku**, None; **T. Okuno**, None; **T. Kobayashi**, None; **T. Ikeda**, None

Corresponding author: Tetsuya Sugiyama, Department of Ophthalmology, Osaka Medical College, 2-7, Daigaku-machi, Takatsuki, Osaka, 569-8686 Japan; tsugiyama@poh.osaka-med.ac.jp.

Investigative Ophthalmology & Visual Science, June 2012, Vol. 53, No. 7
Copyright 2012 The Association for Research in Vision and Ophthalmology, Inc.

Experimental Protocol

LAA, a specific gliotoxic agent, was intravitreally injected to impair glial cells in rabbit eyes. To evaluate the effect of LAA on ONH blood flow in response to IOP elevation and its dose dependence, LAA solution (0.5 mL) of each concentration (2.0 and 6.0 mM) or 0.5 mL saline (as a control) was injected into the vitreous of rabbits. These concentrations were chosen because 1.25 mM DL-α-aminoadipic acid has been shown to cause swelling of Müller cells and astrocytes, whereas neural cells remained intact.[19] In addition, we found that LAA at 20 mM had significant effects on ERG b-waves at 24 hours in the preliminary experiment. The time interval between LAA injection and the main experiment was decided since it was reported that pathologic changes were not observed in the retina, although Müller cells had slightly pale stained nuclei and increased amount of glycogen granules 24 hours after the injection of LAA.[19] Following mydriasis with topical 0.4% tropicamide (Mydrin M; Santen, Osaka, Japan), LAA or saline was injected into the vitreous through the pars plana via a 30-gauge needle under topical anesthesia with 0.4% oxybuprocaine hydrochloride (Benoxil; Santen). IOP, ONH blood flow, and ERG were recorded before and 24 hours after the injection. IOP was measured with a calibrated pneumatonometer (Model 30 Classic; Medtronic Solan, Jacksonville, FL) under topical anesthesia with oxybuprocaine hydrochloride (Benoxil; Santen). Methods for ONH measurement and ERG recording are described in the following text. Twenty-four hours after the intravitreal injection of LAA or saline, we performed the experiment of ONH blood flow measurement during an artificial IOP elevation, then enucleated eyes for histologic analyses after rabbits were euthanized. Right eyes of rabbits were used in the study.

Changes in ONH blood flow in response to elevated IOP were measured by LSFG under topical anesthesia with oxybuprocaine hydrochloride (Benoxil; Santen). After IOP was adjusted to 20 mm Hg for at least 10 minutes, a baseline level of ONH blood flow was measured. IOPs were then elevated to 50 and 70 mm Hg and maintained at each level for 30 minutes. Measurements of ONH blood flow were carried out every 10 minutes at each IOP level. Thus, in addition to the baseline level, three recordings at each IOP level of 50 or 70 mm Hg were recorded, for a total of seven recordings for each rabbit. We compared ONH blood flow in LAA-treated eyes (2.0 and 6.0 mM; $n = 6$) with control animals, who received an intravitreal injection of saline ($n = 7$). Blood pressure was simultaneously monitored at each recording time of ONH blood flow and the relationship between ONH blood flow and OPP was investigated by plotting OPP in the abscissa and relative square blur ratio (SBR) values to the baseline in the ordinate.

IOP Elevation and Measurement of Blood Pressure

A 20-G infusion cannula was inserted into the vitreous cavity through the pars plana under topical anesthesia with 0.4% oxybuprocaine hydrochloride (Benoxil; Santen). This infusion cannula was sutured to the sclera with 5-0 nylon then connected to a bottle of intraocular irrigating solution (BSS PLUS; Alcon Japan, Tokyo, Japan) through a pressure transducer (P10EZ; Gould Statham Instruments, Hatorey, Puerto Rico) for continuous monitoring of actual IOPs. The IOP was artificially elevated by increasing the height of the bottle.

During the experiments, systemic arterial blood pressure and pulse rates were measured on the front leg by an automatic sphygmomanometer (BP-98E, Softron, Tokyo, Japan), which allowed a noninvasive measurement of systemic arterial pressure. A close correspondence between the blood pressure measured on the foreleg by the sphygmomanometer and that obtained through a pressure transducer cannula placed in the femoral artery has been confirmed previously.[20] Systemic blood pressure was measured three times every 10 minutes then averaged to obtain data measured at each time point.

The mean arterial blood pressure (MBP, mm Hg) was calculated as follows: $MBP = DBP + 1/3(SBP - DBP)$, where DBP and SBP were the diastolic and systolic arterial blood pressures, respectively. The OPP (mm Hg) was calculated as follows: $OPP = MBP - IOP - 14$, where -14 was the compensator for the discrepancy in pressure between the femoral artery and the ophthalmic artery in the prone-positioned rabbit.[21,22]

Measurement of ONH Blood Flow

ONH blood flow was measured using LSFG (Softcare Ltd., Fukuoka, Japan), a noninvasive, two-dimensional measurement of tissue circulation. The instrument consists of a fundus camera with a diode laser (wavelength 808 nm), an image sensor (100×100 pixels), and a personal computer. The principles of this method are described in detail elsewhere.[23,24] Briefly, when the ocular fundus is illuminated with a laser, a speckled pattern, the frequency of which varies with velocity of blood flow, appears. The normalized blur (NB) value obtained by this method represents the blurring of the speckle pattern formed by scattered light, which is regarded as a quantitative index of blood velocity. When a near-infrared diode laser is used (wavelength 808 nm), the NB value was positively correlated with capillary circulation in the ONH.[25] The square blur ratio (SBR), another index for quantitative estimation of blood velocity, is proportional to the square of the NB. The SBR is theoretically a more exact measurement proportional to velocity.[24] SBR values were obtained by 60 scans for 2 seconds and averaged through that period in this study. Following mydriasis with topical 0.4% tropicamide (Mydrin M; Santen), rabbits were placed in holding boxes and measurements were performed under topical anesthesia with oxybuprocaine hydrochloride (Benoxil; Santen). The SBR level in the most widely available rectangular area free of visible vessels in the ONH was calculated. The SBR values were measured serially at the same area, and the mean SBR value for five successive measurements at each time point was used.

Recording and Analysis of ERG

To determine selective gliotoxicity of LAA on retinal function, ERG was performed. For recording, a photic stimulator (SLS4100; Nihon-Kohden, Tokyo, Japan), a biophysical amplifier (AVM-10; Nihon-Kohden) and an average (DAT-1100; Nihon-Kohden) were used. Before ERG recordings, rabbits were adapted to the dark for 60 minutes following mydriasis with topical 0.4% tropicamide (Mydrin M; Santen). The ERG was performed under topical anesthesia with oxybuprocaine hydrochloride (Benoxil; Santen) with a 4.7-cal light stimulus set at 20 cm in front of the eye and recordings were made with a gold ring active electrode on the cornea by averaging four responses to the light stimuli at 0.1 Hz. A diffuser was placed before the stimulated eye to ensure a more full-field stimulation and the mean luminance at the corneal surface was 43.8 lux/s. Bandpass filters were set at 1.5 to 100 Hz for a- and b-waves. The amplitudes and the implicit times of the a- and b-waves were measured. Analog data were recorded by a rectilinear pen system and simultaneously stored and digitized using a microcomputer (Maclab 2e; AD Instruments, Castle Hill, Australia). Analysis of the various stored parameters was performed using the microcomputer.

We performed ERG before and 24 hours after intravitreal injection of LAA (2.0 and 6.0 mM; $n = 7$ and 5, respectively) or phosphate-buffered saline (PBS) as controls ($n = 5$) and the amplitudes and the implicit times of the a- and b-waves of LAA-treated eyes were compared with those of the control.

Histologic Examination

To determine the gliotoxic effects of LAA, rabbits were euthanized for histologic examination 24 hours after intravitreal injection of LAA or saline solution. Enucleated eyes were fixed in 2% paraformaldehyde–2.5% glutaraldehyde in 10 mM PBS for 3 hours, then fixed in 4% paraformaldehyde, and embedded in paraffin. A transverse section of each retina (5 μm) was cut parallel to the medullary rays 2 mm directly inferior to the center of the ONH. The section of the retina was stained with hematoxylin and eosin and examined by light microscopy.

Table 1. Intraocular Pressure (IOP) and Square Blur Rate (SBR) in Optic Nerve Head before and 24 Hours after Intravitreal Injection of L-2-Aminoadipic Acid (LAA)

	Before Injection	After Injection	*P* Values
IOP			
Control	15.4 ± 1.3	13.7 ± 1.8	0.56
LAA (2 mM)	15.9 ± 2.9	16.0 ± 3.2	0.97
LAA (6 mM)	15.7 ± 3.0	15.4 ± 2.4	0.78
SBR			
Control	7.3 ± 1.0	7.6 ± 0.5	0.55
LAA (2 mM)	6.2 ± 1.3	6.0 ± 1.7	0.72
LAA (6 mM)	6.8 ± 2.0	6.8 ± 1.5	0.83

Data are expressed as mean ± SD for 6 to 7 rabbits. *P* values obtained by paired *t*-test are shown in this table.

To evaluate retinal damages, cells in the ganglion cell layer (GCL), the inner nuclear layer (INL), and outer nuclear layer (ONL) were counted in transverse sections. For this analysis, six light photomicrographs taken at ×300 magnification, at a distance of approximately 5 mm from the center of the ONH, were obtained in a masked fashion. All cells in each layer in these photographs were counted. Displaced amacrine cells were not excluded from the counts, as described elsewhere.[26] The number of cells was averaged for each eye to obtain data for statistical analysis. Morphologic changes were also examined.

Immunohistochemistry

To evaluate pathologic changes in Müller cells in the transverse section of the retina, each section was subjected to immunohistochemistry with glial fibrillary acidic protein (GFAP), a marker of glial cells. After deparaffinization, each section was treated with a 1% hydrogen peroxide solution for 5 minutes, incubated with primary antibody, mouse monoclonal antibody to GFAP (ab11839, Abcam plc., Cambridge, UK) at 4°C overnight, then with a secondary antibody, biotin-conjugated goat anti-mouse immunoglobulin G (IgG; LSAB2, Dako Cytomation, Glostrup, Denmark) at room temperature for 30 minutes, and streptABComplex/HRP (Dako Cytomation) for 30 minutes. The resulting sections were stained by development with diaminobenzidine (Dojin Chemical, Kumamoto, Japan) and nuclear staining was performed with hematoxylin.

GFAP is normally expressed in retinal astrocytes, but its expression intensity is altered by several pathologic conditions with additional changes in size and number.[15,26] Astrocytes were visualized in flat-mounted retina. Dissected retinas for flat mount were fixed in 4% paraformaldehyde overnight at 4°C, then blocked in 10% normal goat serum and 2% bovine serum albumin for 1 hour and incubated with mouse monoclonal anti-GFAP (Sigma-Aldrich, St. Louis, MO) and goat polyclonal anti-connexin 43 (ab87645; Abcam plc., Cambridge, UK), both diluted 1:500 at 4°C for 3 days. After extensive washing, the retinas were incubated with fluorescein isothiocyanate (FITC)–conjugated secondary goat anti-mouse antibody (Sigma-Aldrich) and Alexa 594–conjugated secondary donkey anti-goat antibody (Sigma-Aldrich), both diluted 1:500 at room temperature for 2 hours. The tissues, mounted on slides, were viewed through the confocal laser-scanning microscope (LSM510; Carl Zeiss, Oberkochen, Germany). The observed area was several parts of the ONH. To quantify the expression of GFAP, images of stained astrocytes were acquired and analyzed using ImageJ image-analysis software (1.43u; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Mean densities of four sites (300 × 300 μm) for each group, selected randomly out of the observed area mentioned earlier, were measured and the averages were compared.

Statistical Analysis

The data are expressed as the mean ± SD or SEM. Statistical analysis was performed using one-way ANOVA or two-way ANOVA for repeated measurements. For comparison of levels at each time of assessment among three groups, Dunnett's test was used. Interactions between ONH blood flow and OPP were analyzed by a single linear regression. The level of significance was set at $P < 0.05$.

Results

Effect of LAA on IOP, Blood Pressure, and Autoregulation of ONH Blood Flow

IOPs and SBR values before and 24 hours after the injection of LAA are shown in Table 1, which detected no significant change in each group. MBPs during IOP elevation are shown in Table 2, in which no significant change was found. Figure 1A demonstrates the changes of SBR values in response to IOP alterations. Since SBR values are relative values, they are expressed relative to the baseline measured at an IOP of 20 mm Hg in % (%SBR). In control eyes with saline injection, the SBR value was maintained at the baseline level when IOP was elevated from 20 to 50 mm Hg, whereas the SBR value significantly ($P < 0.01$, one-way ANOVA) decreased when IOP was elevated from 50 to 70 mm Hg. However, levels were still maintained at 73.4 ± 10.5% (mean ± SEM). In LAA-treated eyes, SBR values were significantly reduced after IOP elevation from 20 to 50 and 70 mm Hg and the effect of LAA on the changes in ONH blood flow was dose dependent. Two-way ANOVA for repeated measurements showed a significant difference between the control and 6.0 mM LAA-treated eyes ($P = 0.0187$, repeated-measures ANOVA). No significant difference was detected between the control and 2.0 mM LAA-treated eyes ($P = 0.599$, repeated-measures ANOVA). Figure 1B demonstrates the changes of SBR values in response to the change in OPP. When OPP was approximately 30–40 mm Hg, relative SBR was maintained in the control eyes, whereas it was obviously decreased in 6.0 mM LAA-treated eyes.

Figure 2 demonstrates linear regression analyses between relative SBR values and OPPs after IOP elevation from 20 to 50 mm Hg. Each dot was plotted using data when IOP was set at 20 or 50 mm Hg in each rabbit of each group. In control eyes, a significant interaction was not observed ($r = -0.18$, $P > 0.05$, Pearson's correlation coefficient). In other words, ONH blood flow did not change in spite of the change in OPP. In contrast, in LAA-treated eyes (LAA: 2.0 and 6.0 mM), significant and positive relationships were recognized between relative SBR values and OPPs ($r = 0.58$, $P = 0.0013$ and $r = 0.57$, $P = 0.0035$,

Table 2. Changes in Mean Blood Pressure (MBP) during the Experiment of IOP Elevation

	Baseline	10 min	20 min	30 min	40 min	50 min	60 min	*P* Values
Control	102.5 (12.4)	103.3 (15.8)	103.0 (14.1)	96.2 (9.8)	101.3 (9.7)	101.1 (13.0)	104.2 (9.4)	0.86
LAA (2 mM)	98.5 (11.6)	97.7 (12.7)	97.1 (13.9)	95.1 (16.4)	101.8 (14.8)	101.3 (15.2)	99.0 (11.5)	0.99
LAA (6 mM)	97.2 (8.9)	96.5 (6.7)	93.6 (6.8)	94.0 (10.0)	97.1 (7.7)	100.2 (6.9)	103.8 (9.2)	0.35

Data are expressed as mean (SD) for 6 to 7 rabbits. *P* values obtained by one-way ANOVA are shown in this table.

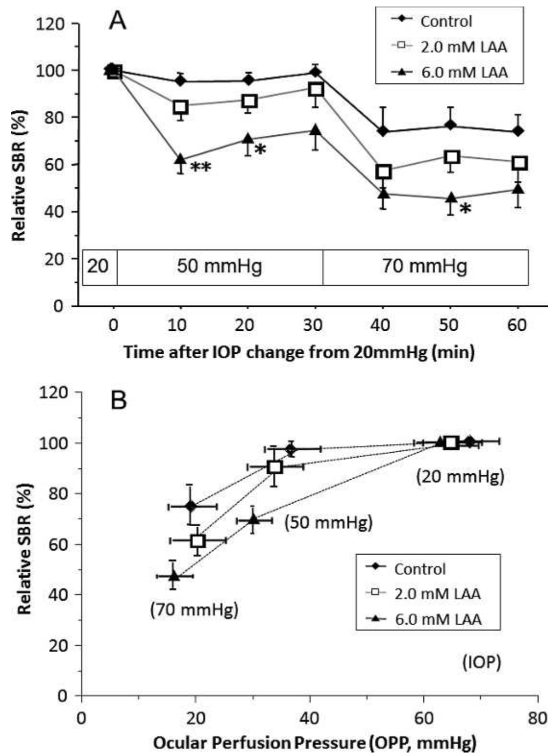


Figure 1. (**A**) Effects of LAA on ONH blood flow in response to IOP elevation. Data are plotted as mean ± SEM for six to seven rabbits. In the control receiving an intravitreal injection of saline, the ONH blood flow was maintained and the minimum level was 73.4 ± 10.5 % to the baseline even after the IOPs were elevated to 70 mm Hg. LAA caused a reduction of the blood flow in a dose-dependent manner after IOP elevation. Two-way repeated-measures ANOVA was significant between control and 6.0 mM LAA-treated eyes (*$P = 0.0187$; **$P < 0.05$; $P < 0.01$ vs. control, Dunnett's test). (**B**) Effects of LAA on ONH blood flow in response to the change in ocular perfusion pressure (OPP). Data are plotted as mean ± SEM for six to seven rabbits. In the control the ONH blood flow was maintained when OPP was 36.8 ± 6.0 mm Hg, whereas it was obviously reduced when OPP was 30.7 ± 3.2 mm Hg in 6.0 mM LAA-treated eyes.

Table 3. Effect of LAA on Electroretinograms (ERGs)

	Control	LAA (2 mM)	LAA (6 mM)	*P* Values
a-wave				
%Amplitude	92 ± 3.5	117 ± 34	112 ± 20	0.66
%Implicit time	97 ± 3.3	110 ± 19	99 ± 5.9	0.27
b-wave				
%Amplitude	84 ± 4.8	100 ± 16	88 ± 12	0.30
%Implicit time	100 ± 15	108 ± 14	104 ± 9.2	0.92

Data are expressed as mean ± SD of relative values to the baseline levels for 5 to 7 rabbits. *P* values obtained by one-way ANOVA are shown in this table.

respectively, Pearson's correlation coefficient), suggesting that ONH blood flow decreased when OPP was lowered.

Alteration of Retinal Function after Intravitreal Injection of LAA

The implicit times and amplitudes of a- and b-waves in ERG before and after LAA injection are shown in Table 3. In LAA-treated eyes, there was no significant reduction in the amplitude or elongation of the implicit time of each wave of ERG compared with control eyes.

Histologic Examination and Immunohistochemistry

Figure 3 shows transverse retinal sections stained with hematoxylin and eosin. There are no apparent changes in GCL, INL, or ONL of LAA-treated eyes. A quantitative assessment of the effect of LAA on GCL, INL, and ONL is shown in Table 4. There were no significant differences in the number of cells in the GCL, INL, or ONL between LAA-treated and control eyes ($P = 0.27$, $P = 0.61$, and $P = 0.19$, respectively; one-way ANOVA).

Figure 4 shows transverse retinal sections stained immunohistochemically with GFAP. Compared with control eyes, foot processes of Müller cells at the inner limiting membrane and

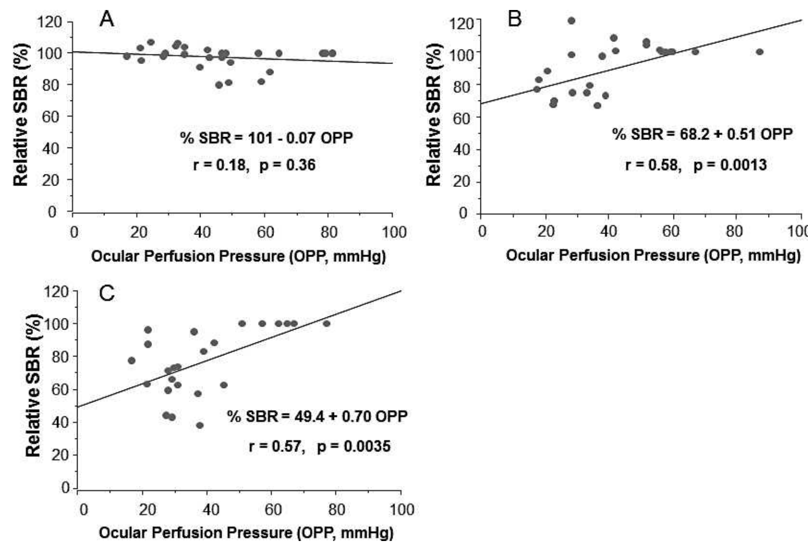


Figure 2. Linear regression analyses between ONH blood flow and OPPs in eyes treated with saline (control, **A**), LAA at 2.0 mM (**B**), and 6.0 mM (**C**). In control eyes, the ONH blood flow was not dependent on OPP ($r = -0.18$, $P > 0.05$, Pearson's correlation coefficient). In LAA (2.0 and 6.0 mM)-treated eyes, significant and positive interactions were found between ONH blood flow and OPP ($r = 0.58$ and 0.57, respectively, $P < 0.01$).

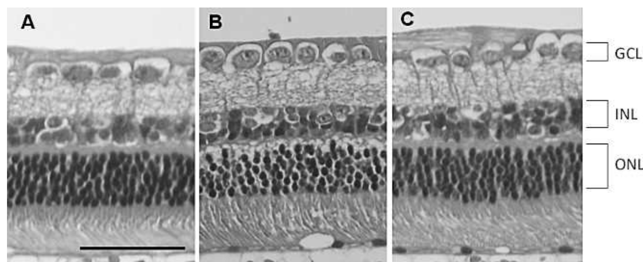


Figure 3. Photomicrographs of transverse sections of the posterior retina stained with hematoxylin and eosin. Sections were obtained from eyes receiving intravitreal injection of saline (**A**), LAA at 2.0 mM (**B**), and 6.0 mM (**C**). No apparent changes were observed in the ganglion cell layer (GCL), inner nuclear cell layer (INL), or outer nuclear layer (ONL) of the retina. Bar = 50 µm.

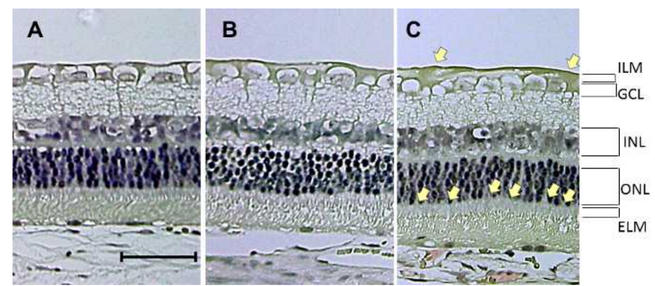


Figure 4. Photomicrographs of transverse sections of the posterior retina stained immunohistochemically with anti–glial fibrillary acidic protein (GFAP) antibody. Sections were obtained from eyes receiving intravitreal injection of saline (**A**), LAA at 2.0 mM (**B**), and 6.0 mM (**C**). Foot processes of Müller cells at the inner limiting membrane (ILM) and external limiting membrane (ELM) were swollen in LAA-treated eyes compared with the control and these changes were more obvious (*arrows*) in eyes treated with LAA at 6.0 mM than those at 2.0 mM (**B**, **C**). Bar = 50 µm.

external limiting membrane were swollen in LAA-treated eyes and these changes were more markedly observed in LAA-treated eyes at 6.0 mM than at 2.0 mM.

Representative changes of immunoreactivity to GFAP on flat-mounted retinas are shown in Figures 5A–5C. Compared with control eyes, processes of astrocytes appeared to be shortened and deformed in LAA-treated eyes. These changes were more markedly observed in LAA-treated eyes at 6.0 mM than at 2.0 mM. On the contrary, immunoreactivity to connexin 43, one of the gap junction proteins and expressed at the surface of vasculature, appeared intact even in LAA-treated eyes at 6.0 mM. Mean densities of GFAP expression in astrocytes were significantly reduced in LAA-treated eyes (Fig. 5D).

Discussion

In the present study, the autoregulation of ONH blood flow was not maintained in eyes with IOP elevation in which glial cells were selectively impaired by LAA treatment. This indicates the possible involvement of glial cells in the autoregulation of ONH blood flow.

In this study, the glial toxicity after intravitreal injection of LAA was demonstrated by histologic examination and immunohistochemistry. No apparent changes were observed in any other cellular elements of the retina other than Müller cells and astrocytes. These results are consistent with previous reports,[17,18,27] which demonstrated that aminoadipic acid at low concentrations caused selective damage to Müller cells and astrocytes without significant effects on the ganglion cells. The implicit time of the ERG b-wave was not prolonged significantly after LAA injections despite the changes of Müller cells and astrocytes. This finding was also consistent with a previous report demonstrating that the electrophysiologic alterations caused by aminoadipic acid at low concentrations were reversible.[18] Our results suggest that the functions of retinal neurons were not substantially affected by LAA at 2.0 or 6.0 mM.

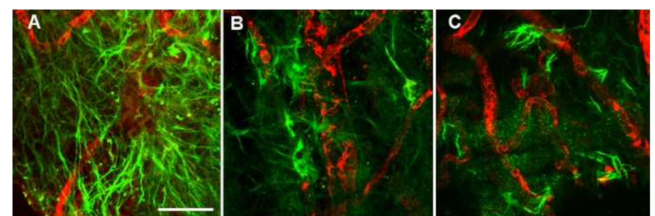
Table 4. Cell Densities (numbers/mm) in the Ganglion Cell Layer (GCL), the Inner Nuclear Layer (INL), and the Outer Nuclear Layer (ONL)

	Control	LAA (2 mM)	LAA (6 mM)	*P* Value
GCL	32.6 ± 4.3	37.4 ± 4.1	37.4 ± 6.3	0.27
INL	268.0 ± 16.8	276.8 ± 16.7	279.3 ± 20.6	0.61
ONL	1412.4 ± 67.4	1409.4 ± 55.5	1330.3 ± 80.4	0.19

Data are expressed as mean ± SD for 5 to 7 rabbits. *P* values obtained by one-way ANOVA are shown in this table.

The current study showed that ONH blood flow was maintained when IOP was elevated to 50 mm Hg in the control eyes, which is consistent with previous reports.[28–32] In contrast, ONH blood flow in LAA-treated eyes was decreased with a reduction of OPP during IOP elevation and a positive relationship was shown between ONH blood flow and OPP. These results indicate that the autoregulation of ONH blood flow was impaired, at least partly due to disordered glial cells.

In previous reports, morphologic changes and activation of glial cells in human glaucoma,[15] experimental glaucoma,[14,27,33] and experimental diabetes[34] were demonstrated. Astrocyte reactivation may be a primary cellular response triggered by elevated IOP or ischemia and/or a secondary cellular response triggered by axonal degeneration.[14] The cell processes of



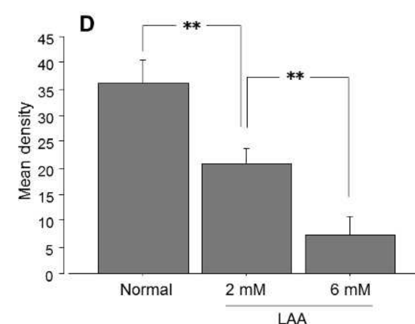


Figure 5. Astrocytes stained immunohistochemically with anti-GFAP antibody (*green*) and connexin 43 (*red*) in the ONH of flat-mounted retina. Retinas were dissected from eyes receiving an intravitreal injection of saline (**A**), LAA at 2.0 mM (**B**), and 6.0 mM (**C**). Compared with the control group (**A**), astrocyte processes in LAA-treated eyes (**B**, **C**) seem to be shortened and deformed. These changes were more marked in eyes treated with LAA at 6.0 mM (**C**) than at 2.0 mM (**B**). Bar = 50 µm. Mean densities of GFAP expression in astrocytes were quantitatively compared in each group. One-way ANOVA revealed that there were significant differences ($P < 0.0001$) between three groups (**D**: mean ± SD, $n = 4$, each, $^{**}P < 0.01$, Bonferroni's test).

astrocytes are connected to each other via gap junctions,[35,36] which are built mainly of connexin43. These form a functional syncytium that allows astrocytes to communicate and maintain control of the ionic and metabolic homeostasis in the ONH. It was reported that astrocytes in the ONH decrease gap junction communication under conditions of elevated IOP.[37] Closure of gap junctions will interrupt the continuity of astrocyte intercellular communication, causing loss of cell–cell contact and loss of homeostatic regulation.[38] Actually we have already reported that disruption of gap junctions may be involved in the impairment of autoregulation in ONH blood flow.[39] Astrocytes have been shown to participate in blood flow regulation at the gliovascular interface in accordance with neuronal activities in the central nervous system, where astrocytic network couplings with gap junctions surround the vessel walls.[40–42] Neuronal activity evokes localized changes in blood flow in the central nervous system, which is termed neurovascular coupling. Regarding eyes, it has been reported that glial cells also contribute to this neurovascular coupling through gap junctions.[43–45] Another study using LSFG previously reported that a calcium channel antagonist impaired autoregulation of ONH circulation after an acute increase in IOP.[31] It suggested the influx of Ca^{2+}-related vascular smooth muscle relaxation may also play a role in the autoregulation, even though neurovascular coupling, glial cells, and gap junction are intact. Nevertheless, we speculate that glial cells may have a crucial role in the autoregulation of ONH blood flow through gap junctions, based on the current and the above-cited studies.[40–45]

One limitation of the current study is a relatively small sample size such as six or seven; the difference in SBR changes between control and 2 mM LAA-treated eyes might be detected if their sample size would be increased. Another limitation is that we cannot exclude completely any mechanisms other than changes in glial cells, which might be involved in impaired autoregulation in the ONH blood flow. Also we did not determine the mechanism the autoregulation of ONH blood flow was impaired by the gliotoxic compound. We did not demonstrate the interruption of glial intercellular communication at the molecular level. In addition, we should further investigate whether the disordered autoregulation of ONH blood flow is involved in retinal ganglion cell death, as observed in glaucoma. Also, there are differences regarding ONH structure and blood supply to the ONH between rabbits and primates or humans, despite similarities in the arterial supply. Therefore, our findings may not directly apply to the ONH circulation of primates or humans. Further studies are required to address this issue.

In conclusion, this study demonstrates that the autoregulation of ONH blood flow was impaired in LAA-treated eyes and suggests the possible involvement of glial cells in the autoregulation of ONH blood flow. The current study may provide a new approach for future studies regarding the mechanisms of autoregulation of ONH blood flow.

References

1. Moore D, Harris A, WuDunn D, et al. Dysfunctional regulation of ocular blood flow: a risk factor for glaucoma? *Clin Ophthalmol*. 2008;2:849–861.
2. Tielsch LM, Katz J, Sommer A, et al. Hypertension, perfusion pressure, and primary open-angle glaucoma. *Arch Ophthalmol*. 1995;113:216–221.
3. Bonomi L, Marchini G, Maffaffa M, et al. Vascular risk factors for primary open angle glaucoma: the Egna–Neumarkt Study. *Ophthalmology*. 2000;107:1287–1293.
4. Leske MC, Heijl A, Hyman L, et al. Predictors of long-term progression in the early manifest glaucoma trial. *Ophthalmology*. 2007;114:1965–1972.
5. Leske MC, Wu SY, Hennis A, et al. Risk factors for incident open-angle glaucoma. The Barbados Eye Studies. *Ophthalmology*. 2008;115:85–93.
6. Harris A, Ciulla TA, Chung HS, Martin B. Regulation of retinal and optic nerve blood flow. *Arch Ophthalomol*. 1998;116: 1491–1495.
7. Orgul S, Gugleta K, Flammer J. Physiology of perfusion as it relates to the optic nerve head. *Surv Ophthalmol*. 1999;43: S17–S26.
8. Flammer J, Orgul S. Optic nerve blood-flow abnormalities in glaucoma. *Prog Retin Eye Res*. 1998;17:267–289.
9. Evans DW, Harris A, Garrett M, et al. Glaucoma patients demonstrate faulty autoregulation of ocular blood flow during posture change. *Br J Ophthalmol*. 1999;83:809–813.
10. Emre M, Orgul S, Gugleta K, Flammer J. Ocular blood flow alteration in glaucoma is related systemic vascular dysregulation. *Br J Ophthalmol*. 2004;88:662–666.
11. Okuno T, Sugiyama T, Kojima S, et al. Diurnal variation in microcirculation of ocular fundus and visual field change in normal-tension glaucoma. *Eye*. 2004;18:697–702.
12. Grieshaber MC, Flammer J. Blood flow in glaucoma. *Curr Opin Ophthalmol*. 2005;16:79–83.
13. Feke GT, Pasquale LR. Retinal blood flow response to posture change in glaucoma patients compared with healthy subjects. *Ophthalmology*. 2008;115:246–252.
14. Hernandez MR. The optic nerve head in glaucoma: role of astrocytes in tissue remodeling. *Prog Retinal Eye Res*. 2000; 19:297–321.
15. Wang L, Cioffi GA, Dong J, Fortune B. Immunohistologic evidence for retinal glial cell changes in human glaucoma. *Invest Ophthalmol Vis Sci*. 2002;43:1088–1094.
16. Olney JW, de Gubareff T, Collins JF. Stereospecificity of the gliotoxic and anti-neurotoxic actions of alpha-aminoadipate. *Neurosci Lett*. 1980;19:277–282.
17. Pedersen OØ, Karlsen RL. Destruction of Müller cells in the adult rat by intravitreal injection of D,L-α-aminoadipic acid. An electron microscopic study. *Exp Eye Res*. 1979;28:569–575.
18. Bonaventure N, Roussel G, Wioland N. Effect of DL-α-amino adipic acid on Müller cells in frog and chicken retinae in vivo: relation to ERG b wave, ganglion cell discharge and tectal evoked potentials. *Neurosci Lett*. 1981;27:81–87.
19. Ishikawa Y, Mine S. Aminoadipic acid toxic effects on retinal glial cells. *Jpn J Ophthalmol*. 1983;27:107–118.
20. Kida T, Oku H, Sugiyama T, Ikeda T. The mechanism and change in the optic nerve head circulation in rabbits after glucose loading. *Curr Eye Res*. 2001;22:95–101.
21. Bill A. Blood pressure in the ciliary arteries of rabbits. *Exp Eye Res*. 1963;2:20–24.
22. Cioffi G, Granstam E, Alm A. Ocular circulation. In: Kaufman P, Alm A, eds. *Adler's Physiology of the Eye*. 10th ed. St. Louis: Mosby; 2003:747–784.
23. Tamaki Y, Araie M, Kawamoto E, Fujii H. Noncontact, two-dimensional measurement of retinal microcirculation using laser speckle phenomenon. *Invest Ophthalmol Vis Sci*. 1994; 35:3825–3834.
24. Sugiyama T, Araie M, Riva CE, Schmetterer L, Orgul S for the Ocular Blood Flow Research Association. Use of laser speckle flowgraphy in ocular blood flow research. *Acta Ophthalmol*. 2010;88:723–729.
25. Sugiyama T, Utsumi T, Azuma I, Fujii H. Measurement of optic nerve head circulation: comparison of laser speckle and hydrogen clearance methods. *Jpn J Ophthalmol*. 1996;40: 339–343.
26. Goto W, Oku H, Okuno T, Sugiyama T, Ikeda T. Amelioration of endothelin-1-induced optic nerve head ischemia by topical bunazosin. *Curr Eye Res*. 2005;30:81–91.

27. Kanamori A, Nakamura M, Nakanishi Y, Yamada Y, Negi A. Long-term glial activity in rat retinas ipsilateral and contralateral to experimental glaucoma. *Exp Eye Res*. 2005;81:48–56.
28. Geijer C, Bill A. Effect of raised intraocular pressure on retinal, prelaminar, laminar, and retro-laminar optic nerve blood flow in monkeys. *Invest Ophthalmol Vis Sci*. 1979;18:1030–1042.
29. Sossi N, Anderson DR. Effect of elevated intraocular pressure on blood flow. Occurrence in cat optic nerve head studies with iodoantipyrine I125. *Arch Ophthalmol*. 1983;101:98–101.
30. Riva CE, Cranstoun SD, Petrig BL. Effect of decreased ocular perfusion pressure on blood flow and the flicker-induced flow response in the cat optic nerve head. *Microvasc Res*. 1996;52: 258–269.
31. Takayama J, Tomidokoro A, Ishii K, et al. Time course of the change in optic nerve head circulation after an acute increase in intraocular pressure. *Invest Ophthalmol Vis Sci*. 2003;44: 3977–3985.
32. Tamaki Y, Araie M, Kawamoto E, Eguchi S, Fujii H. Noncontact, two-dimensional measurement of tissue circulation in choroids and optic nerve head using laser speckle phenomenon. *Exp Eye Res*. 1995;60:373–383.
33. Wang X, Tay SS, Ng YK. An immunohistochemical study of neuronal and glial cell reactions in retinae of rats with experimental glaucoma. *Exp Brain Res*. 2000;132:476–484.
34. Rungger-Brandle E, Dosso AA, Leuenberger PM. Glial reactivity, an early feature of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2000;4:1971–1980.
35. Rose CR, Ransom BR. Gap junctions equalize intracellular Na^+ concentration in astrocytes. *Glia*. 1997;20:299–307.
36. Zahs KR, Ceelen PW. Gap junctional coupling and connexin immunoreactivity in rabbit retinal glia. *Vis Neurosci*. 2006;23: 1–10.
37. Malone P, Miao H, Parker A, Juarez S, Hernendez MR. Pressure induces loss of gap junction communication and redistribution of connexin 43 in astrocytes. *Glia*. 2007;55:1085–1098.
38. Hernandez MR, Miao H, Lukas T. Astrocytes in glaucomatous optic neuropathy. *Prog Brain Res*. 2008;173:353–373.
39. Shibata M, Oku H, Sugiyama T, et al. Disruption of gap junctions may be involved in impairment of autoregulation in optic nerve head blood flow of diabetic rabbits. *Invest Ophthalmol Vis Sci*. 2011;52:2153–2159.
40. Paulson OB, Newman EA. Does the release of potassium from astrocyte endfeet regulate cerebral blood flow? *Science*. 1987; 237:896–898.
41. Alkayed NJ, Narayanan J, Gebremedhin D, Medhora M, Roman RJ, Harder DR. Molecular characterization of an arachidonic acid epoxygenase in rat brain astrocytes. *Stroke*. 1996;27:971–979.
42. Simard M, Arcuino G, Takano T, Liu QS, Nedergaard M. Signaling at the gliovascular interface. *J Neurosci*. 2003;23: 9254–9262.
43. Metea MR, Newman EA. Glial cells dilate and constrict blood vessels: a mechanism of neurovascular coupling. *J Neurosci*. 2006;26:2862–2870.
44. Buerk DG, Riva CE, Cranstoun SD. Frequency and luminance-dependent blood flow and K^+ ion changes during flicker stimuli in cat optic nerve head. *Invest Ophthalmol Vis Sci*. 1995;36:2216–2227.
45. Gugleta K, Fuchsjager-Mayrl G, Orgul S. Is neurovascular coupling of relevance in glaucoma? *Surv Ophthalmol*. 2007; 52:S139–S143.