Hyperglycemia Accelerates Impairment of Vasodilator Responses to Acetylcholine of Retinal Blood Vessels in Rats

Asami Mori, Orie Saigo, Masayuki Hanada, Tsutomu Nakahara, and Kunio Ishii

Department of Molecular Pharmacology, Kitasato University School of Pharmaceutical Sciences, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

Received November 29, 2008; Accepted April 2, 2009

Abstract. We previously reported that vascular endothelial functions in both retinal and systemic circulation are impaired 6–8 weeks after induction of hyperglycemia with streptozotocin (STZ) in rats. However, it remains to be elucidated whether the period required for the onset of endothelial dysfunction is different, depending on vascular beds and severity of hyperglycemia. In this study, we examined the effects of several vasodilators on the diameter of retinal blood vessel and blood pressure in Control, STZ (STZ treatment alone), and STZ + Glc (STZ treatment plus D-glucose feeding) rats. The overall structures of the retina and the retinal capillary network were also evaluated. The vasodilator effects of acetylcholine on retinal arterioles were significantly reduced in the STZ + Glc group, but not in the STZ group, 2 weeks after induction of hyperglycemia. There were no significant differences in acetylcholine-induced decreases in blood pressure among the three experimental groups. The responses to NOR3, forskolin, and adenosine were unaffected by hyperglycemia. The retinal thickness was significantly reduced in the STZ + Glc group. No significant changes were observed in the morphology and the density of retinal capillary network by immunohistochemical techniques. These results suggest that endothelium-dependent vasodilatory mechanisms of retinal arterioles are more vulnerable than those of peripheral resistance vessels to the effects of hyperglycemia. Hyperglycemia shortens the period required for onset of retinal endothelial dysfunction, depending on its severity.

Keywords: diabetes, endothelium, nitric oxide, retinal hemodynamics

Introduction

Diabetic retinopathy is a leading cause of blindness in industrialized countries and is the most common complication of diabetes. During the initial stage of diabetic retinopathy (nonproliferative diabetic retinopathy), no symptoms or only mild vision problems appear. However, nonproliferative diabetic retinopathy often proceeds to an advanced proliferative stage of diabetic retinopathy in which new blood vessels grow on the surface of the retina. These new blood vessels are abnormal and have weaker walls. Therefore, they easily break and bleed into the vitreous cavity and thereby lead to serious vision problems (1–3). Thus, proliferative retinopathy is a much more serious form of the disease and can lead to blindness.

Tight control of blood glucose levels is a key point in preventing the incidence and progression of diabetic retinopathy; however, additional effective treatments are also needed. Abnormalities of retinal circulation contribute to the pathogenesis of diabetic retinopathy; therefore, the normalization of the abnormalities would be one of the strategies for slowing the progression of this disease. To develop novel therapeutics and strategy for improving retinal circulation, it is important to understand the mechanisms underlying the alternations of retinal vascular function in diabetes. In our previous studies using streptozotocin (STZ)-treated rats as an experimental diabetic animal model, the effects of several vasodilators on the diameters of retinal blood vessels and blood pressure were examined 6–8 weeks after a single injection of STZ. At this stage of diabetes, both increase in retinal arteriolar diameter and decrease in systemic blood pressure caused by acetylcholine...
(ACh) were significantly diminished (4, 5). Therefore, it appears that vascular endothelial functions in retinal and systemic circulation are impaired in STZ-induced diabetic rats. However, retinal and systemic vascular endothelial cells may display a differential vulnerability to the effects of hyperglycemia because endothelial cell characteristics markedly differ depending on the vascular beds. If this hypothesis is correct, the period required for the onset of endothelial dysfunction would be different among the vasculatures and severity of hyperglycemia would affect the periods.

To test this hypothesis, we examined the vasodilator effects on retinal and systemic circulation at very early stage of diabetes (2-week duration of diabetes) and attempted to induce more severe hyperglycemia in rats by giving 5% D-glucose solution as drinking water after injection of STZ. The effects of severe hyperglycemia on the overall structures of the retina and the retinal capillary network were also evaluated.

Materials and Methods

Animal model of diabetes

All experiments were performed in accordance with the Guidelines for Animal Experiments in Kitasato University adopted by the Committee on the Care and Use of Laboratory Animals of Kitasato University and tenets of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Prior to the experiments, male Wistar rats weighing 160 – 170 g were maintained at least 1 week on standard rat chow and tap water ad libitum under a 12:12-h dark cycle. Hyperglycemia was induced by a single intravenous injection of STZ (65 mg/kg) (Sigma, St. Louis, MO, USA) dissolved in sodium citrate buffer (pH 4.5). Age-matched control rats (Control group) were treated with an injection of an equal volume of vehicle (n = 28). The STZ-treated animals (n = 42) were divided into two experimental groups: STZ group (n = 12) and STZ + Glc group (n = 30). The rats of STZ + Glc group were given 5% D-glucose solution as drinking water after STZ treatment. In our preliminary studies, several vehicle-treated rats were given 5% D-glucose solution as drinking water. However, the animals maintained normal blood glucose levels, and the retinal vasodilator responses to ACh were unaffected by the two-week feeding of D-glucose. Therefore, in the present study, experiments were performed on the three groups (Control, STZ, and STZ + Glc groups). Plasma glucose was determined with a commercially available enzyme kit (Glucose Test Wako; Wako Pure Chemical, Osaka). The experiments were performed 2 weeks after the injection.

In vivo experimental procedures

The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) (Nacalai Tesque, Inc., Kyoto). After disappearance of the corneal reflex, each animal was placed on a heating pad. A tracheotomy was performed for artificial ventilation. Catheters were inserted into the femoral and jugular veins for administration of drugs. The left femoral artery was cannulated for measurement of arterial pressure, which was recorded on a thermal pen recorder (WT-645G; Nihon Kohden, Tokyo) via a pressure transducer (DX-360, Nihon Kohden) and a preamplifier (AP-610G, Nihon Kohden). Heart rate (HR) was measured with a cardiometer (AT-601G, Nihon Kohden) triggered by the blood pressure pulse. Mean arterial pressure (MAP) and HR were digitized at 1 Hz (SCIENCE LINK II; Keisoku Giken, Utsunomiyia) and stored on the hard disk of a personal computer (PowerBook 165C; Apple Japan, Tokyo). To prevent movement of the eye and consistently measure diameter of retinal blood vessels, rats were treated with tetrodotoxin (50 μg/kg, i.v.) (Nacalai Tesque) under artificial ventilation with room air (stroke volume: 10 mL/kg and frequency: 80 strokes/min) using a rodent respirator (SN-480-7, Sinano, Tokyo) (4). Blood pressure was decreased by treatment with tetrodotoxin; therefore, methoxamine (approx. 30 μg·kg⁻¹·min⁻¹) (Sigma) was continuously injected into the jugular vein at a constant rate by means of a syringe pump (Harvard Apparatus, South Natick, MA, USA) to maintain adequate systemic circulation.

Acetylcholine (0.3 – 10 μg·kg⁻¹·min⁻¹) (Sigma), NOR3 ((±E)-ethyl-2-[(E)-hydroxyamino]-5-nitro-3-hexenamide) (0.5 – 10 μg·kg⁻¹·min⁻¹) (Dojin, Osaka), forskolin (1 – 30 μg·kg⁻¹·min⁻¹) (Sigma), or adenosine (50 – 300 μg·kg⁻¹·min⁻¹) (Sigma) was infused into the femoral vein using a syringe pump (Harvard Apparatus). The doses of ACh and adenosine that have no significant effect on HR were chosen on the basis of our previous studies (4, 5).

Measurement of diameters of retinal blood vessels

To protect the eye, hydroxyethylcellullose (SCOP-ISOL 15®; Senju Pharmaceutical, Osaka) was dropped onto the cornea. The optic disc was centered and focused in the field of view. Sodium fluorescein (10% solution, 0.8 mL/kg) and brilliant blue 6B (5% solution, 0.8 mL/kg) were injected into the right femoral vein to enhance contrast of blood vessels. Fundus images were captured with a Finepix S3 pro (Fuji Photo Film, Co., Ltd., Tokyo) that was equipped with a bore scope-type objective lens for small animals (Model 01, Magnification ×20; Scalar, Tokyo) and stored on the hard disk of a laboratory computer system (Power Macintosh G3-
300DT, Apple). The diameters of retinal blood vessels were measured as described previously (4).

**Histological evaluation of the eye**

After the end of the in vivo experiment, both eyes were enucleated for histological evaluation. Enucleated eyes were immersed for 12 h in the fixative mixture (37.5% ethanol, 9.3% formaldehyde, 12.5% acetic acid, and 3% glutaraldehyde) at room temperature. Fixed retinal tissues were embedded in paraffin, and 5-μm horizontal sections through the optic disk of the eye were cut. The sections were stained with hematoxylin and eosin and subject to morphometry as previously described (6). The overall structure of the retina was evaluated by measuring the thickness of the retinal layers. Measurements of total retinal thickness and thicknesses of the inner plexiform layer (IPL), inner nuclear layer (INL), and outer nuclear layer (ONL) were performed in the same topographic region of the retina. Averages for these measurements taken in three adjacent areas within 1 mm of the optic nerve were calculated. We did all of the morphometrical analysis in a blind fashion.

**Quantitative assessment of retinal capillary density**

Measurements of retinal capillary density were conducted in a separate group of animals (Control: n = 6, STZ: n = 6, and STZ + Glc: n = 6). Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). The chest was opened rapidly and the vasculature was perfused for 5 min at a pressure of 120 mmHg with fixative (1% paraformaldehyde in phosphate-buffered saline, pH 7.4) from an 18-gauge cannula inserted into the aorta via an incision in the left ventricle. The right atrium was incised to create a route for the fixative to exit. After the perfusion, eyes were removed and stored in fixative for 24 h at 4°C. The retina was separated from the lens, vitreous, and pigment epithelium with 4 radial cuts and then incubated in blocking solution (5% normal hamster serum) in PBS containing 0.3% Triton X-100 (PBS/0.3% Triton X-100) for 0.5 – 1 h at room temperature. To visualize the vascular network, the retinas were incubated with a mouse monoclonal anti-rat endothelial cell antigen (RECA)-1 antibody (endothelial cell marker) (1:20 dilution; Serotec, Oxford, UK) overnight, followed by incubation with a Cy3-conjugated donkey antibody against mouse immunglobulins (1:400 dilution; Jackson ImmunoResearch, West Grove, PA, USA). Retinas were rinsed in PBS/0.3% Triton X-100 and the retinal flat mounts were prepared with a mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA, USA). Images (×10 objective, tissue region 1,091 × 1,449 μm) were taken by using a fluorescent microscope system BZ-9000 (Keyence, Osaka). The retinal capillary networks in the images captured from five regions of retina in each animal were analyzed using ImageJ (http://rsb.info.nih.gov/ij/). Based on fluorescence intensities ranging from 0 to 255, blood vessels were distinguished from background by empirically determining threshold values that included only the blood vessels in the specimens. The area density of blood vessels stained with anti-RECA antibody was calculated as the proportion of pixels having a fluorescence intensity value equal to or greater than the corresponding threshold. Mean values were calculated for retinal regions in each rat and from these values overall means were calculated for each group (6 rats per group).

**Data analyses**

Statistical comparison of paired data was performed using Student’s t-test, and multigroup data were analyzed by ANOVA (one-way or two-way) followed by the Bonferroni’s post-test using PRISM4 software (GraphPad Software, Inc., La Jolla, CA, USA). A P value smaller than 0.05 was considered to be statistically significant. All values are presented as the mean ± S.E.M.

**Results**

**Plasma glucose levels and body weights**

The experiments were performed 2 weeks after injection of STZ or the vehicle. The plasma glucose levels and body weights of rats used in this study are summarized in Fig. 1. The STZ-treated rats (Fig. 1A)
showed elevated blood glucose levels and reduced body weight gain (Fig. 1B) compared to the age-matched controls. The plasma glucose level of the STZ + Glc group was significantly higher than that in the STZ group, whereas there was no significant difference in body weights between the two groups.

**Baseline values of MAP, diameter of retinal blood vessels, and HR**

Baseline values of MAP and diameter of retinal blood vessels were adjusted to the same ranges among the experimental groups by changing methoxamine infusion rates (MAP: Control, 109 ± 1 mmHg, n = 22; STZ, 108 ± 2 mmHg, n = 6; STZ + Glc, 107 ± 1 mmHg, n = 24; retinal arteriolar diameter: Control, 35.8 ± 1.0 μm, n = 22; STZ, 34.5 ± 2.7 μm, n = 6; STZ + Glc, 37.5 ± 1.1 μm, n = 24; retinal venular diameter: Control, 50.7 ± 1.2 μm, n = 22; STZ, 53.0 ± 3.2 μm, n = 6; STZ + Glc, 52.0 ± 1.0 μm, n = 24). However, baseline HR values were significantly lower in the STZ (281 ± 8 beats/min, n = 6) and STZ + Glc (298 ± 6 beats/min, n = 24) groups than in the Control group (330 ± 6 beats/min, n = 22). There was no significant difference in HR between the STZ and STZ + Glc groups.

**In vivo vascular responses**

In our previous studies, we found that ACh increases the diameter of retinal blood vessels and decreases blood pressure in rats. Both vascular responses were reduced 6 – 8 weeks after STZ treatment (4, 5). Therefore, we first examined the effects of ACh (0.3 – 10 μg·kg⁻¹·min⁻¹, i.v.) on diameters of retinal blood vessels and blood pressure in the three experimental groups (Control, STZ, and STZ + Glc). In control rats, ACh (0.3 – 10 μg·kg⁻¹·min⁻¹, i.v.) increased the diameters of retinal blood vessels and decreased MAP in a dose-dependent manner (Figs. 2 and 3). The vasodilator responses of retinal venules were smaller than those of retinal arterioles (Figs 3: A and B). There was no significant difference in vasodilator responses to ACh between the Control and STZ groups. The vasodilator responses of retinal arterioles in the STZ + Glc group were smaller than those in the Control and STZ groups (Fig. 3A). The vasodilator effect on retinal venules was unaffected in the STZ + Glc group (Fig. 3B). No significant differences in ACh-induced decreases in MAP were observed among the three groups (Fig. 3C). The doses of ACh did not change HR (Fig. 3D).

To determine whether the diminished response to ACh of retinal arterioles is attributed to the impairment of a signaling pathway evoked by nitric oxide (NO) and by activation of adenylyl cyclase, we examined the effects of the NO donor NOR3 (0.5 – 10 μg·kg⁻¹·min⁻¹, i.v.) and the adenylyl cyclase activator forskolin (1 – 30 μg·kg⁻¹·min⁻¹, i.v.) in the Control and STZ + Glc groups. As shown in Fig. 4, NOR3 (0.5 – 10 μg·kg⁻¹·min⁻¹, i.v.), like ACh, increased the diameter of retinal blood vessels and decreased MAP without significantly changing HR. However, the vascular responses to NOR3 were not different between the Control and STZ + Glc groups (Fig. 4: A, B, and C). Forskolin (1 – 30 μg·kg⁻¹·min⁻¹, i.v.) increased diameters of retinal blood vessels and HR and decreased MAP. These responses were unchanged in the STZ + Glc group (Fig. 5).

We previously demonstrated that activation of the NO pathway contributes to retinal arteriolar dilation and depressor response to adenosine in rats in vivo (5). To determine whether the endothelial NO-mediated vasodilatory mechanisms are impaired in the STZ + Glc group, we next examined the effects of adenosine. Adenosine (50 – 300 μg·kg⁻¹·min⁻¹, i.v.) increased diameters of retinal blood vessels and decreased MAP without affecting HR. These responses in the Control group were not different from those in the STZ + Glc group (Fig. 6).
Morphological changes in retina

Representative microscopic photographs of retinal sections presented in Fig. 7A indicate that the degree of reduction in retinal thickness depends on the blood glucose level. Values of total retinal thickness in Control, STZ, and STZ + Glc groups were 132 ± 9 μm (n = 6), 124 ± 6 μm (n = 6), and 106 ± 7 μm (n = 6), respectively. Significant reduction was observed between the Control and STZ + Glc groups (P < 0.05). We measured the thickness of the three retinal layers, IPL, INL, and ONL. Primarily, the reduction in thickness occurred in the IPL of STZ + Glc group (Fig. 7B).

Retinal capillary density

Representative images of the central retinas in the Control, STZ, and STZ + Glc groups are shown in Fig. 8A. There was no significant difference in the density of retinal capillaries among the three experimental groups (Fig. 8B).

Discussion

Streptozotocin-induced diabetic rats have been frequently used to study the underlying mechanisms of diabetes-induced complications and also to study the effect of various therapies proposed for the treatment of the complications. Our previous studies demonstrated that vascular endothelial functions in both retinal and systemic circulation were impaired 6–8 weeks after induction of hyperglycemia with a single injection of STZ (4, 5). In the present study, we found that the vasodilator effects of ACh on retinal arterioles were significantly reduced in rats treated with a combination of STZ treatment and D-glucose feeding, but not in rats treated with STZ alone, 2 weeks after induction of hyperglycemia. Despite the impairment of retinal vaso-
dilator responses, depressor responses to ACh were unaffected in the STZ + Glc group. There was no significant difference in responses to NOR3, an NO donor, and forskolin, an activator of adenylyl cyclase, between the Control and STZ + Glc groups. These results suggest that the endothelium in retinal arterioles is more susceptible than that in peripheral resistance vessels to hyperglycemia.

It has been well known that ACh stimulates production and release of endothelium-derived relaxing factors (EDRFs), including NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF), and thereby dilates blood vessels in an endothelium-dependent manner. The endothelium-dependent vasodilatory mechanism(s) could be impaired in various types of vascular beds of diabetic animals (7–9). In ocular vasculatures, both in vitro and in vivo studies have shown that endothelial NO contributes, at least in part, to the vasodilator responses to ACh (4, 10). Using the isolated perfused rat eyes, Yu et al. demonstrated that the vasodilator effect of ACh was diminished at 21 weeks after induction of hyperglycemia with STZ. The diminished response was reversed by treatment with tetrahydrobiopterin, an essential cofactor in NO synthesis (11). On the other hand, our previous in vivo studies suggested that NO-dependent vasodilatory mechanism(s) might be preserved in the rat retinal arterioles 6–8 weeks after a single injection of STZ (4, 5). Instead, the production of and/or reduced vascular responsiveness to EDHF appeared to be attenuated (4, 5). Because the underlying mechanisms responsible for the endothelial dysfunction may depend on the severity of hyperglycemia as well as its duration, severe hyperglycemia induced by the combination of STZ treatment and D-glucose feeding might impair the endothelial NO-mediated vascular functions of retinal blood vessels. However, the vaso-
dilation of retinal arterioles induced by adenosine, which activates the NO pathway (5), were unaffected in the STZ + Glc group. Thus, the endothelial NO-dependent vasodilatory mechanism(s) in the retinal arterioles seems to be preserved even after 2 weeks of severe hyperglycemia.

We found that in the very early stage of diabetes, the mechanism(s) of ACh-induced vasodilation of retinal arterioles, possibly the EDHF-mediated pathway, is impaired. At present, the underlying mechanism for the impairment remains unclear. Several mechanisms such as increase in aldose reductase (12), activation of protein kinase C (13), upregulation of inflammatory mediators (14), and increase in formation of advanced glycation end products (15) are thought to promote the diabetic vascular complications. Therefore, these pathways may contribute to the endothelial dysfunction observed in the early stage of diabetes.

With regard to EDHF, several putative candidates have been reported including potassium ions (K⁺) (16, 17), epoxyeicosatetraenoic acid (18, 19), hydrogen peroxide (H₂O₂) (20, 21), and C-type natriuretic peptide (22). In addition to these diffusible factors, non-diffusible, contact-mediated mechanisms, such as flow of hyperpolarizaton via myoendothelial gap junctions, have been proposed (23). However, which candidate of EDHF plays an important role in the retinal vasculature remains to be established. Because the nature of EDHF is dependent on vascular beds, identification of molecules serving as an EDHF in rat retinal arterioles is needed in future studies.

The present study indicates that the degree of retinal morphological changes depends on the severity of hyperglycemia. Generally, changes in electroretinograms are observed in diabetic patients and animals as an early event, preceding any visible morphological abnormalities of retina (24 – 27). The retinal morphological changes, such as reduction in number of ganglion cells and thickness of retinal layers, were observed several months after the onset of hyperglycemia (28 – 30). In the present study, the retinal thickness was significantly reduced at 2 weeks after induction of severe hyperglycemia in
the STZ + Glc group. These results suggest that hyperglycemia shortens the period required for onset of retinal neuronal dysfunction as well as endothelial dysfunction, depending on its severity.

On the other hand, no changes in the morphology and density of the retinal capillary network were detected in this study. The formation of new blood vessel results from the abnormalities of retinal circulation and subsequent retinal ischemia; therefore, the period of severe hyperglycemia found here to impair the ACh-induced vasodilation (2 weeks) might be too short to induce neovascularization in the retina.

As previously reported (4, 31), baseline HR of diabetic rats was lower than that of controls. Rats were denervated by treatment with TTX, and baseline values of MAP were adjusted to the same ranges among the experimental groups. Therefore, it is likely that intrinsic cardiac pacemaker properties are altered in diabetic rats. Interestingly, even at 2 weeks after a single injection of STZ, baseline HR was decreased compared to controls and no further significant reduction occurred in the STZ + Glc group. Thus, the cardiac pacemaker cells may be more susceptible than endothelial cells to hyperglycemia.

Long-term of hyperglycemia produces the cataracts in the lens of experimental animals and patients with diabetes. Indeed, the combination of STZ treatment and D-glucose feeding established mature cataracts in the majority of diabetic rats within 2 months (32). If severe cataracts are developed, it is impossible to examine in vivo retinal vascular responses using the methodology adopted in this study. However, at 2 weeks after induction of the severe hyperglycemia, only small cataracts in the periphery of the lens were observed in 50% of lenses (32). Small cataracts in such regions did not affect the accuracy of measurements of the diameter of retinal blood vessels.

In summary, we found that endothelium-dependent vasodilatation of retinal arterioles are more vulnerable than those of peripheral resistance vessels to the effects of hyperglycemia. The impairment of endothelium-dependent vasodilatory mechanisms may contribute to the abnormal retinal hemodynamics and consequently play an important role in pathogenesis of diabetic retinopathy. Therefore, early treatment for the impaired retinal circulation would be critical to slowing the progression of the disease. Because the induction of more severe hyperglycemia by the combination of STZ treatment and D-glucose feeding shortens the period required for the development of retinal endothelial dysfunction and neuronal degeneration in rats, the animal models would be useful for screening potential therapeutic agents that prevent or slow the progression of diabetic retinopathy.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (No. 20590900, T.N.); Suzuken Memorial Foundation (T.N.); and by a Kitasato University Research Grant for Young Researchers (A.M., T.N.).

References