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Hyperglycemia Impairs Acetylcholine-Induced Vasodilation of Retinal Arterioles Through Polyol Pathway–Independent Mechanisms in Rats

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Abstract. We previously reported that acetylcholine (ACh)–induced vasodilation of retinal arterioles is diminished in diabetic rats; however, the underlying mechanism(s) of this phenomenon has not been fully elucidated. To determine the role of the polyol pathway in the diabetes-induced retinal vascular dysfunction, we investigated the effect of GP-1447, an inhibitor of aldose reductase, on the attenuation of ACh-induced vasodilation of retinal arterioles seen in diabetic rats. Male Wistar rats were treated with streptozotocin (STZ) and experiments were performed 2 weeks later. The STZ-treated animals were given drinking water containing 5% D-glucose to shorten the term for the development of retinal vascular dysfunction. Treatment with GP-1447 was initiated immediately after STZ treatment and continued throughout the 2-week experimental period. The attenuation of retinal vascular responses to ACh were not modified by treatment with GP-1447, whereas the aldose reductase inhibitor completely prevented diabetes-induced thinning of the retina and sorbitol accumulation in the retina and the lens. These results suggest that mechanisms that are independent of the polyol pathway may contribute to the onset of retinal endothelial dysfunction, although the pathway plays an important role in morphological changes of retina and formation of cataracts in diabetic rats.

Keywords: aldose reductase, diabetes, endothelium, polyol pathway, retinal circulation

Introduction

Diabetic retinopathy is the leading cause of vision loss in adults of industrialized countries and is the most common complication of diabetes. Both functional abnormalities and histological changes of the retinal vasculature contribute to the development of this disease (1, 2). Vascular endothelial cells play an important role in regulation of vascular function, and abnormalities of retinal circulation are frequently observed in experimental diabetic animals and patients with diabetes mellitus (3 – 5).

We previously found that intravenously administered acetylcholine (ACh) increases the retinal arteriolar diameter but decreases systemic blood pressure in rats (6, 7). Both responses to ACh were significantly diminished 6 – 8 weeks after induction of hyperglycemia in streptozotocin (STZ)–treated rats (6, 7). More recently, we showed that in an early stage of diabetes (2-week duration of diabetes) the vasodilation of retinal arterioles, but not the depressor response, to ACh were significantly reduced by inducing more severe hyperglycemia by giving D-glucose as drinking water after STZ treatment (8). These results suggest that both retinal and systemic circulation could be impaired in diabetes and endothelium-dependent vasodilatory mechanisms of retinal arterioles seem to be more vulnerable than those of peripheral resistance vessels to the effects of hyperglycemia. However, the mechanism(s) underlying the onset and development of retinal endothelial dysfunction remains to be elucidated.

Several mechanisms are thought to promote the diabetic vascular complications, for example, increase in aldose reductase (9), activation of protein kinase C (10), upregulation of inflammatory mediators (11), increase in formation of advanced glycation end products (12), and so on. As a first step toward clarifying the underlying mechanism(s) of the retinal endothelial dysfunction, we sought to determine whether increase in aldose reductase

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contributes to the diminished retinal vascular responses to ACh seen in the early stage of diabetes.

Aldose reductase is the first enzyme in the polyol pathway, converting excess glucose to sorbitol, which is then further metabolized to fructose by sorbitol dehydrogenase. Previous studies using aldose reductase–deficient mice and inhibitors of this enzyme clearly showed that the polyol pathway is responsible for several events in the pathogenesis of diabetic retinopathy including blood-retinal barrier breakdown, loss of pericytes, neuro-retinal apoptosis, glial reactivation, and neovascularization (13 – 15). In the present study, we investigated the effects of the aldose reductase inhibitor GP-1447 (16, 17) on the diabetes-induced attenuation of retinal vasodilator responses to ACh in rats. We also evaluated the effects of this inhibitor on the morphological changes of retina and accumulation of sorbitol in the retinas and the lenses of diabetic rats.

Materials and Methods

Animals and treatments

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 Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

Prior to the experiments, male Wistar rats (6 – 7-week-old) were maintained at least 1 week on standard rat chow and tap water ad libitum under a 12:12-h dark cycle. The animals were divided into four groups: non-diabetes (n = 15); non-diabetes treated with the aldose reductase inhibitor GP-1447 (n = 11); diabetes (n = 13); diabetes treated with GP-1447 (n = 11). Diabetes was induced by a single intravenous injection of STZ (65 mg/kg) (Nacalai Tesque, Inc., Kyoto) dissolved in sodium citrate buffer (pH 4.5). Age-matched control rats were treated with an injection of an equal volume of vehicle. The STZ-treated animals were given drinking water containing 5% D-glucose following injection of STZ to shorten the term for the development of retinal vascular dysfunction (8). The GP-1447 (27 mg/mL in stock solution) was diluted in distilled water (final concentration: 0.25 mg/mL) for the non-diabetic rats or diluted in 5% D-glucose solution (final concentration: 0.066 mg/mL) for the diabetic animals and provided ad libitum in the drinking water. The treatment with GP-1447 started immediately after injection of STZ or the vehicle. The experiments were performed 2 weeks later. The averaged water intakes in non-diabetic and diabetic rats were 237 ± 14 (n = 26) and 1,629 ± 42 (n = 24) $mL \cdot kg^{-1} \cdot day^{-1}$, respectively. Therefore, it is estimated that the daily intakes of GP-1447 in non-diabetic and diabetic rats were 100.7 ± 0.8 and 106.0 ± 1.1 mg/kg, respectively. Plasma glucose was determined with a commercially available enzyme kit (Glucose Test Wako; Wako Pure Chemical, Osaka).

In vivo experimental procedures

The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) (Nacalai Tesque). 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 cardiotachometer (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, Utsunomiya) and stored on the hard disk of a personal computer (PowerBook 165C; Apple Japan, Tokyo). To prevent movement of the eye and obtain images of the retinal blood vessels that are captured at the same angle, rats were treated with tetrodotoxin (50 μg/kg, i.v.) (Nacalai Tesque) under artificial ventilation with room air (stroke volume of 10 mL/kg; frequency of 80 strokes/min) using a rodent respirator (SN-480-7; Sinano, Tokyo) (7, 8). Blood pressure was decreased by treatment with tetrodotoxin; therefore, methoxamine (approx. 30 $\mu g \cdot kg^{-1} \cdot min^{-1}$) (Sigma-Aldrich, St. Louis, MO, USA) was continuously injected into the jugular vein at a constant rate by means of a syringe pump (Model 1140-001; Harvard Apparatus, South Natick, MA, USA) to maintain adequate systemic circulation. After hemodynamic parameters reached stable levels, ACh (0.3 – 10 $\mu g \cdot kg^{-1} \cdot min^{-1}$) (Sigma-Aldrich) was infused into the femoral vein using a syringe pump (Harvard Apparatus). The doses of ACh that have no significant effect on HR were chosen on the basis of our previous studies (6, 7).

Measurement of diameter of retinal blood vessels

Fundus images were captured with a Finepix S3 pro (Fuji Photo Film, Co., Ltd., Tokyo) that was equipped with the bore scope-type objective lens for small animals (Model 01, Magnification ×20; Scalar, Tokyo) as described previously (6 – 8). Briefly, hydroxyethylcellulose (SCOPISOL 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. The fundus images were stored on the hard disk of a laboratory computer system (Power Macintosh G3-300DT, Apple Japan).

The retinal blood vessel diameter was measured as described previously (6 – 8). In brief, the digitized fundus images were processed using image processing software (Photoshop 7; Adobe Systems, Inc., San Jose, CA, USA). To make the analysis easier, the greatest contrast of retinal blood vessels was obtained by altering the brightness of the green channel image. After intensifying the contrast of the retinal blood vessels, the region (120 × 240 μm) containing a retinal arteriole in the fundus image (2,820 × 4,230 μm) was selected. Blood vessels were distinguished from background by determining a certain threshold value for each image. The diameter of the vessel was calculated by dividing the vessel's area by its length in the selected area (NIH image 1.6.2; National Institutes of Health, Bethesda, MD, USA).

Histological evaluation of the eye

For histological evaluation, the eyes were enucleated and 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 eyes 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 subjected to morphometry as described previously (8). The overall structure of the retina was assessed by measuring the thickness of the retinal layers. Thicknesses of the inner plexiform layer (IPL), inner nuclear layer (INL), and outer nuclear layer (ONL) were measured in the same topographic region of the retina. Averages for these measurements taken in four adjacent areas within 1 mm of the optic nerve were calculated. We did all of the morphometrical analyses in a blind fashion.

Measurement of sorbitol in the retina and the lens

The contents of sorbitol in the retinas and the lenses were measured as described previously (16). Briefly, the tissues were homogenized in 16% perchloric acid (0.04 mL/mg tissue) and then neutralized with 2 M potassium carbonate. The homogenates were centrifuged at 5,500 × *g* for 10 min. The contents of sorbitol in the supernatants were measured with a commercially available kit (F-kit, D-sorbitol/xylitol; R-Biopharm AG, Darmstadt, Germany). The tissue sorbitol content was expressed as μmol of sorbitol per gram of wet weight of tissue (μmol/g).

Data analyses

The significance of the difference among mean values was evaluated with GraphPad Prism™ (GraphPad, San Diego, CA, USA) by one-way ANOVA followed by the Scheffé correction. When comparing the responses to vasodilators among groups, two-way ANOVA was used. A *P*-value of less than 0.05 was considered to represent a statistically significant difference. 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 measured just before and 2 weeks after the injection are summarized in Table 1. Plasma glucose levels in diabetic rats were significantly higher than those in non-diabetic rats. Body weights of all groups significantly increased compared to the initial weights; however, the weight gain rate of the STZ-treated rats became significantly lower than that of the non-diabetic rats. Treatment with GP-1447 did not affect the glucose levels and body weights of non-diabetic and diabetic rats.

Baseline values of MAP, retinal arteriolar diameter, and HR

Baseline values of MAP and retinal arteriolar diameter were adjusted to the same ranges among the experimental

Table 1. Plasma glucose concentration and body weight measured just before (initial) and 2 weeks (final) after the injection

		Plasma glucose (mg/dL)		Body weight (g)	
		Initial	Final	Initial	Final
Non-diabetes	(n = 15)	123 ± 3	122 ± 3	147 ± 2	260 ± 3*
Non-diabetes + GP-1447	(n = 11)	123 ± 2	120 ± 3	148 ± 3	267 ± 3*
Diabetes	(n = 13)	123 ± 4	811 ± 34*#	146 ± 2	192 ± 6*#
Diabetes + GP-1447	(n = 11)	125 ± 3	818 ± 33*§	152 ± 3	201 ± 5*§

The experiments were performed 2 weeks after injection of STZ or the vehicle. Values are represented as the mean ± S.E.M. *P < 0.05 vs. the corresponding initial value. #P < 0.05 vs. Non-diabetes group. §P < 0.05 vs. Non-diabetes + GP-1447 group.

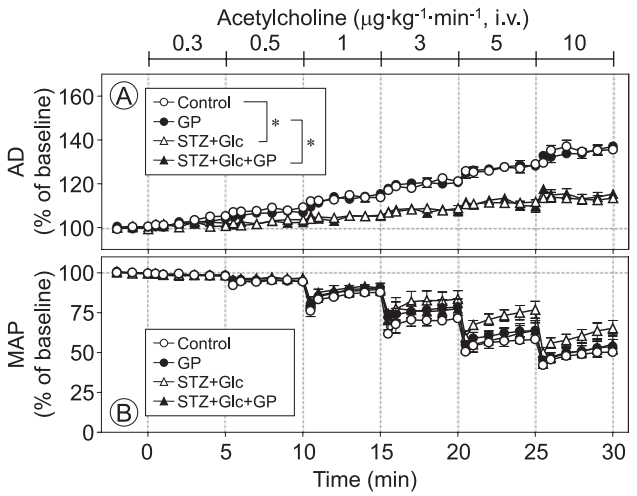

Fig. 1. Changes in diameter of retinal arterioles (AD) (A) and mean arterial pressure (MAP) (B) induced by intravenous infusion of acetylcholine (0.3 – 10 μg·kg^{-1}·min^{-1}) in non-diabetes, non-diabetes with GP-1447, diabetes, and diabetes with GP-1447 groups. Data are expressed as percentages of the baseline values measured just before starting the infusion. Each point with a vertical bar represents the mean ± S.E.M. from 5 animals. *P < 0.05.

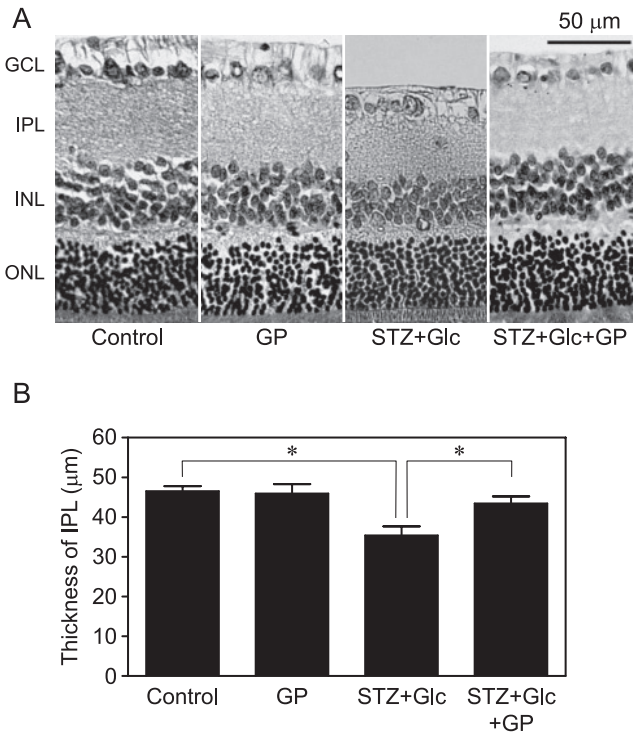

Fig. 2. Morphological changes in the retina. A: Representative photographs of retinal sections in non-diabetes, non-diabetes with GP-1447, diabetes, and diabetes with GP-1447 groups. Thickness of the inner plexiform layer (IPL) was significantly reduced in diabetic rats. B: GP-1447 showed a significant protective effect against the reduction in the thickness of IPL. Each column with a vertical bar represents the mean ± S.E.M. from 8 – 13 animals. *P < 0.05. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

groups by changing methoxamine infusion rates (MAP: non-diabetes, 108 ± 0 mmHg, n = 5; non-diabetes + GP-1447, 108 ± 0 mmHg, n = 5; diabetes, 107 ± 0 mmHg, n = 5; diabetes + GP-1447, 108 ± 1 mmHg, n = 5; retinal arteriolar diameter: non-diabetes, 38.0 ± 1.2 μm, n = 5; non-diabetes + GP-1447, 39.6 ± 1.5 μm, n = 5; diabetes, 38.9 ± 1.7 μm, n = 5; diabetes + GP-1447, 36.9 ± 1.9 μm, n = 5). However, baseline values of HR were significantly (P < 0.05) lower in the diabetic groups (diabetes, 296 ± 5 beats/min, n = 5 and diabetes + GP-1447, 293 ± 9 beats/min, n = 5) than in the non-diabetic groups (non-diabetes, 340 ± 3 beats/min, n = 5 and non-diabetes + GP-1447, 339 ± 8 beats/min, n = 5).

In vivo vascular responses

ACh (0.3 – 10 μg·kg^{-1}·min^{-1}, i.v.) increased the retinal arteriolar diameter but decreased MAP in a dose-dependent manner (Fig. 1). The ACh-induced vasodilation of retinal arterioles was impaired in diabetic rats. The impairment of vascular responses were unaffected by treatment with GP-1447 (Fig. 1A). The depressor responses to ACh showed a tendency to be attenuated in the STZ + Glc group, but these did not reach a statistical significance (Fig. 1B). No significant differences in the depressor responses were observed among the four groups. The doses of ACh did not change HR in any of the groups (data not shown).

Morphological changes in the retina

Representative photographs of retinal sections indicate that diabetes reduced the thickness of the retinal layer (Fig. 2A). The reduction in thickness occurred in IPL, whereas thicknesses of INL and ONL were unchanged. GP-1447 showed a significant protective effect against the diabetes-induced retinal damage (Fig. 2B).

Content of sorbitol in the retina and the lens

The sorbitol content in the retinas of diabetic rats was significantly higher than that of control rats. The increase in sorbitol content of the retinas was completely prevented by treatment with GP-1447 (Fig. 3A). Similarly, the alteration in sorbitol content of the lenses that occurs in diabetic rats was abolished by GP-1447 (Fig. 3B). GP-1447 did not affect the levels of sorbitol in the tissues of non-diabetic rats.

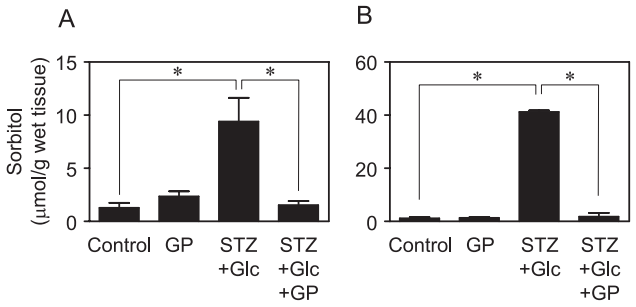

Fig. 3. Contents of sorbitol in retinas (A) and lenses (B) in non-diabetes, non-diabetes with GP-1447, diabetes, and diabetes with GP-1447 groups. Each point with a vertical bar represents the mean ± S.E.M. from 6 – 7 animals. *$P < 0.05$.

Discussion

The present study demonstrates that the aldose reductase inhibitor GP-1447 failed to ameliorate the impaired ACh-induced vasodilation of retinal arterioles, whereas it prevented the thinning of retinal layer in diabetic rats. The enhanced accumulation of sorbitol in the retinas and the lenses from diabetic rats was abolished by GP-1447 treatment. These results suggest that mechanisms that are independent of the polyol pathway may contribute to the onset of retinal endothelial dysfunction, although the pathway plays an important role in diabetes-induced retinal neuron damage in rats.

Previous studies using aldose reductase–deficient animals and aldose reductase inhibitors suggested that activation of the polyol pathway could be an important mechanism for the events in the pathogenesis of diabetic retinopathy, including blood-retinal barrier breakdown, loss of pericytes, neuro-retinal apoptosis, glial reactivation, and proliferation of blood vessels (13, 14). Consistent with these studies, the present study using GP-1447 also indicates that aldose reductase plays an important role in the diabetes-induced changes in retinal thickness and sorbitol content in the retina. In addition, our previous study using the same diabetic model demonstrated that the development of cataracts was completely prevented by inhibition of aldose reductase with GP-1447 (18). Thus the contribution of the polyol pathway to the onset and development of diabetic retinopathy and cataracts is suggested; however, the impairment of ACh-induced retinal vasodilation seems to be mediated by a pathway that involves aldose reductase–independent mechanisms.

Several studies have demonstrated that aldose reductase is mainly present in the pericytes but not in the endothelial cells of retinal blood vessels (19 – 22), although it has also been reported that aldose reductase is present in both endothelial cells and pericytes (23, 24). Our results suggest that the expression levels of aldose reductase might be too low in endothelial cells of rat retinal blood vessels to affect the cellular function through a significant sorbitol accumulation under hyperglycemia. The results obtained from a recent study on cell lines of rat retinal capillary pericytes and endothelial cells may support this hypothesis (25). On the other hand, in rabbit aorta and rat skeletal muscle arterioles, the polyol pathway contributes to the hyperglycemia-induced endothelial dysfunction (26 – 28). Therefore, the role of aldose reductase in endothelial cells may differ depending on the vascular beds.

It has been well known that ACh stimulates production and release of endothelium-derived relaxing factors (EDRFs), including nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF), and thereby dilates blood vessels in an endothelium-dependent manner. Our previous studies suggested that the vasodilator responses to ACh of rat retinal arterioles are mediated by both NO and EDHF, and endothelial NO-dependent vasodilatory mechanism(s) could be preserved 6 – 8 weeks after a single injection of STZ, whereas the production of and/or reduced vascular responsiveness to EDHF is attenuated (6, 7). Furthermore, we found that the endothelial NO-dependent vasodilatory mechanism(s) in the retinal arterioles seems to be preserved even after 2 weeks of severe hyperglycemia induced by the combination of STZ treatment and D-glucose feeding (8). Therefore, the EDHF-mediated vasodilation of retinal blood vessels may be impaired by the polyol pathway–independent mechanisms. With regard to EDHF, several putative candidates have been reported (29 – 33). Because which candidate of EDHF plays an important role in the rat retinal vasculature remains to be established, identification of molecules serving as an EDHF in this vasculature is needed in future studies.

Dysfunction of retinal neuronal cells and abnormalities of the retinal vascular system have been observed in various types of diabetic models. However, there seems to be no conclusive evidence that supports a cause-and-effect relationship between the neuronal and vascular changes. For example, the elevation of retinal vascular permeability was observed in STZ-treated rats from 3 to 10 days after the induction of diabetes (34). In patients with diabetes mellitus, the flicker light–induced vasodilation of retinal blood vessels was already diminished before the clinical appearance of diabetic retinopathy (35). These studies indicate that endothelial dysfunction in the retinal vascular system could serve as early signs for development of diabetic retinopathy. On the other hand, others suggest that neurodegenerative events may precede the vascular changes (36 – 38). In the present study, under

blockade of the polyol pathway with GP-1447, diabetes impaired ACh-induced vasodilation of retinal arterioles, but reduction in thickness of IPL and enhancement of sorbitol accumulation in the diabetic retina were markedly prevented. Therefore, it is unlikely that the neuronal damage detected in the inner retina is associated with retinal vascular dysfunction at the early stage of diabetes in rats.

In summary, we found that the aldose reductase inhibitor GP-1447 showed protective effects against the diabetes-induced retinal damage and completely prevented the enhancement of sorbitol accumulation in the retina, whereas it failed to ameliorate the impaired retinal vascular responses to ACh. Thus, aldose reductase inhibitors would have a potential to prevent the initial stage of the development of diabetic retinopathy. However, it should be noted that the present findings suggest that aldose reductase inhibitors may fail to prevent the abnormalities of retinal hemodynamics in diabetic status.

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