Methylglyoxal Impairs $\beta_2$-Adrenoceptor-Mediated Vasodilatory Mechanisms in Rat Retinal Arterioles

Mari Akagawa, Asami Mori, Kenji Sakamoto, and Tsutomu Nakahara*

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

Received October 24, 2017; accepted November 17, 2017

Methylglyoxal, a highly reactive dicarbonyl compound, is formed as a by-product of glycolysis and plays an important role in the pathogenesis of diabetic complications, including diabetic retinopathy. However, it remains to be determined how methylglyoxal affects the regulatory mechanisms of retinal blood flow. In this study, we examined the effects of methylglyoxal on $\beta_2$-adrenoceptor-mediated vasodilatory mechanisms in rat retinal arterioles. The retinal vasodilator responses were assessed by measuring the diameter of retinal arterioles in the fundus images. Intravitreal injection of methylglyoxal significantly diminished the vasodilation of retinal arterioles induced by the $\beta_2$-adrenoceptor agonist salbutamol. The vasodilator effect of BMS-191011, a large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channel opener, on retinal arterioles was also attenuated by methylglyoxal. In contrast, methylglyoxal had no significant effect on retinal vasodilator response to forskolin. Methylglyoxal attenuated retinal vasodilator response to salbutamol under blockade of BK$_{Ca}$ channels with iberiotoxin, an inhibitor of the channels. These results suggest that methylglyoxal attenuates $\beta_2$-adrenoceptor-mediated retinal vasodilation by impairing the coupling of the $\beta_2$-adrenoceptor to the guanine nucleotide-binding protein (Gs protein) and the function of the BK$_{Ca}$ channel. Increased methylglyoxal in the eyes may contribute to the impairment of regulatory mechanisms of retinal blood flow in patients with diabetic retinopathy.

Key words adenyl cyclase; $\beta_2$-adrenoceptor; diabetic complication; large-conductance Ca$^{2+}$-activated K$^+$ channel; retina

Diabetic retinopathy, the most common complication of diabetes mellitus, is the main cause of acquired blindness in industrialized countries. Under hyperglycemic conditions, increased inflammatory cell adhesion to retinal blood vessels, decreased retinal blood flow, and breakdown of the blood–retinal barrier are frequently observed in experimental diabetic animals and patients with diabetes.$^{1,2}$ These abnormalities of retinal vasculature contribute to the onset and progression of diabetic retinopathy.$^{3-5}$

Methylglyoxal, a highly reactive dicarbonyl compound, is formed as a by-product of glycolysis and normally detoxified by the glyoxalase enzyme system. Methylglyoxal reacts with lysine, cysteine, and arginine residues in proteins and forms advanced glycation end products (AGEs).$^6$ Modification of proteins with methylglyoxal changes their functions, e.g., methylglyoxal-induced modification of proteins associated with the formation of reactive oxygen species leads to an increase in oxidative stress.$^6$

Plasma levels of methylglyoxal are higher in patients with diabetes (4–400 $\mu$M) than in healthy controls (approximately 0.5 $\mu$M).$^7-11$ Excessive methylglyoxal reduces the contractility of isolated blood vessels such as thoracic aorta and superior mesenteric arteries.$^{12}$ In the retinal vascular system, methylglyoxal exhibits cytotoxic effects on pericytes by inducing apoptosis through oxidative stress$^{13}$ and enhances vascular permeability.$^{14}$ Thus, high levels of methylglyoxal may contribute to the pathogenesis of diabetes-related vascular diseases, including diabetic retinopathy.

Our previous studies on rats have shown that the activation of the sympathetic nervous system could increase retinal blood flow by elevating the level of circulating adrenaline.$^{15,16}$ Adrenaline stimulates both $\alpha$- and $\beta$-adrenoceptors on rat retinal blood vessels,$^{16}$ and the vasoconstrictor action of adrenaline, which is mediated by $\alpha_1$-adrenoceptors, is usually masked by the vasodilator action mediated by $\beta$-adrenoceptors.$^{16}$ Under diabetic conditions, $\beta_2$-adrenoceptor-mediated vasodilatory mechanisms were markedly impaired, while the vascular function of $\alpha_1$-adrenoceptors was preserved in the retina.$^{15,17}$ Therefore, the impairment of the $\beta_2$-adrenoceptor-mediated signaling pathway may shift the balance of vasodilator response to vasoconstrictor response, thereby decreasing retinal blood flow, and may consequently contribute to the progression of diabetic retinopathy.

Based on these evidences, we hypothesized that methylglyoxal may impair $\beta_2$-adrenoceptor-mediated vasodilatory mechanisms in retinal blood vessels. To test this hypothesis, we investigated the effects of methylglyoxal on vasodilation of retinal blood vessels induced by salbutamol, an agonist of $\beta_2$-adrenoceptors. We previously demonstrated that salbutamol dilates rat retinal blood vessels by activating large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channels,$^{18}$ and the impairment of BK$_{Ca}$ channel function is attributable to the diminished retinal vasodilator response to salbutamol in diabetic rats.$^{19}$ Therefore, we also examined the effects of methylglyoxal on retinal vasodilator response to BMS-191011, a BK$_{Ca}$ channel opener.

MATERIALS AND METHODS

Drugs The following reagents were used: forskolin, methoxamine hydrochloride, methylglyoxal, salbutamol hemisulfate salt (Sigma-Aldrich, St. Louis, MO, USA), and methoxamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA).
U.S.A.); iberiotoxin (Peptide Institute, Osaka, Japan); 3-[(5-chloro-2-hydroxyphenyl)methyl]-5-[4-(trifluoromethyl)phenyl]-1,3,4-oxadiazol-2(3H)-one (BMS-191011) (Tocris Bioscience, Ellisville, MO, U.S.A.).

Salbutamol and methoxamine were dissolved in saline and diluted with saline to appropriate concentrations for infusion. Methylglyoxal was also diluted with saline. BMS-191011 was diluted with saline to appropriate concentrations for infusion.

Animals Forty-four male Sprague–Dawley rats (8–9 weeks old) were obtained from Charles River Breeding Laboratories (Kanagawa, Japan) and maintained in a room with constant temperature (22±2°C), humidity (55±5%), and a 12-h light/dark cycle and allowed free access to standard rat chow and tap water.

All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research and the Regulations for the Care and Use of Laboratory Animals in Kitasato University adopted by the Institutional Animal Care and Use Committee of Kitasato University.

Experimental Procedures The surgical procedures, the techniques for fundus photography and the methods for retinal arteriolar diameter measurement have been previously described (for further details, see Supplementary Materials and Methods). Experimental Protocols

Protocol 1: Effects of Methylglyoxal on Salbutamol-Induced Responses

We first examined the effect of intravitreal injection of methylglyoxal on the retinal vasodilator response to salbutamol. Intravitreal injections were performed as previously described. Methylglyoxal (0.5 or 2 µmol) or vehicle (saline), in a total volume of 5 µL, was injected into the vitreous cavity of the left eye before the surgical procedures and tetrodotoxin (TTX) treatment. The doses of methylglyoxal were selected based on our preliminary studies; 2 µmol/eye was the highest dose. Assuming a vitreous volume of 56 µL for the rats, the vitreal concentration of methylglyoxal just after the intravitreal injection (2 µmol/eye) is estimated to be 33 mM. About 90 min after the intravitreal injection, salbutamol (0.03–3 µg/kg/min) was injected into the femoral vein using a syringe pump (Model 1140-001, Harvard Apparatus).

Protocol 2: Effects of Methylglyoxal on BMS-191011-Induced Responses

We previously demonstrated that the activation of BKca channels is involved in the retinal vasodilator response to salbutamol in rats. The BKca channel opener BMS-191011-induced vasodilation of retinal arterioles was diminished in diabetic rat retina. Therefore, we next examined the effect of methylglyoxal (2 µmol) on BMS-191011 (10–100 µg/kg/min, intravenously (i.v.))-induced vasodilation of retinal arterioles.

Protocol 3: Effects of Methylglyoxal on Forskolin-Induced Responses

β2-Adrenoceptors are coupled to guanine nucleotide-binding proteins (Gs proteins), which activate adenylyl cyclase. Therefore, we examined the effects of methylglyoxal (2 µmol) on the adenylyl cyclase activator forskolin (1–10 µg/kg/min, i.v.)-induced vasodilation of retinal arterioles.

Protocol 4: Effects of Methylglyoxal on Salbutamol-Induced Responses in the Presence of Iberiotoxin

To determine whether methylglyoxal impairs the coupling of β2-adrenoceptor to the Gs protein, we examined the effect of methylglyoxal (2 µmol) on salbutamol-induced vasodilation of retinal arterioles under blockade of BKca channels with iberiotoxin, an inhibitor of BKca channels. Iberiotoxin (20 µmol) was injected into the vitreous cavity, and then, the eyes were treated with methylglyoxal (2 µmol) or vehicle (saline) as described in Protocol 1.

Data Analysis The statistical analyses were performed using the PRISMS6 software (GraphPad Software, San Diego, CA, U.S.A.). The Mann–Whitney U test and Tukey’s test were used for the comparisons between two groups and among more than two groups, respectively. The responses to the vasodilators between groups were compared using a two-way ANOVA. A p value of <0.05 was considered statistically significant. All values are presented as mean±standard error (S.E.).

RESULTS

Baseline values of retinal arteriolar diameter, mean arterial pressure (MAP), and heart rate (HR) in each experi-

| Groups                  | AD (µm)   | MAP (mmHg) | HR (beats/min) |
|-------------------------|-----------|------------|----------------|
| Protocol 1 (salbutamol) |           |            |                |
| Vehicle (n=8)           | 37.6±1.3  | 117±1      | 340±7          |
| Methylglyoxal (0.5 µmol, n=4) | 42.8±2.7  | 114±1      | 370±10         |
| Methylglyoxal (2 µmol, n=4) | 40.8±2.4  | 115±2      | 350±10         |
| Protocol 2 (BMS-191011) |           |            |                |
| Vehicle (n=4)           | 39.5±3.6  | 111±3      | 366±9          |
| Methylglyoxal (2 µmol, n=6) | 48.6±2.7  | 110±1      | 350±8          |
| Protocol 3 (forskolin)  |           |            |                |
| Vehicle (n=5)           | 38.7±0.9  | 116±2      | 333±4          |
| Methylglyoxal (2 µmol, n=5) | 40.6±1.3  | 120±1      | 324±8          |
| Protocol 4 (salbutamol in the presence of iberiotoxin) | | | |
| Vehicle (n=4)           | 39.0±0.5  | 112±1      | 356±16         |
| Methylglyoxal (2 µmol, n=4) | 45.5±2.40  | 116±1      | 392±6          |

Values are means±S.E. These values were measured just before starting the infusion of salbutamol, BMS-191011 or forskolin. a) p<0.05 vs. the corresponding control value.
mental group are summarized in Table 1. The MAP and HR values were adjusted to approximately 110–120 mmHg and 340–370 beats/min by infusing methoxamine. Retinal arteriolar diameter tended to increase in methylglyoxal-treated groups, and the increase reached statistical significance ($p<0.05$) in the iberiotoxin plus methylglyoxal-treated group.

Effects of Methylglyoxal on Salbutamol-Induced Responses

Salbutamol (0.03–3 $\mu$g/kg/min, i.v.) increased the diameter of retinal arterioles and decreased MAP in a dose-dependent manner (Figs. 1A, B). However, doses of salbutamol tested in this study showed no significant increase in HR (Fig. 1C). Treatment with methylglyoxal (0.5 and 2 $\mu$mol) significantly prevented salbutamol-induced vasodilation of retinal arterioles (Fig. 1A), e.g., at 3 $\mu$g/kg/min, changes in the retinal arteriolar diameter in vehicle- and methylglyoxal (0.5 and 2 $\mu$mol/eye)-treated groups were 38.0 ± 2.4% ($n=8$), 30.0 ± 2.7% ($n=4$, $p>0.05$), and 20.8 ± 2.5% ($n=4$, $p<0.05$), respectively. Intravitreal injection of methylglyoxal did not affect salbutamol-induced depressor responses (Fig. 1B), indicating that the amount of methylglyoxal leaked out of the eye into the systemic circulation is a very small.

Effects of Methylglyoxal on BMS-191011-Induced Responses

BMS-191011 (10–100 $\mu$g/kg/min, i.v.) increased the diameter of retinal arterioles but had no significant effect on MAP and HR (Fig. 2). Changes in the retinal arteriolar diameter induced by BMS-191011 were significantly reduced by methylglyoxal treatment (e.g., at 100 $\mu$g/kg/min; vehicle, 22.2 ± 0.7%, $n=4$ vs. methylglyoxal, 2.0 ± 2.2%, $n=6$, $p<0.05$).

Effects of Methylglyoxal on Forskolin-Induced Responses

Forskolin (1–10 $\mu$g/kg/min) also increased the diameter of retinal arterioles with depressor responses and tachycardia (Fig. 3). However, methylglyoxal had no significant effect on forskolin-induced responses.

Effects of Methylglyoxal on Salbutamol-Induced Responses in the Presence of Iberiotoxin

Increases in the retinal arteriolar diameter induced by salbutamol in iberiotoxin (20 pmol)-treated eyes were smaller than those in control (saline-treated) eyes (Fig. 4A vs. Fig. 1A), e.g., at
3 μg/kg/min, changes in the retinal arteriolar diameter of salmon- and iberiotoxin-treated groups were 38.0 ± 2.4% (n = 8) and 24.4 ± 1.7% (n = 4, p < 0.01), respectively. Methylglyoxal attenuated the retinal vasodilator response to salbutamol in the presence of iberiotoxin (changes in retinal arteriolar diameter at 3 μg/kg/min; iberiotoxin, 24.4 ± 1.7%, n = 4 vs. iberiotoxin+salbutamol, 8.9 ± 4.2%, n = 4, p < 0.05). Intravitreal injections of iberiotoxin plus methylglyoxal had no significant effect on salbutamol-induced MAP and HR responses (Figs. 4B, C).

DISCUSSION

We previously demonstrated that retinal vasodilator responses to β2-adrenoceptor agonists are, at least in part, mediated by activating BKCas channels in rats, and the BKCas channel-mediated vasodilatory mechanism is impaired by diabetes. In patients with diabetes, the plasma concentration of methylglyoxal, a reactive glucose metabolite, is high. As a first step toward clarifying the pathological role of methylglyoxal in diabetic retinas, this study aimed to determine how methylglyoxal affects the regulatory mechanisms of retinal vasomotor tone. The results suggest that methylglyoxal acts directly on β2-adrenoceptors and BKCas channels and consequently impairs β2-adrenoceptor-mediated vasodilator mechanisms in retinal blood vessels.

β2-Adrenoceptors are usually coupled to Gs proteins, which activate adenylyl cyclase to form cAMP from ATP. In addition to the basic mechanisms, the activation of BKCas channels contributes to β2-adrenoceptor-mediated vasodilation of rat retinal arterioles. The results obtained from experiments with BMS-191011 and forskolin suggest that the BKCas channel function is impaired by methylglyoxal, whereas the adenylyl cyclase-cAMP pathway-mediated vasodilator mechanism is preserved when higher levels of methylglyoxal are present in the retina. However, the salbutamol-induced vasodilator response observed under blockade of BKCas channels with iberiotoxin, a BKCas channel inhibitor, was significantly reduced by methylglyoxal. Therefore, the coupling of β2-adrenoceptor to the Gs protein may also be impaired by methylglyoxal.

The mechanisms by which methylglyoxal impairs the coupling of β2-adrenoceptor to the Gs protein and the function of BKCas channel remain to be elucidated. Methylglyoxal is a highly reactive dicarbonyl compound and thus may bind to cell surface proteins, including extracellular domains of β2-adrenoceptors and BKCas channels. Methylglyoxal reacts with lysine, cysteine, or arginine residues in proteins. Such extracellular modification of receptors and channels would change their functions. Thus, methylglyoxal injected into the vitreous cavity may directly interact with some of the extracellular components of β2-adrenoceptors and BKCas channels on retinal vascular smooth muscle cells, thereby impairing their functions.

In this study, methylglyoxal significantly prevented retinal vasodilator responses to all vasodilators tested except forskolin. Mammalian adenylyl cyclases contain two conserved cytoplasmic regions (C1 and C2), which form the catalytic core. Forskolin penetrates the cell membrane and binds in a pocket at the interface of the C1 and C2 domains. On the contrary, water-soluble substances typically do not cross the cell membrane, and the pathways and mechanisms of methylglyoxal, a water-soluble molecule, across the plasma membrane remain to be fully clarified. The finding that intravitreal injection of methylglyoxal had no significant effect on forskolin-induced retinal vasodilation may indicate that methylglyoxal did not cross the retinal vascular cell membrane or the length of exposure of retinal vascular cells to methylglyoxal (ca. 90 min) was insufficient to penetrate the cell membrane. Forskolin-induced relaxation of airway smooth muscle is partly mediated by activating BKCas channels. However, iberiotoxin failed to affect retinal vasodilator response to forskolin (unpublished observations); therefore, the contribution of the adenylyl cyclase-cAMP pathway to activation of BKCas channels on rat retinal vascular cells is unlikely. This also explains why methylglyoxal did not affect forskolin-induced retinal vasodilation.

Previous studies demonstrated that methylglyoxal (420 μM) attenuates noradrenaline-induced contraction of rat aorta and mesenteric artery by activating BKCas channels. On the other hand, our present results suggest that methylglyoxal impairs the BKCas channel function in retinal arterioles. The reasons for the differences are unclear; but may be due to the vascular bed studied (retinal vs. peripheral), or differences in concentrations of methylglyoxal. However, baseline diameter of retinal arterioles tended to increase in methylglyoxal-treated groups, methylglyoxal may affect the contractility of retinal blood vessels. Therefore, it would be interesting to examine the effects of methylglyoxal on the contractile responses of retinal blood vessels in future studies.

Methylglyoxal is produced in the process of glucose metabolism and its levels are elevated under diabetic conditions. An accumulation of methylglyoxal in the retina may contribute to the onset and progression of diabetic retinopathy. It has been
reported that plasma concentrations of methylglyoxal in diabetic patients range from 4–400 μM. However, the concentrations of methylglyoxal in the vitreous humor and in the retinal tissue have not been previously reported. Therefore, methylglyoxal doses were selected on the basis of the results of studies examining short-term impacts of methylglyoxal. The changes in the β2-adrenoceptor system observed in non-diabetic rats exposed to methylglyoxal resembled those in diabetic rats, but the severity of dysfunction was greater in methylglyoxal-treated retinas than in diabetic retinas. The results indicate that the severity of dysfunction was greater in methylglyoxal-treated retinas than in diabetic retinas. The results indicate that the concentration of methylglyoxal tested (an estimated vitreous methylglyoxal peak concentration: ca. 33 μM) may be higher than that achieved in diabetic retinas. Furthermore, diabetic retinopathy is associated with chronic exposure to hyperglycemia. It would be important to examine the effects of chronic treatment with lower concentrations of methylglyoxal on the regulatory mechanisms of retinal circulation.

In summary, our study provides the first pharmacological evidence suggesting that intravitreal methylglyoxal impairs β2-adrenoceptor-mediated vasodilatory mechanisms in retinal blood vessels. The levels of methylglyoxal could be enhanced under diabetic conditions, and circulating adrenaline plays an important role in the regulation of retinal blood flow. Therefore, methylglyoxal may contribute to retinal vascular dysfunction in diabetes mellitus, leading to the progression of diabetic retinopathy.

**Acknowledgments** This study was supported by the Kitasato University Research Grant for Young Researchers (A.M.), Suzuken Memorial Foundation (A.M.), and the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant Numbers: 15K08242 A.M. and 26460103 T.N.).

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

**REFERENCES**

1. Bursell SE, Clermont AC, Shiba T, King GL, Uusitalo H. Evaluating retinal circulation using video fluorescein angiography in control and diabetic rats. *Curr. Eye Res.*, 11, 287–295 (1992).

2. Clermont AC, Bursell SE. Retinal blood flow in diabetes. *Microcirculation*, 14, 49–61 (2007).

3. De La Cruz IP, Gonzalez-Correa JA, Guerrero A, de la Cuesta FS. Pharmacological approach to diabetic retinopathy. *Diabetes Metab. Res. Rev.*, 20, 91–113 (2004).

4. Peke GT, Buzney SM, Ogawara H, Fujio N, Goger DG, Spack NP, Gabbay KH. Retinal circulatory abnormalities in type 1 diabetes. *Invest. Ophthalmol. Vis. Sci.*, 35, 2968–2975 (1994).

5. Ghirlanda G, De Leo MA, Caputo S, Cercone S, Greco AV. From functional to microvascular abnormalities in early diabetic retinopathy. *Diabetes Metab. Rev.*, 13, 15–35 (1997).

6. Yim HS, Kang SO, Hah YC, Chock PB, Yim MB. Free radicals generated during the glycation reaction of amino acids by methylglyoxal. A model study of protein-cross-linked free radicals. *J. Biol. Chem.*, 270, 28228–28233 (1995).

7. McLellan AC, Thornalley PJ, Benn J, Sonksen PH. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin. Sci.*, 87, 21–29 (1994).

8. Oto Y, Aoki S, Ohnishi K, Yasuda T, Kawano K, Tsukada Y. Increased serum levels of advanced glycation end products in NIDDM patients with diabetic complications. *Diabetes Care*, 21, 1027 (1998).

9. Odani H, Shinzato T, Matsumoto Y, Usami J, Maeda K. Increase in three alpha, beta-dicarbonyl compound levels in human uremic plasma: specific in vivo determination of intermediates in advanced Maillard reaction. *Biochem. Biophys. Res. Commun.*, 256, 89–93 (1999).

10. Lapolla A, Flamini R, Dalla Vedova A, Senesi A, Reitano R, Fedele D, Basso E, Seraglia R, Traldi P. Glyoxal and methylglyoxal levels in diabetic patients: quantitative determination by a new GC/MS method. *Clin. Chem. Lab. Med.*, 41, 1166–1173 (2003).

11. Kong X, Ma MZ, Huang K, Qin L, Zhang HM, Yang Z, Li XY, Su Q. Increased plasma levels of the methylglyoxal in patients with newly diagnosed type 2 diabetes. *J. Diabetes*, 6, 535–540 (2014).

12. Mukohda M, Yamawaki H, Nomura H, Okada M, Hara Y. Methylglyoxal inhibits smooth muscle contraction in isolated blood vessels. *J. Pharmacol. Sci.*, 109, 305–310 (2009).

13. Kim J, Son JW, Lee JA, Oh YS, Shin SH. Methylglyoxal induces apoptosis mediated by reactive oxygen species in bovine retinal pericytes. *J. Korean Med. Sci.*, 19, 95–100 (2004).

14. Kim J, Kim CS, Lee YM, Jo H, Shin SH, Kim JS. Methylglyoxal induces hyperpermeability of the blood-retinal barrier via the loss of tight junction proteins and the activation of matrix metalloproteinases. *Graefes Arch. Clin. Exp. Ophthalmol.*, 250, 691–697 (2012).

15. Nakazawa T, Sato A, Mori A, Saito M, Sakamoto K, Nakahara T, Ishii K. β2-adrenoceptor-mediated vasodilation of retinal blood vessels is reduced in streptozotocin-induced diabetic rats. *Vasc. Pharmacol.*, 49, 77–83 (2008).

16. Mori A, Nakahara T, Sakamoto K, Ishii K. Role of β2-adrenoceptors in regulation of retinal vascular tone in rats. *Naunyn Schmiedebergs Arch. Pharmacol.*, 384, 603–608 (2011).

17. Mori A, Miwa T, Sakamoto K, Nakahara T, Ishii K. Pharmacological evidence for the presence of functional β2-adrenoceptors in rat retinal blood vessels. *Naunyn Schmiedebergs Arch. Pharmacol.*, 382, 119–126 (2010).

18. Mori A, Takei T, Sakamoto K, Nakahara T, Ishii K. 4-Hydroxy-2-nonenal attenuates β2-adrenoceptor-mediated vasodilation of rat retinal arterioles. *Naunyn Schmiedebergs Arch. Pharmacol.*, 388, 575–582 (2015).

19. Mori A, Suzuki S, Sakamoto K, Nakahara T, Ishii K. Vasodilation of retinal arteries induced by activation of BK<sub>Ca</sub> channels is attenuated in diabetic rats. *Eur. J. Pharmacol.*, 669, 94–99 (2011b).

20. Mori A, Sajio O, Hanada M, Nakahara T, Ishii K. Hyperglycemia accelerates impairment of vasodilator responses to acetylcholine of retinal blood vessels in rats. *J. Pharmacol. Sci.*, 110, 160–168 (2009).

21. Nakazawa T, Kaneko Y, Mori A, Saito M, Sakamoto K, Nakahara T, Ishii K. Attenuation of nitric oxide- and prostaglandin-independent vasodilation of retinal arterioles induced by acetylcholine in streptozotocin-treated rats. *Vasc. Pharmacol.*, 46, 153–159 (2007).

22. Berkowitz BA, Lukaszew RA, Mullins CM, Penn JS. Impaired hyaloidal circulation function and uncoordinated ocular growth patterns in experimental retinopathy of prematurity. *Invest. Ophthalmol. Vis. Sci.*, 39, 391–396 (1998).

23. Tesmer JJ, Sunahara RK, Gilman AG, Sprang SR. Crystal structure of the catalytic domains of adenylyl cyclase in a complex with Gsα: GTPyS. *Science*, 278, 1907–1916 (1997).

24. Zhang G, Liu Y, Ruoho AE, Hurley JH. Structure of the adenylyl cyclase catalytic core. *Nature*, 386, 247–253 (1997).

25. Hiramatsu T, Kume H, Kotlikoff MI, Takagi K. Role of calcium-activated potassium channels in the relaxation of tracheal smooth muscles by forskolin. *Clin. Exp. Pharmacol. Physiol.*, 21, 367–375 (1994).