Role of Epoxycosatrienoic Acids in Acetylcholine-Induced Dilation of Rat Retinal Arterioles in Vivo

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INTRODUCTION

Vascular endothelial cells play a crucial role in the regulation of retinal vascular tone by releasing several vasodilatory substances, including nitric oxide (NO), prostaglandin I2, and endothelium-derived hyperpolarizing factor (EDHF). We previously demonstrated that both NO and prostaglandin I2 were key regulators of retinal vascular tone. However, the presence of NO- and prostaglandin-independent, endothelium-dependent hyperpolarization (EDH)-related vasodilatory mechanism(s) was suggested in the retinal vasculature. The underlying mechanism of EDH-related retinal vasodilation is yet not fully understood.

Several candidates for EDHF have been proposed, including K+i, epoxyeicosatrienoic acid (EET), C-type natriuretic peptide, and hydrogen peroxide. Moreover, there is strong evidence to support that hyperpolarization in endothelial cells can be transmitted to subjacent smooth muscle cells via gap junctions. The underlying mechanism of EDH varies depending on species, agonists, and the type of vasculature used.

For example, endothelial hyperpolarization mediated by intermediate-conductance KCa (IKCa) channels and small-conductance KCa (SKCa) channels play a critical role in initialzing the EDH-related dilation of conduit and resistance-sized arteries in many species including humans. In contrast, in rat retinal arterioles, the large-conductance, Ca2+-activated K+ channels (BKCa) channel, but not the IKCa and SKCa channels, appear to be primarily responsible for the EDH-related dilation.

CYP epoxide-derived EETs cause vascular smooth muscle relaxation by activating BKCa channels and membrane hyperpolarization. Therefore, the purpose of the present study was to determine the role of EETs in acetylcholine (ACH)-induced dilation of rat retinal arterioles in vivo.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee for Kitasato University (Approval No. I07-1). All animal procedures and experiments were performed in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Animals A total of 47 male Wistar rats (8 to 10 weeks old) were used in this study. Rats were maintained in an animal room controlled at a constant condition (temperature, 22 ± 2°C; relative humidity, 55 ± 5%; 12 h dark–light cycle), with free access to standard rat chow and tap water.

Reagents The following reagents were used: acetylcholine chloride, indomethacin, Nω-nitro-l-arginine methyl ester (l-NAME), methoxamine hydrochloride (Sigma-Aldrich, St. Louis, MO, U.S.A.), 14,15-epoxyeicosapentaenoic acid (14,15-EE-5(Z)-E), 14,15-EE-4(Z)-E, 17-octadecynoic acid (17-ODYA) (Cayman Chemical Co., Ann Arbor, MI, U.S.A.), iberiotoxin (Peptide Institute, Osaka, Japan), and pentobarbital sodium, tetrodotoxin (Nacalai Tesque, Kyoto, Japan). 17-ODYA was dissolved in ethanol and further diluted with saline (final concentration of ethanol, 1.4%). 14,15-EE-5(Z)-E as a solution in ethanol was further diluted with saline (final concentration of ethanol, 66%). 14,15-EE-5(Z)-E as a solution in ethanol was evaporated and reconstituted in saline. Indomethacin was dissolved in 0.24% sodium carbonate (Na2CO3). All other
drugs were dissolved in saline.

General Preparations Surgical procedures were performed under anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneally) as reported previously. Heart rate was measured with a cardiotachometer triggered by the blood pressure pulse. Mean arterial pressure and heart rate were digitized at 1 Hz (15BXW-H4; Dacs Giken, Okayama, Japan). To capture fundus images at the same angle throughout the experiment by preventing movement of the eye, rats were treated with tetrodotoxin (50 µg/kg, intravenously (i.v.)) under artificial ventilation with room air. Methoxamine hydrochloride was infused into the jugular vein at approx. 20 µg/kg/min using a syringe pump (Model 1140-001, Harvard Apparatus, South Natick, MA, U.S.A.) to compensate the decrease in mean arterial pressure after treatment with tetrodotoxin. In rats treated with L-NAME and indomethacin, we used the lower dose of methoxamine hydrochloride (approx. 7 µg/kg/min) because L-NAME increases blood pressure. Additional pentobarbital sodium (10 mg/kg) were injected as needed.

Experimental Protocols Protocol 1: Roles of CYP Epoxynogenase-Derived EETS in ACh-Induced Dilation of Retinal Arterioles

We first examined the roles of CYP epoxynogenase-derived EETS in ACh-induced dilation of the retinal arterioles. It has been shown that 17-ODYA, an inhibitor of CYP epoxynogenase, and 14,15-EE-5(Z)-E, a structural analog of 14,15-EET, prevented ACh-induced dilation in several vascular beds. Ten microliters of either 17-ODYA (1.4 nmol), 14,15-EE-5(Z)-E (2 nmol) or the vehicle (1.4% ethanol for 17-ODYA and 66% methanol for 14,15-EE-5(Z)-E) was injected into the vitreous cavity of the left eye as previously reported. Intravitreal injection was performed before the surgical procedures and tetrodotoxin treatment described above. Approximately 90 min after the intravitreal injection, ACh (0.3–10 µg/kg/min) was infused into the femoral vein using a syringe pump (Model 1140-001, Harvard Apparatus). The doses of ACh shown to have no obvious effect on heart rate in our previous results were chosen. Protocol 2: Roles of EETs in the EDH-Related Component of ACh-Induced Dilation of Retinal Arterioles

14,15-EE-5(Z)-E has been shown to prevent the EDH-related dilation of some arteries. To determine the role of EETs in the EDH-related vasodilator response, we examined effects of 14,15-EE-5(Z)-E (2 nmol/eye) on the ACh-induced dilation of the retinal arterioles under inhibition of NO synthases and cyclooxygenases with l-NAME (30 mg/kg, i.v.) plus indomethacin (5 mg/kg, i.v.). The doses of l-NAME and indomethacin were chosen on the basis of our previous results. Treatment of rats with l-NAME and indomethacin was performed approximately 20 min before the ACh infusion was started. Protocol 3: Effects of Intravitreal Injection of 14,15-EET on the Diameter of Retinal Arterioles

EETs have been reported to induce vascular smooth muscle relaxation through the activation of BKCa channels. Therefore, we examined whether 14,15-EET dilates retinal arterioles and whether the response is prevented by the BKCa channel inhibitor iberiotoxin. In addition, we examined whether the 14,15-EET-induced dilation of retinal arterioles is blocked by 14,15-EE-5(Z)-E. In these experiments, 14,15-EET (0.5 nmol/eye) and either 14,15-EE-5(Z)-E (2 nmol/eye), iberiotoxin (20 pmol/eye), or the vehicle were intravitreally injected.

In previous studies, we found that the dose of iberiotoxin almost completely blocked the BKCa channel opener BMS-191011-induced dilation of retinal arterioles. In preliminary studies, we found that 14,15-EE-5(Z)-E did not affect the retinal vasodilator responses to BMS-191011 (10–100 µg/kg/min, i.v.).

Measurement of the Diameter of the Retinal Arterioles

We assessed the changes in the diameter of the retinal arterioles in ocular fundus images as described in our previous reports. Briefly, images of the fundus were captured before and during intravenous infusion of ACh by using a digital camera equipped with a bore scope-type objective lens for small animals (Model 01; Scalar, Tokyo, Japan). A region (120 × 240 pixels, 120 × 240 µm) of the fundus image (2820 × 4230 pixels, pixel size = 1 µm), which includes a retinal arteriole, was selected. The diameter of each arteriole was measured for evaluation of the retinal vasodilator response. This original fundus camera system allows us to capture repeatedly high-quality, reproducible ocular fundus images of rats for several hours.

The retinal arteriolar diameter, mean arterial pressure, and heart rate were expressed as a percentage (%) of the baseline values (the mean values of the data obtained from time = 2 to 0 min).

Statistical Analyses All values were presented as the mean ± standard error of the mean (S.E.M.). An unpaired t-test and one-way ANOVA followed by Tukey’s post-test, were used for the comparisons between two groups and among more than two groups, respectively. When comparing the responses to ACh, two-way repeated measured ANOVA followed by Bonferroni’s post-test was used (PRISM6, GraphPad Software, San Diego, CA, U.S.A.). A p value of <0.05 was considered statistically significant.

RESULTS

There were no significant differences in the parameters measured just before the start of ACh infusion among the experimental groups in each protocol (Table 1). Representative images of the fundus captured at baseline and 0.5 min after initiation of the intravenous infusion of ACh (10 µg/kg/min) are shown in Fig. 1, with an obvious dilation of the retinal arterioles.

ACh (0.3–10 µg/kg/min, i.v.) increased the diameter of the retinal arterioles, but decreased the mean arterial pressure in a dose-dependent manner (Figs. 2A, B). Consistent with our previous findings, the doses of ACh had no obvious effect on heart rate (Fig. 2C). Intravitreal injection of 17-ODYA (1.4 nmol/eye) reduced the ACh-induced increase in the diameter of the retinal arterioles, without affecting the depressor response (Figs. 2A, B). 17-ODYA completely prevented the retinal vasodilator responses to ACh at lower doses, whereas at higher doses, 17-ODYA-insensitieve component of vasodilatation was observed (Fig. 2A).

Intravitreal injection of 14,15-EE-5(Z)-E (2 nmol/eye) also reduced the ACh-induced increase in the diameter of the retinal arterioles, without affecting the depressor response (Figs. 3A, B). 14,15-EE-5(Z)-E also completely prevented the retinal vasodilator responses to ACh at lower doses (Fig. 3A). Thus, the contribution of CYP epoxynogenase-derived EETS seems to be more important at lower doses of ACh.
The vasodilator responses to ACh under treatment with L-NAME and indomethacin are shown in Fig. 4. The ACh-induced dilation of the retinal arterioles (at 10 µg/kg/min, 17.8 ± 2.6%, n = 5) was significantly smaller than that seen in absence of L-NAME and indomethacin (see Fig. 2A; at 10 µg/kg/min, 32.4 ± 4.5%, n = 5). The NO- and prostaglandin-independent component of vasodilation was attenuated by 14,15-EE-5(Z)-E (Fig. 4A).

An intravitreal injection of 14,15-EET (0.5 nmol/eye) increased the diameter of the retinal arterioles without affecting mean arterial pressure and heart rate (Fig. 5). The vasodilator responses of the retinal arterioles were prevented by 14,15-EE-5(Z)-E and iberiotoxin (Fig. 5A).

**DISCUSSION**

We previously reported that the dilator response to ACh is mediated by NO-dependent and NO-independent mechanisms in retinal arterioles in rats in vivo.6-7) NO causes retinal vasodilator response through the cyclooxygenase-1/prostaglandin I₂/prostanoid IP receptor/cAMP signaling pathway8 and the activation of 4-aminopyridine-sensitive Kᵥ channels9) in rats. Thus, NO and prostaglandin I₂ are key regulators of retinal vascular tone1-5) (Fig. 6). In the present study, we found that both 17-ODYA, an inhibitor of CYP epoxygenase, and 14,15-EE-5(Z)-E, an antagonist of EETs, attenuated the ACh-induced dilation of rat retinal arterioles. The attenuation of ACh-induced dilation by 14,15-EE-5(Z)-E was also observed under treatment with L-NAME plus indomethacin. These results suggest that the NO- and prostaglandin-independent component of ACh-induced retinal vasodilator response is mediated partly by an EDH mechanism, through CYP epoxygenase-derived EETs, in rats (Fig. 6).

Multiple mechanisms have been proposed to explain the dilator action of EETs.11,12) However, at present, the mechanisms by which EETs activate BK Ca channels in rat retinal arterioles are unknown. In rat cerebral arteries, endothelium-derived EETs activate transient receptor potential vanilloid 4 (TRPV4) channels in smooth muscle cells.20,21) Activation of TRPV4 channels facilitates the Ca²⁺ influx, stimulating ryanodine receptor in the sarcoplasmic reticulum generating Ca²⁺ sparks. The Ca²⁺ sparks activate BK₉ channels resulting in smooth muscle cell hyperpolarization and relaxation. We recently demonstrated that activation of TRPV4 channels facilitates the Ca²⁺ influx, stimulating ryanodine receptor in the sarcoplasmic reticulum generating Ca²⁺ sparks. The Ca²⁺ sparks activate BK₉ channels resulting in smooth muscle cell hyperpolarization and relaxation. We recently demonstrated that activation of TRPV4 channels dilates rat retinal arterioles partly through activation of BK₉ channels.22)

Depending on the vascular bed and animal species, TRPV4 channels are expressed in both vascular endothelial and smooth muscle cells.21) BK₉ channels are expressed in smooth

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**Table 1. Baseline Values of Retinal Arteriolar Diameter, Mean Arterial Pressure and Heart Rate in Rats**

| Protocol 1-1 | Retinal arteriolar diameter (µm) | Mean arterial pressure (mmHg) | Heart rate (beats/min) |
|--------------|---------------------------------|-----------------------------|----------------------|
| Vehicle (n = 5) | 33.7 ± 1.2 | 113 ± 2 | 411 ± 12 |
| 17-ODYA (n = 5) | 37.1 ± 1.4 | 110 ± 2 | 420 ± 8 |

| Protocol 1-2 | Retinal arteriolar diameter (µm) | Mean arterial pressure (mmHg) | Heart rate (beats/min) |
|--------------|---------------------------------|-----------------------------|----------------------|
| Vehicle (n = 5) | 35.6 ± 2.4 | 110 ± 2 | 391 ± 18 |
| 14,15-EE-5(Z)-E (n = 5) | 37.4 ± 3.0 | 113 ± 3 | 397 ± 19 |

| Protocol 2 | Retinal arteriolar diameter (µm) | Mean arterial pressure (mmHg) | Heart rate (beats/min) |
|------------|---------------------------------|-----------------------------|----------------------|
| L-NAME + indomethacin + vehicle (n = 5) | 33.9 ± 2.0 | 130 ± 4 | 377 ± 10 |
| L-NAME + indomethacin + 14,15-EE-5(Z)-E (n = 6) | 33.6 ± 1.8 | 136 ± 4 | 360 ± 7 |

| Protocol 3 | Retinal arteriolar diameter (µm) | Mean arterial pressure (mmHg) | Heart rate (beats/min) |
|------------|---------------------------------|-----------------------------|----------------------|
| Vehicle (n = 5) | 45.2 ± 1.1 | 112 ± 1 | 385 ± 9 |
| Vehicle + 14,15-EE-5(Z)-E (n = 5) | 45.6 ± 3.0 | 113 ± 3 | 396 ± 15 |
| Vehicle + iberiotoxin (n = 6) | 52.6 ± 2.0 | 110 ± 1 | 393 ± 9 |

Values are means ± S.E.M. These values were measured just before starting the infusion of ACh.

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**Fig. 1. Representative Fundus Images at Baseline and 0.5 min after Initiating Intravenous Infusion of ACh (10 µg/kg/min) in Rats**

The increase in diameter of the retinal arteriole in the selected region was 37.5%.
muscle cells of rat retinal blood vessels. These results suggest that EETs stimulate TRPV4 channels in vascular smooth muscle cells, resulting in activation of BK<sub>Ca</sub> channels, leading to dilation of retinal arterioles. However, EETs could directly influence the activity of BK<sub>Ca</sub> channels. Therefore, we cannot exclude the possibility that EETs directly activates BK<sub>Ca</sub> channels in retinal arterioles.

It has been reported that four EET regioisomers (5,6-, 8,9-, 8,11-, and 11,12-) are potent vasodilators. In this study, we investigated the role of EETs in retinal arteriolar function and their potential involvement in the regulation of retinal blood flow.

Fig. 2. Effect of 17-ODYA (1.4 nmol/eye) on ACh (0.3–10 µg/kg/min)-Induced Responses in Rats
A; retinal arteriolar diameter, B; mean arterial pressure, C; heart rate. Intravitreal injection of 17-ODYA reduced the ACh-induced dilation of retinal arterioles, without affecting the depressor response. n = 5. *p < 0.05.

Fig. 3. Effect of 14,15-EE-5(Z)-E (2 nmol/eye) on ACh (0.3–10 µg/kg/min)-Induced Responses in Rats
A; retinal arteriolar diameter, B; mean arterial pressure, C; heart rate. Intravitreal injection of 14,15-EE-5(Z)-E reduced the ACh-induced dilation of retinal arterioles, without affecting the depressor response. n = 5. *p < 0.05.

Fig. 4. Effect of 14,15-EE-5(Z)-E (2 nmol/eye) on ACh (0.3–10 µg/kg/min)-Induced Responses in Rats Treated with L-NAME (30 mg/kg, i.v.) and Indomethacin (5 mg/kg, i.v.)
A; retinal arteriolar diameter, B; mean arterial pressure, C; heart rate. Intravitreal injection of 14,15-EE-5(Z)-E reduced the ACh-induced, EDH-related dilation of the retinal arterioles. n = 5–6 *p < 0.05.

Fig. 5. Effect of 14,15-EET on Retinal Arteriolar Diameter, Mean Arterial Pressure and Heart Rate in the Presence of 14,15-EE-5(Z)-E or Iberiotoxin
A; retinal arteriolar diameter, B; mean arterial pressure, C; heart rate. Rats were injected intravitreally with 14,15-EET (0.5 nmol/eye) and either 14,15-EE-5(Z)-E (2 nmol/eye), iberiotoxin (20 pmol/eye), or the vehicle. Both 14,15-EE-5(Z)-E and iberiotoxin reduced 14,15-EET-induced dilation of retinal arterioles. n = 5–6. *p < 0.05.
11,12-, and 14,15-EET) are synthesized from arachidonic acid by CYP epoxygenases in vascular endothelial cells. A recent study on rat cerebral artery smooth muscle cells has shown that 14,15-EET increased BKCa channel current density and that the response was prevented by iberiotoxin, an inhibitor of BKCa channels. In rat retinal arterioles, the activation of BKCa channels, but not IKCa and SKCa channels, appears to be an important mechanism for the ACh-induced, EDH-related dilation. Therefore, we examined the effects of 14,15-EET on rat retinal arterioles. As a result, we found that 14,15-EET dilated rat retinal arterioles and that this response was prevented by iberiotoxin. Thus, 14,15-EET seems to represent a transferable EDHF in rat retinal arterioles. However, the potency and actions of EET regioisomers and the underlying mechanisms are not the same in all vascular tissues. Therefore, further studies using regioisomer-selective agonist/antagonists are needed to determine whether other EET regioisomers (5,6-, 8,9-, and 11,12-EET) are responsible for the ACh-induced dilation of retinal arterioles in future.

The present study demonstrated that CYP epoxygenase-derived EETs contribute to the ACh-induced, EDH-related dilation of rat retinal arterioles. However, the contribution of EETs seems to be more important at lower doses of ACh, and higher doses of ACh induced 17-ODYA- and 14,15-EE-5(Z)-E-insensitive retinal vasodilator response. Thus, the contribution of EETs may vary depending on the dose of ACh. In addition, EETs could modulate the gap junctions. To clarify these possibilities, further studies are needed to determine: 1) whether other EDHF candidates are involved in the ACh-induced dilation of retinal arterioles; and 2) whether EETs facilitate the transmission of ACh-induced hyperpolarization from the endothelial cells to the adjacent smooth muscle cells through gap junctions. Furthermore, the mechanism of EDH-related dilation depends on agonists used. It would be interesting to investigate whether other agonists induce EDH-related retinal vasodilator response via the same mechanism described above.

The endothelium-dependent vasodilatory mechanism(s) could be impaired in various types of vascular beds of diabetic animals. We have previously shown that retinal vasodilator response to acetylcholine is diminished in diabetic rats. The EDH-related, rather than NO-dependent, vasodilatory mechanisms were impaired in retinal arterioles of diabetic rats. The impairment of BKCa channel function seemed to be responsible for the attenuation of ACh-induced vasodilation observed in diabetic retina. A reduced production and/or bioavailability of EETs may contribute to the impaired EDH-mediated responses in some vascular beds of diabetic animals. The inhibition of soluble epoxide hydrolase (sEH), an enzyme responsible for degradation of EETs, augmented ACh-induced, EDH-related relaxation possibly resulting from elevated EET levels in mesenteric arteries of type 2 diabetic mice. Therefore, sEH inhibitors may restore the impaired retinal vascular function by increasing EETs levels in diabetic rats.

In summary, the present study provides the first evidence suggesting that CYP epoxygenase-derived EETs are responsible for the NO- and prostaglandin-independent component of ACh-induced dilation of retinal arterioles in rats in vivo. EETs may function as an EDHF in rat retinal arterioles.
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Conflict of Interest  The authors declare no conflict of interest.

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