Vasodilator Effects of Elcatonin, a Synthetic Eel Calcitonin, on Retinal Blood Vessels in Rats

Asami Mori, Hironori Suzawa, Kenji Sakamoto, 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.

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The aim of this study was to examine the effects of elcatonin, a synthetic derivative of eel calcitonin, on rat retinal blood vessels, and to determine how diabetes affects the retinal vascular responses. Ocular fundus images were captured with an original high-resolution digital fundus camera in vivo. The retinal vascular responses were evaluated by measuring the diameter of retinal blood vessels contained in the digital images. Both systemic blood pressure and heart rate were continuously recorded. Elcatonin increased the diameter of retinal blood vessels but decreased mean blood pressure in a dose-dependent manner, whereas it had no significant effect on heart rate. A diminished retinal vasodilator response and significant pressor response to elcatonin were observed in rats injected intravenously with N^2-nitro-l-arginine methyl ester, a nitric oxide (NO) synthase inhibitor. Intravitreal injection of indomethacin, a non-selective cyclooxygenase (COX) inhibitor, and SQ22536, an adenyl cyclase inhibitor, markedly attenuated the vasodilator effects of elcatonin on retinal blood vessels. The retinal vasodilator responses to elcatonin were unaffected 2 weeks after the induction of diabetes by a combination of streptozotocin treatment and D-glucose feeding. These results suggest that elcatonin dilates rat retinal blood vessels via NO- and COX-dependent mechanisms and that the adenyl cyclase-adenosine 3',5'-cyclic monophosphate system plays a major role in the vasodilator mechanisms. The retinal vasodilatory effects of elcatonin seem to be preserved at early stages of diabetes.

Key words adenyl cyclase; cyclooxygenase; nitric oxide; prostaglandin; retinal blood vessel

Diabetic retinopathy is a leading cause of blindness in industrialized countries and is the most common complication of diabetes. Tight control of blood glucose levels decreases the risk of diabetic retinopathy onset and progression; however, additional effective treatments are still needed. The narrowing of retinal blood vessels and reduction in retinal blood flow are associated with the onset of diabetic retinopathy, and the impairment of retinal circulation contributes to the pathogenesis of the disease.1–4 Therefore, one strategy for preventing the disease or reducing its severity is to improve the impaired retinal circulation.

Our previous studies demonstrated that adenosine 3',5'-cyclic monophosphate (cAMP)-elevating agents dilate retinal blood vessels more effectively than peripheral resistance vessels.5,6 For example, prostaglandin (PG) I₂ and forskolin (an activator of adenylyl cyclase), which elevate intracellular cAMP levels, dilate retinal arterioles at doses much lower than that required to decrease systemic blood pressure.7 A cAMP analog decreased systemic blood pressure less than that by an analog of guanosine 3',5'-cyclic monophosphate (cGMP), though they produced a comparable vasodilation of retinal blood vessels.8 These results suggested that cAMP-elevating agents could be effective drugs for improving retinal circulation.

Calcitonin, a polypeptide hormone secreted from parafollicular (C) cells of the mammalian thyroid gland into general circulation, regulates blood Ca^{2+} levels and bone metabolism by acting on osteoclasts.9 Therefore, calcitonin is used clinically to treat hypercalcemia9 and osteoporosis.10,11 Calcitonin also has the ability to relieve pain associated with a variety of disorders, including osteoporosis.12–16 These actions are mediated by increased cAMP and intracellular Ca^{2+} levels evoked by the stimulation of calcitonin receptors coupled to G proteins.15–19

Cutaneous flushing is one of the most common side effects of calcitonin and the effects of elcatonin, a synthetic analog of eel calcitonin,20 might be closely associated with plasma levels of vasoactive intestinal peptide.21 Previous studies have shown that calcitonin increased renal blood flow22 and improved peripheral circulatory disturbance in patients with Raynaud’s syndrome.23 In addition, elcatonin attenuated noradrenaline-induced contractions of plantar arteries isolated from rats with chronic constriction injury of the sciatic nerve.24 Despite its vasodilator effect on the peripheral circulatory system, systemic administration of calcitonin had no significant effect on systemic blood pressure.25–27 Thus, the vasodilator action of calcitonin appears to vary between vascular beds. Retinal blood vessels have different characteristics compared to blood vessels in other peripheral circulatory beds.28,29 Therefore, we hypothesized that calcitonin shows vasodilator effects on retinal blood vessels.

To test this hypothesis, we examined the effects of elcatonin on retinal blood vessels in rats and determined the contribution of nitric oxide (NO)- and cyclooxygenase (COX)-dependent mechanisms to the elcatonin-induced responses. In addition, we determined how the retinal vascular responses to elcatonin were affected in an experimental model of diabetes induced by a combination of streptozotocin (STZ) injection and D-glucose feeding.28

MATERIALS AND METHODS

Reagents The following reagents were used: fluorescein sodium salt, indomethacin, methoxamine hydrochloride, N^2-

* To whom correspondence should be addressed. e-mail: nakaharat@pharm.kitasato-u.ac.jp

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nitro-L-arginine methyl ester (L-NAME), SQ22536 (Sigma-Aldrich, St. Louis, MO, U.S.A.), pontamine sky blue 6B (Tokyo-Kasei Kogyo, Tokyo, Japan), hydroxyethylcellulose (Scopisol 15®), Senju Pharmaceutical, Osaka, Japan), pentobarbital sodium, STZ, tetrodotoxin (TTX) (Nacalai Tesque, Kyoto, Japan), and elcatonin (Asahi Kasei Pharma Corporation, Tokyo, Japan).

Elcatonin was dissolved in 0.01 mM sodium acetate buffer (pH 5.6) containing 0.02% bovine serum albumin and 0.9% sodium chloride and further diluted in the same buffer (4 or 40 U/mL). L-NAME was dissolved in saline (30 mg/mL). Indomethacin and SQ22536 were dissolved in dimethyl sulfoxide (DMSO) (10 and 100 mM for indomethacin and SQ22536, respectively) and further diluted in saline (1 and 10 mM for indomethacin and SQ22536, respectively).

Animals Forty-four male Wistar rats (6–9 weeks old) were maintained in a room with constant temperature (22±2°C), humidity (55±5%), and a 12-h light/dark cycle and were 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 for Kitasato University.

Experimental Procedures Surgical procedures were performed under general anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneally (i.p.)) following the procedure described in our previous reports.5,50 Digitized mean arterial pressure (MAP) and heart rate (HR) were recorded, and eye movements for the capture of fundus images at the same angle throughout the experiment. Therefore, the anesthetized rats were treated with TTX (50 µg/kg, intravenously (i.v.)) under artificial ventilation with room air (stroke volume, 10 mL/kg; frequency, 80 strokes/min) using a rodent respirator (SN-480-7, Shinano, Tokyo, Japan). To compensate for the decrease in blood pressure induced by TTX, methoxamine was continuously infused using a syringe pump (Model 1140-001, Harvard Apparatus, South Natick, MA, U.S.A.). The dose of methoxamine was ca. 40 µg/kg/min (ca. 5.5 µg/kg/min for L-NAME-treated rats).

Experimental Protocols Protocol 1: To investigate the role of NO in elcatonin-induced responses, we examined the effects of the NO synthase inhibitor L-NAME on elcatonin-induced changes in the diameter of retinal blood vessels, MAP, and HR. L-NAME (30 mg/kg, i.v.) was administered, and methoxamine infusion was started 45 min later. After hemodynamic parameters had reached a stable level (ca. 15 min later), elcatonin (1–30 U/kg/min) was injected into the femoral vein using a syringe pump (Model 1140-001, Harvard Apparatus).

Protocol 2: Our previous findings suggest that NO dilates rat retinal blood vessels through the COX-1/PG/cAMP-mediated signaling pathway.5,31) (Fig. 1). Therefore, we examined the role of the PG/cAMP system in the elcatonin-induced vasodilation of retinal blood vessels. The COX inhibitor indomethacin (1 mM), the adenylyl cyclase inhibitor SQ22536 (10 mM), or the vehicle (10% DMSO) in a total volume of 10 µL was injected into the vitreous cavity of the left eye prior to the surgical procedures and the TTX treatment described above. Intravitreal injection was performed as previously reported.6,52,53) About 90 min following the intravitreal injection, elcatonin (1–30 U/kg/min, i.v.) was infused using a syringe pump (Model 1140-001, Harvard Apparatus). The timing of administration and the dose of each compound for “Protocol 1” and “Protocol 2” were selected based on results of our previous studies.6,51)
nal vasodilator responses to acetylcholine and β2-adrenoceptor agonists, but not to forskolin and NO donors, were markedly diminished 2 weeks following the onset of diabetes.28,29,33)

Fundus Photography and Measurement of Retinal Blood Vessel Diameter Fundus photography was performed as described in our previous studies.5,31) Briefly, sodium fluorescein (10%, 0.8 mL/kg) and pontamine sky blue 6B (5%, 0.8 mL/kg) solutions were injected into the right femoral vein to enhance blood vessel contrast. To prevent drying of the eye, hydroxyethylcellulose (Scopisol 15®) was administered to the cornea. Fundus images were captured before and during i.v. infusion of elcatonin, using a digital camera (EOS7D, Canon, Tokyo, Japan) equipped with a bore scope-type objective lens for small animals (Model 01; Scalar, Tokyo, Japan). Fundus images (5184×3456 pixels, pixel size=1 µm) were stored on the hard disk of a laboratory computer system. A region of the fundus image containing an arteriole or a venule was selected (140×280 pixels, 140×280 µm), and the vessel diameter in the same region was measured throughout the experiment. The retinal vascular response was evaluated by measuring changes in the diameter. The diameter of the retinal blood vessel was expressed as a percentage of the baseline value, just before the infusion of elcatonin.

Data Analyses All values are presented as mean±standard error (S.E.) To compare baseline values between 2 groups and more than 2 groups, we used unpaired t-test and Tukey’s test, respectively. When comparing the responses between groups, two-way ANOVA was used (PRISM6, GraphPad Software, San Diego, CA, U.S.A.). A p value <0.05 was considered statistically significant.

RESULTS

Baseline values of retinal blood vessel diameter, MAP, and HR are summarized in Table 1. There were no significant differences in the baseline values between experimental groups.

Effects of L-NAME on Elcatonin-Induced Responses Elcatonin (1–30 U/kg/min, i.v.) increased the diameter of retinal blood vessels but decreased MAP in a dose-dependent manner (Figs. 2A–C), whereas it had no significant effect on HR (Fig. 2D). The vasodilator effects of elcatonin on retinal blood vessels were significantly attenuated by L-NAME (e.g., vasodilation of retinal arterioles induced by 30 U/kg/min elcatonin; vehicle, 23.9±3.3% vs. L-NAME, 7.0±1.0%) (Figs. 2A, B). In rats treated with L-NAME, elcatonin produced a significant pressor response (e.g., increases in MAP induced by 30 U/kg/min elcatonin; vehicle, 23.9±3.3% vs. L-NAME, 7.0±1.0%) (Figs. 2A, B). In rats treated with L-NAME, elcatonin produced a significant pressor response (e.g., increases in MAP induced by 30 U/kg/min elcatonin; vehicle, 23.9±3.3% vs. L-NAME, 7.0±1.0%) (Figs. 2A, B).

![Fig. 2. Effects of Intravenous Injection of L-NAME (30 mg/kg) on Changes in Retinal Arteriolar Diameter (AD, A), Retinal Venular Diameter (VD, B), Mean Arterial Pressure (MAP, C) and Heart Rate (HR, D) Induced by Intravenous Infusion of Elcatonin (1–30 U/kg/min)](image)

Table 1. Baseline Values of Retinal Arteriolar Diameter (AD), Retinal Venular Diameter (VD) Mean Arterial Pressure (MAP) and Heart Rate (HR) in Rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>AD (µm)</th>
<th>VD (µm)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Saline (n=4)</td>
<td>33.8±0.8</td>
<td>50.9±2.5</td>
<td>115±1</td>
<td>326±12</td>
</tr>
<tr>
<td>L-NAME (n=5)</td>
<td>36.1±1.0</td>
<td>48.8±2.8</td>
<td>113±0</td>
<td>316±8</td>
</tr>
<tr>
<td><strong>Experiment 2-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (10% DMSO, n=4)</td>
<td>34.3±2.0</td>
<td>50.6±1.9</td>
<td>115±2</td>
<td>321±6</td>
</tr>
<tr>
<td>Indomethacin (n=5)</td>
<td>35.0±1.3</td>
<td>50.8±1.8</td>
<td>114±2</td>
<td>337±13</td>
</tr>
<tr>
<td><strong>Experiment 2-2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (10% DMSO, n=7)</td>
<td>55.4±2.3</td>
<td>65.7±2.9</td>
<td>115±1</td>
<td>352±16</td>
</tr>
<tr>
<td>SQ22536 (n=7)</td>
<td>52.2±3.2</td>
<td>59.3±0.8</td>
<td>117±1</td>
<td>356±10</td>
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<tr>
<td><strong>Experiment 3</strong></td>
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</tr>
<tr>
<td>Non-diabetes (n=6)</td>
<td>45.1±4.1</td>
<td>73.0±9.2</td>
<td>114±1</td>
<td>335±7</td>
</tr>
<tr>
<td>Diabetes (n=6)</td>
<td>53.7±5.3</td>
<td>76.1±10.5</td>
<td>112±1</td>
<td>329±7</td>
</tr>
</tbody>
</table>

Values are means±S.E. These values were measured just before starting the infusion of elcatonin.
Effects of Indomethacin and SQ22536 on Elcatonin-Induced Responses

Intravitreal injection of indomethacin significantly attenuated elcatonin-induced vasodilation of retinal blood vessels (e.g., vasodilation of retinal arterioles induced by 30 U/kg/min elcatonin; vehicle, 26.1 ± 3.0% vs. indomethacin, 3.8 ± 1.1%) (Fig. 3). However, it had no significant effects on MAP and HR responses to elcatonin (data not shown). Similar observations were made with SQ22536 (Fig. 4).

Characteristics of Diabetic Rats

On the day of experimentation, plasma glucose levels in diabetic rats were significantly higher than those in non-diabetic rats. In contrast, the body weights of diabetic rats were lower than that of non-diabetic rats (Table 2). Baseline values were adjusted to the same ranges between non-diabetic and diabetic groups by changing the methoxamine infusion rates (Table 1).

Effects of Diabetes on Elcatonin-Induced Responses

There were no significant differences in retinal vasodilator responses to elcatonin between non-diabetic and diabetic rats (Figs. 5A, B). The effects of elcatonin on MAP and HR were also unaffected by diabetes (Figs. 5C, D).

Table 2. Plasma Glucose Concentration and Body Weight Measured Just before (Initial) and 2 Weeks (Final) after the Injection

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma glucose (mg/dL)</th>
<th>Body weight (g)</th>
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<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Non-diabetes (n=6)</td>
<td>120±8</td>
<td>110±4</td>
</tr>
<tr>
<td>Diabetes (n=6)</td>
<td>114±7</td>
<td>824±47b</td>
</tr>
</tbody>
</table>

Values are mean±S.E. The experiments were performed 2 weeks after injection of streptozotocin or the vehicle. a) p<0.05 vs. the corresponding initial value. b) p<0.01 vs. non-diabetes group.
DISCUSSION

The present study demonstrates that elcatonin increased the diameter of retinal blood vessels in a dose-dependent manner. The elcatonin-induced vasodilatation of retinal blood vessels was significantly reduced by L-NAME and indomethacin. In addition, SQ22536, an inhibitor of adenylyl cyclase, almost completely prevented the retinal vasodilator responses. These results suggest that elcatonin dilates rat retinal blood vessels via NO- and COX-dependent mechanisms, and that the adenylyl cyclase-cAMP system plays an important role in the vasodilator mechanisms.

The calcitonin receptor is a member of a subfamily of the G-protein coupled receptor superfamily and couples to adenylyl cyclase and phospholipase C. The current study shows that SQ22536 almost completely prevented the elcatonin-induced vasodilation of retinal blood vessels. Thus, the adenylyl cyclase-cAMP system seems to play an important role in the retinal vasodilator response to elcatonin. However, in rat retinal blood vessels, the stimulatory effect of elcatonin on the adenylyl cyclase-cAMP system is likely to be indirectly mediated by NO- and COX-dependent mechanisms, because retinal vasodilator responses to elcatonin were prevented by L-NAME and indomethacin. In most vascular beds, NO dilates blood vessels by activating soluble guanylyl cyclase and elevating intracellular cGMP levels, whereas the NO-induced vasodilator response in rat retinal blood vessels is mediated mainly through the COX-1/PG/ET pathway. Therefore, the NO-dependent component of the elcatonin-induced response is likely mediated by stimulation of the COX/cAMP pathway, not that of soluble guanylyl cyclase/cGMP pathway. Presently, the mechanism by which elcatonin stimulates the NO-mediated signaling pathway remains unclear.

We have shown that retinal blood vessels have different characteristics compared to blood vessels in other peripheral circulatory beds. The present study also provides evidence suggesting that responses evoked by the stimulation of calcitonin receptors may differ between retinal and peripheral vasculatures because elcatonin dilated retinal blood vessels in an NO- and COX-dependent manner, whereas it decreased blood pressure in an NO-dependent manner. Interestingly, in the presence of L-NAME, elcatonin exhibited a significant pressor effect. These results indicate that the contractile action of elcatonin may usually be counteracted by its vasodilatory action, involving NO. Peripheral administration of elcatonin increased plasma renin activity but did not change blood pressure in rats. Perhaps, contractile action due to increased plasma renin activity may be counteracted by an intact NO-mediated vasodilatory effect.

To address how diabetes affects the elcatonin-induced vasodilation of retinal blood vessels, we examined the effects of elcatonin in an experimental model of diabetes induced by a combination of STZ injection and d-glucose feeding. In the same model, we found that the vasodilator effects of elcatonin on retinal blood vessels are unaffected after a 2-week duration of diabetes. At the stage of this model, retinal vasodilator responses to acetylcholine and β2-adrenoceptor agonists, but not to forskolin and NO donors, are diminished. The NO- and prostaglandin-independent, possibly endothelium-derived hyperpolarizing factor (EDHF)-mediated, component of acetylcholine-induced vasodilation of retinal blood vessels is affected in diabetic rats. Therefore, the vasodilatory effects of elcatonin are unlikely to be mediated by EDHF or β2-adrenoceptor stimulation. The vasodilatory mechanisms of elcatonin in retinal blood vessels seem to be preserved at the early stage of diabetes.

It is well known that endothelial function in systemic circulation is impaired in diabetic animals. Therefore, elcatonin might cause a significant pressor response in diabetic rats as demonstrated in L-NAME-treated rats; however, we did not observe any significant pressor effect of elcatonin in our diabetic model. In the model, depressor responses to acetylcholine are not affected after 2 weeks of diabetes; therefore, endothelial NO-mediated vasodilatory mechanisms in peripheral resistance vessels may be preserved at early stages of diabetes. Thus, an intact endothelium-dependent vasodilatory effect may counteract the contractile action of elcatonin.

In summary, the present study provides the first pharmacological evidence indicating that elcatonin dilates retinal blood vessels. Impairment of retinal circulation could contribute to the pathogenesis of diabetic retinopathy. Therefore, elcatonin could become a candidate therapeutic for preventing progression of diabetic retinopathy by improving retinal circulation. However, the detailed mechanism of the retinal vasodilator response to elcatonin remains unclear, and calcitonin may stimulate tumor angiogenesis through the calcitonin receptor. Further studies are needed to address these points.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

8) Suzuki H, Nakamura I, Takahashi N, Ikuhara T, Matsuzaki K,


