Vasodilation of Retinal Arteriole Mediated by Corticotropin-Releasing Factor Receptor is Impaired in Streptozotocin-Induced Diabetic Rats

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We investigated the vasodilator responses of retinal arterioles induced by stimulating corticotropin-releasing factor receptors in non-diabetic and diabetic rats. Male Wistar rats were treated with streptozotocin (65 mg/kg, i.v.) and experiments were performed 6—8 weeks later. Rats were treated with tetrodotoxin (50 µg/kg, i.v.) to eliminate any nerve activity and prevent movement of the eye and infused with a mixture of norepinephrine and epinephrine to maintain adequate systemic circulation under artificial ventilation. Fundus images were captured with an original high-resolution digital fundus camera system. The vasodilator responses of retinal arterioles were assessed by measuring changes in diameters of retinal arterioles in response to urocortin and urocortin 2. Both urocortin (0.03—1.0 µmol/kg, i.v.) and urocortin 2 (0.1—3.0 µmol/kg, i.v.) increased diameters of retinal arterioles and decreased systemic blood pressure in a dose-dependent manner. The responses to urocortins were reduced in diabetic rats. These results suggest that urocortin and urocortin 2 play as vasodilators in retinal and peripheral resistance arterioles. The impairment of vasodilation mediated by the corticotropin-releasing factor receptors may contribute to the alteration of retinal and systemic circulation in the diabetic state.

Key words urocortin; diabetes; retinal arteriole; streptozotocin

Diabetic retinopathy is the most common complication of diabetes and is a leading cause of blindness in industrialized countries. A progression of histological and physiological abnormalities of the retinal circulation leads to the blindness that results from diabetic retinopathy.1,2) The vascular abnormalities, such as an alteration of responsiveness of retinal blood vessels and breakdown of the blood–retinal barrier, have been widely documented in patients and experimental animals with diabetes.1−5) Circulating hormones and local factors released from endothelial cells6,7) and retinal tissues8,9) might play an important role in the maintenance of vascular function in retinal circulation, because of lack of autonomic innervation. Therefore, it is important to examine effects of diabetes on vascular responses to circulating hormones and local factors in retinal circulation.

Urocortin (now known as urocortin 1) is a 40-amino acid peptide that belongs to the family of corticotropin-releasing factor (CRF),10) which is also known as corticotropin-releasing hormone.11,12) Recently, two 38-amino acid isoforms of urocortin are identified (i.e., urocortin 2 and urocortin 3).13,14) The actions of these peptides are mediated through the activation of two G protein-coupled receptor subtypes that are coupled to adenylyl cyclase; corticotropin-releasing factor type 1 (CRF1) receptor and corticotropin-releasing factor type 2 (CRF2) receptor.15−17) CRF shows higher affinity for CRF1 than CRF2 receptors, whereas urocortin has an equal affinity for CRF1 and CRF2 receptors. Both urocortin 2 and urocortin 3 bind selectively to CRF2 receptors.18)

Urocortins exhibit several in vitro cardiovascular effects including hypotension and tachycardia.19−23) In addition, the in vitro studies have demonstrated that urocortins relax the isolated blood vessel preparations, such as coronary, basilar, renal and tail arteries, etc.24−31) These cardiovascular effects of urocortins were predominantly mediated by CRF2 receptors.20−22,32−34) However, it remains to be determined the effects of stimulation of CRF2 receptors on retinal circulation and how diabetes affects the vasodilator actions on retinal blood vessels. Therefore, in the present study, we examined 1) whether urocortin and urocortin 2 dilate retinal arterioles in vivo and 2) whether diabetes affects the in vivo vasodilator responses to urocortins of retinal arterioles using streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Animal Model of Diabetes Male Wistar rats weighting 230—250 g were maintained on standard rat chow and tap water ad libitum with 12 : 12-h dark cycle in a quiet environment. Diabetes was induced by a single intravenous injection of streptozotocin (65 mg/kg) dissolved in sodium citrate buffer (pH 4.5). Age-matched control rats were treated with an injection of an equal volume of vehicle. Induction of diabetes was confirmed with blood glucose measurements (>300 mg/dl) 24 h after streptozotocin injection. Plasma glucose was determined with a commercially available enzyme kit (Glucose Test Wako, Wako Pure Chemical, Osaka, Japan). The experiments were performed 6—8 weeks after the injections, because a cataract, which is a clouding of the lens within the eye, is formed in rats with a longer duration of diabetes. This study was 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.

Experimental Procedures The rats were anesthetized with diethyl ether. 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 both the femoral 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, Japan), via a pressure transducer (DX-360, Nihon Kohden) and a preamplifier (AP-

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610G, Nihon Kohden). Heart rate was measured with a cardio
tachometer (AT-601G, Nihon Kohden) triggered by the
blood pressure pulse. Arterial pressure and heart rate were
digitized at 1 Hz using SCIENCE LINK II (Keisoku Giken,
Utsunomiya, Japan) and stored on the hard disk of a dedi-
cated laboratory computer system (PowerBook 165C, Apple
Japan, Tokyo, Japan).

Images of the retinal blood vessels that are captured at
slightly different angles result in different diameter measure-
ments, therefore, prevention of movement of the eye was
very important. In the preliminary study, we found that
tetrodotoxin (50 μg/kg, i.v.) completely prevents movement
of the eye for several hours. The dose of tetrodotoxin abol-
ished the pressor response to spinal cord stimulation and
the acetylcholine-induced reflex tachycardia observed in
anesthetized rats for ca. 6 h. Thus, we decided to use
tetrodotoxin (50 μg/kg, i.v.) to prevent movement of the eye
under artificial ventilation with room air (stroke volume:
10 ml/kg and frequency: 80 strokes/min) using a rodent respi-
ator (SN-480-7; Sinano, Tokyo). Since blood pressure was
decreased after injection of tetrodotoxin, a mixture solution
of norepinephrine and epinephrine (1:9) was continuously
infused by a syringe pump (Harvard Apparatus, South Natick,
MA, U.S.A.) to maintain systemic blood pressure at the baseline
level. The infusion rates of norepinephrine and epinephrine
were 0.3—0.5 μg/kg/min and 2.7—4.5 μg/kg/min, respec-
tively. After hemodynamic parameters reached a stable,
urocortin (0.03—1.0 μmol/kg, i.v.) or urocortin 2 (0.1—
3.0 μmol/kg, i.v.) was injected into the femoral vein.

**Measurement of Diameters of Retinal Blood Vessels**

To protect the eye, 0.3% sodium hyalurate (Santen Phar-
maeutical, Osaka, Japan) 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, i.v.) and bril-
liant blue 6B (5% solution, 0.8 ml/kg, i.v.) were injected into
the right femoral vein to enhance the contrast of blood ves-
sels. Retinal images were captured with a digital camera
(D1x, Nikon, Tokyo, Japan) that was equipped with the bore
type-objective lens for small animals (Model 01, Mag-
nification X20; Scalar, Tokyo, Japan) and stored on the hard
disk of a dedicated laboratory computer system (Power Mac-
intosh G3-300DT, Apple Japan, Tokyo, Japan).

The digitized retinal images were processed using an
image processing software (Photoshop 5, Adobe, Systems
Inc, San Jose, CA, U.S.A.) as reported previously.36,37
Briefly, we selected the green channel image, which could
provide the greatest contrast among three individual color
channels (red, green and blue). To make the analysis easier,
we obtained the greatest contrast of retinal blood vessels by
altering the brightness of the green channel image. After
intensifying the contrast of the blood vessels, we selected a re-
gion (120×240 μm) including a retinal arteriole. Blood ves-
sel was distinguished from background by determining a cer-
tain threshold value for each image. Mean diameter of vessel
was calculated by dividing the total pixels for vessel by pix-
els for length of vessel in the selected area (NIH image
The diameters measured from any particular animal cannot
be directly compared with another animal because the im-
ages captured from the retina are subjected to image magnifi-
cation, depending on the distance from the camera to the
retina, and also the refractive condition of the eye.38 Therefore,
the diameter of retinal arteriole was expressed as per-
centage of the baseline value just before drug administration
and percent changes in diameter were compared between ani-
mals.

**Drugs**

The following drugs were used: urocortin (rat),
urocortin 2 (mouse) (Peptide Institute, Osaka, Japan); (+)
epinephrine (+) bitartrate, (−)norepinephrine bitartrate,
streptozotocin (Sigma, St. Louis, MO, U.S.A.); tetrodotoxin
(Wako Pure Chemical, Osaka, Japan). Norepinephrine and
epinephrine were dissolved in saline containing 0.01% l-
ascorbic acid. Urocortin and urocortin 2 were initially dis-
solved in 1% acetic acid and distilled water, respectively, and
further diluted in saline (final concentration of acetic acid
was below 0.03%).

**Statistical Analyses**

Data are presented as means±
S.E.M. The signficance of the difference between mean values
was evaluated with GraphPad Prism™ by unpaired t-test
with Welch’s correction if necessary. When comparing con-
centration-dependent relationships, two-way analysis of vari-
cence (Two-way ANOVA) was used. ED50 values (the dose re-
quired to produce 50% of maximal response) were estimated
by non-linear regression analysis. A p value smaller than
0.05 was considered to be statistically significant.

**RESULTS**

At the time of the experiment, all streptozotocin-treated
rats exhibited hyperglycemia with blood glucose concentra-
tions (634±14 mg/dl, n = 12) significantly higher than those
of the age-matched control rats (142±6 mg/dl, n = 13,
p < 0.05). The final weight of the diabetic rats (280±6 g,
n = 12) was significantly lower than that of the control rats
(427±9 g, n = 13, p < 0.05). There was no significant differ-
ence in mean arterial pressure between experimental groups
under baseline conditions, because a mixture solution of nor-
epinephrine and epinephrine was infused to recover blood
pressure to normal level (non-diabetic, 102±13, p < 0.05,
101±11, p < 0.05, n = 12—13). Baseline heart rate values
were significantly lower in the diabetic group than in the age-
matched controls (non-diabetic, 481±8 beats/min vs. dia-
betic, 422±7 beats/min, p < 0.05, n = 12—13).

Urocortin (0.03—1.0 μmol/kg, i.v.) increased diameters of
retinal arterioles and decreased arterial pressure in a dose-de-
pendent manner (Fig. 1). The ED50 values for urocortin-in-
duced vasodilation of retinal arterioles and depressor re-
ponses were 0.11±0.02 μmol/kg and 0.16±0.03 μmol/kg,
respectively (n = 7). Diabetes significantly reduced the va-
sodilator responses of retinal arterioles and depressor re-
ponses to urocortin (ED50 values for vasodilation of retinal
arteriole; non-diabetic, 0.16±0.03 μmol/kg vs. dia-
betic, 0.37±0.08 μmol/kg; p < 0.01, n = 7).

Similarly, urocortin 2 (0.1—3.0 μmol/kg, i.v.) dose-de-
pendently dilated retinal arterioles and decreased arterial
pressure (Fig. 2). These responses to urocortin 2 were also
attenuated in diabetic rats, compared with controls. The ED50
values for vasodilation of retinal arterioles to urocortin 2
were not significantly different between non-diabetic and dia-
The present study demonstrates that urocortin and urocortin 2 increased the diameters of retinal arterioles and decreased blood pressure in diabetic rats and the age-matched controls. These results suggest that urocortin and urocortin 2 act as vasodilators in retinal circulation, as well as systemic circulation. However, the vasodilator responses to urocortins observed in diabetic rats were significantly smaller than those in controls.

The vasodilator effects of urocortin and urocortin 2 are mainly mediated through CRF2 receptors that are reported to be present on vascular smooth muscle and endothelial cells.20—22,34,39) The activation of the CRF2 receptors present on vascular smooth muscle cells increases intracellular cAMP levels, thereby dilating blood vessels.27,39) On the other hand, activation of CRF2 receptors on endothelial cells stimulates production/release of endothelium-derived relaxing factors, such as nitric oxide.39) Opening of K+ channels may be involved in the urocortins-induced vasodilations through the endothelium-dependent or -independent mechanism.24,31,40) All of these mechanisms might contribute to the in vivo responses to urocortin and urocortin 2 observed in the present study. Therefore, the attenuation of vasodilator responses to urocortins by diabetes are possibly due to dysfunction of endothelial cells, impairment of relaxant mechanisms in vascular smooth muscle, or alteration in CRF2 receptor itself.

In the ocular vasculature, acetylcholine-induced vasodilator responses were markedly impaired in streptozotocin-induced diabetic rats.41,42) We also found that, in a previous study, vasodilation of retinal arterioles to acetylcholine, but not to forskolin and sodium nitroprusside, was reduced in streptozotocin-induced diabetic rats.37) These results suggest that diabetes impairs endothelium-dependent vasodilation of retinal blood vessels. Therefore, if CRF2 receptor-mediated vasodilation is endothelium-dependent, diabetes would reduce the vasodilator effect of urocortin or urocortin 2 on retinal arterioles by impairing endothelial cell functions. The present results support this hypothesis.

Because of the highly limited passage of the peptides through blood–retinal barrier, intravenously injected urocortins might preferentially bind to the receptors present on endothelial cells. On the other hand, the retinal–blood barrier is impaired in diabetes and, as a result, vascular permeability of retinal blood vessels is increased.1—3) The incomplete blood–retinal barrier in diabetic state may allow the peptides to access the vascular smooth muscle. Therefore, we cannot exclude the possibility that, in diabetic state, endothelium-independent mechanism(s) may be also involved in the CRF receptors-mediated vasodilation of retinal arterioles. Moreover, effects of diabetes on expression of CRF receptors on endothelial cells and vascular smooth muscle cells of rat re-
nal arterioles are unknown. At the present time, the exact mechanism(s) underlying vasodilator effects of urocortin and urocortin 2 on retinal arterioles in vivo is unclear; however, our results strongly suggest that CRF2 receptors-mediated vasodilatory mechanisms on retinal circulation are attenuated by diabetes.

The present study also indicates that the depressor responses to urocortin and urocortin 2 were significantly attenuated in diabetic rats. These results suggest that CRF2 receptors-mediated vasodilatory mechanisms on peripheral resistance arterioles is impaired in diabetic rats. Regarding the effect of diabetes on the cardiovascular actions of urocortins, Sanz et al. have shown that urocortin-induced relaxation of renal arteries was impaired in diabetic female rats, but not diabetic male rats. They concluded that the reduction in production-release of nitric oxide might contribute to the impairment of relaxation to urocortin of renal arteries from diabetic females. However, the effects of diabetes on responses to urocortins in other vascular preparations have not been established. As mentioned above, the mechanisms of the vasodilation of urocortins vary depending on vascular beds, species and experimental preparations; therefore, more extensive studies should be required to determine the effect of diabetes on CRF receptor-mediated vasodilator responses.

The vasodilator responses to urocortin were greater than those to urocortin 2 in the present study. However, the urocortins of different species (rat urocortin and mouse urocortin 2) were used because of the limited commercial availability. Therefore, it is difficult to compare the potency of those to urocortin 2 in the present study. However, the urocortins vary depending on vascular beds, species and experimental preparations; therefore, more extensive studies should be required to determine the effect of diabetes on CRF receptor-mediated vasodilator responses.

In conclusion, the present study showed that, in streptozotocin, because rats with a longer duration of diabetes forms cataract in their eyes. Therefore, our current results would represent changes in vascular function in relatively early stages of diabetes. Nevertheless, our study clearly indicated that diabetes impairs the CRF receptors-mediated vasodilation of retinal arterioles, as well as peripheral resistance vessels, even at six to eight weeks after induction of diabetes. This could conceivably contribute to early steps of diabetes-induced microvascular complications, including diabetic retinopathy.

In conclusion, the present study showed that, in streptozotocin-induced diabetic rats, vasodilation of retinal arterioles induced by urocortin or urocortin 2 is impaired. The impairment of vascular responses induced by stimulation of CRF receptors on retinal circulation may have some implications for the mechanisms by which diabetes leads to development of diabetic retinopathy.

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REFERENCES