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Tissue kallikrein (kallidinogenase) protects against retinal ischemic damage in mice

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L-N5-(1-iminoethyl) ornithine
dihydrochloride (PubChem CID: 2733507)

ABSTRACT

Ocular ischemic syndrome is likely stem from retinal ischemia, and which causes visual disorder. The pathological mechanism of ocular ischemic syndrome is still unknown, therefore the optimal treatment for ocular ischemic syndrome remains to be established. Then, this study aimed to evaluate the effects of tissue-derived kallidinogenase in retinal ischemia protection in mice. In the present study, the effects of tissue-derived kallidinogenase (1 or 10 μg/kg, i.v.) on ischemia/reperfusion-induced retinal damage in mice were examined by histological, electrophysiological, and permeability analyses. In addition, we assessed phosphorylation of endothelial nitric oxide synthase (eNOS) and nuclear factor-kappa B (NF-κB), which is closely-involved in ischemic injury and permeability. Moreover, the neuroprotective effect of kallidinogenase in an in vitro model of ischemia induced by oxygen–glucose deprivation or hypoxia was examined. The results indicated that kallidinogenase significantly prevented the decrease in ganglion cell number induced by ischemia/reperfusion. Electroretinogram measurements showed that kallidinogenase significantly prevented the ischemia/reperfusion-induced reductions in a- and b-wave amplitudes seen 5 days after ischemia/reperfusion. Moreover, kallidinogenase significantly inhibited the permeability increase induced by ischemia/reperfusion. Similar to the results in vivo, kallidinogenase significantly inhibited the retinal ganglion cell death induced by oxygen–glucose deprivation. Also, kallidinogenase significantly suppressed the hypoxia-induced increase in permeability. However, these effects observed in vitro disappeared when an eNOS inhibitor was used concurrently. These findings suggest that kallidinogenase may prevent ischemia/reperfusion-induced retinal damage, might be through eNOS activation.

1. Introduction

The symptoms of ocular ischemic syndrome, such as amaurosis fugax, are likely to stem from retinal ischemia and lead to irreversible retinal cell death (Sivalingam et al., 1991). The pathological mechanism of ocular ischemic syndrome is still unknown. Therefore, the optimal treatment for ocular ischemic syndrome remains to be established.

Kallidinogenase is also known as kallikrein. Kalliginogenase is derived from blood plasma or tissue and has a serine protease capable of cleaving kininogen to release vasoactive kinins (Bhoola et al., 1992). These kinins activate bradykinin B2 receptors and ultimately activate second messengers (Regoli and Barabe, 1980; Regoli et al., 1998). Kallidinogenase has been reported to protect against cerebral ischemia through anti-oxidant (Xia et al., 2004), anti-inflammatory (Xia et al., 2006), and anti-apoptotic pathways (Chao et al., 2006). Moreover, kallidinogenase improves choroidal and retinal circulatory disorders by activating bradykinin receptors coupled to nitric oxide release (Yamaguchi et al., 1999), and protects against chronic ischemic injury to the retina through *a* pathway that involves endothelin-1 (Nagano et al., 2007). Recently, it has been reported that kallidinogenase normalizes retinal vasopermeability (Kato et al., 2009) and inhibits retinal and choroidal neovascularization via the cleavage of vascular endothelial growth factor (VEGF)-165 or VEGF-164 (Fukuhara et al., 2013; Nakamura et al., 2011). Therefore, we hypothesized that kallidinogenase might reverse the retinal damages in retinal ischemic model (Ishizuka et al., 2013b; Ogishima et al., 2011).

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2. Materials and methods

2.1. Animals

In the present study, 8- to 9-week-old-male ddY mice (Japan SLC, Hamamatsu, Japan) were used (Ogishima et al., 2011). They were kept under controlled lighting conditions (12 h: 12 h light/dark). All 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. All experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

2.2. Retinal ischemia model

Anesthesia was induced by 2.0–3.0% isoflurane and maintained by 1.0–1.5% isoflurane (both in 70% N_2O/30% O_2) using an animal anesthesia machine (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). Body temperature was maintained at 37.0–37.5 °C with NS-TC 10 (Neuroscience Inc., Tokyo, Japan). The pterygopalatine artery (PPA) and external carotid artery (ECA) ligations that caused retinal ischemia were performed as described in our previous report (Ogishima et al., 2011). After 5 h of ischemia, ECA and PPA ligatures were removed under anesthesia.

2.3. Treatment with kallidinogenase

Kallidinogenase was obtained from Sanwa Kagaku Kenkyusho Co., Ltd. (Aichi, Japan) and suspended in saline immediately before use. Tissue-derived kallidinogenase at a dose of 1 or 10 μg/kg or an identical volume of saline was intravenously administered just after ligation, 2 h after reperfusion, and then twice a day for 2 days after reperfusion. We selected these doses by the information that intravenous administration of kallidinogenase at a dose of 7 μg/kg/day normalized a retinal permeability in streptozotocin (STZ)-treated rat (Kato et al., 2009). The injection volume was adjusted to 10 ml/kg body weight.

2.4. Electroretinogram (ERG) recording

Electroretinogram (ERG) analysis is used to evaluate visual function deficits: a-waves, b-waves, and OPs reflect the functionality of photoreceptors, Müller and bipolar cells, and amacrine cells in the inner retinal layer, respectively. ERG recordings were performed as described in our previous report (Imai et al., 2010). ERG readings were obtained 5 days after the ischemia/reperfusion process. Scotopic ERG was used to evaluate retinal function. Beforehand, mice were kept in a completely dark room for 24 h. Anesthesia was induced by the intraperitoneal administration of a mixture of ketamine (120 mg/kg; Daiichi-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg; Bayer Health Care, Osaka, Japan). To dilate the pupils, each eye was treated with one drop of 1% tropicamide and 2.5% phenylephrine (Santen Pharmaceutical Co., Ltd., Osaka, Japan), respectively. Flash ERG of the left eyes of dark-adapted mice was performed using a golden-ring electrode (Mayo, Aichi, Japan), a reference electrode (Nihon Kohden, Tokyo, Japan), and a neutral electrode (Nihon Kohden). All measurements were obtained under dim red light, and mice were kept warm throughout the entire process. A-wave and b-wave amplitudes were defined as described in our previous report (Ishizuka et al., 2013a). Oscillatory potential (OP) was measured as the time between the a- and b-wave peaks. The factors analyzed were OP number (OP2, OP3, OP4, OP5), and flash intensity (0.98 log cds/m^2). In the present study, OPs were isolated by the band-pass filter. OP amplitudes were measured using ERG at all frequencies (0.3–500 Hz). The four OPs have been enumerated on each trace.

2.5. Histology

Mice were euthanized under anesthesia after the ERG recordings. The enucleated left eyes were immersed in a fixative solution containing 4% paraformaldehyde for at least 24 h at 4 °C. Eight paraffin-embedded sections (thickness, 5 μm) were cut horizontally and stained with hematoxylin and eosin. The damage induced by retinal ischemia was evaluated as described below; 3 sections in each eye were used for the morphometric analysis. The number of cells in the ganglion cell layer (GCL) in the area extending from 375 to 625 μm from the optic disc (nasal and temporal portions), as well as the thickness of the inner plexiform layer (IPL) and inner nuclear layer (INL) were measured at 2 points per section on light microscopic images; values were then averaged. Data from 3 sections were averaged for each eye. The results were used to evaluate the cell count in the GCL as well as IPL and INL thickness.

2.6. Retinal blood vessel permeability

The permeability of mouse retinal vessels was determined as described previously (Nakamura et al., 2012). Under deep anesthesia with isoflurane, each mouse was injected in the jugular vein with 0.5 ml of PBS containing 100 mg/ml Hoechst 33342 (molecular mass, 616 Da; Sigma-Aldrich, St. Louis, MO, USA) and 20 mg/ml FITC-dextran (molecula rmass, 2000 kDa; Sigma-Aldrich). The isolated retinas were flat mounted and observed by confocal microscopy (FluoView FV10; Olympus, Tokyo, Japan). Moreover, to quantify retinal permeability using Metamorph (Universal Imaging Corp., Downingtown, PA, USA), fluorescent images were photographed (200×0.144 mm^2) using an epifluorescence microscope (BX50; Olympus) fitted with a CCD camera (DP30VW; Olympus). Points at which Hoechst 33342 that did not merge with FITC-dextran at a distance of 1 mm from the optic disc were identified on the photographs in a masked fashion by a single observer (F.I.). For each eye, data from all four areas examined were analyzed (total area, 2.304 mm^2, about 15% of the total retinal area). To calculate retinal permeability, the following equation was used:

$$\text{Permeability rate} = (\text{Hoechst 33342 area} - \text{area of FITC} - \text{dextran and7 Hoechst 33342 co} - \text{localization})/(\text{FITC} - \text{dextran area})$$

This equation indicates the ratio of the leakage to vascular area and is used at previous report (Nakamura et al., 2012).

2.7. Western blot analysis

Mice were euthanized by cervical dislocation; the eyeballs were then removed quickly. The retinas were carefully separated from the eyeballs and immediately frozen in dry ice. To extract protein, the tissue was homogenized in RIPA cell-lysis buffer (product no. R0278; Sigma-Aldrich) containing protease inhibitor (cocktail of products no. P5726 and P00441; Sigma-Aldrich) using a Physcotron homogenizer (Microtec Co. Ltd., Chiba, Japan). The lysate was centrifuged at 12,000 g for 20 min. The protein concentration of the supernatant was measured with bovine serum albumin using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA). A mixture of equal parts protein and 10% 2-mercaptoethanol-containing sample buffer (Nakalai Tesque, Osaka, Japan) was subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA, USA) was used to transfer the separated protein. Transfers were blocked for 1 h at room temperature with

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