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Apelin-36 is protective against N-methyl-D-aspartic-acid-induced retinal ganglion cell death in the mice

Kenji Sakamoto*, Yuta Murakami, Shohei Sawada, Hiroko Ushikubo, Asami Mori, Tsutomu Nakahara, Kunio Ishii

Department of Molecular Pharmacology, Kitasato University School of Pharmaceutical Sciences, Tokyo 108-8641, Japan

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ABSTRACT

Retinal ganglion cell death in glaucoma is caused at least in part by a large Ca^{2+} influx through N-methyl-D-aspartic acid (NMDA) receptors. Apelin is a peptide originally found in the tissue extracts of bovine stomach. Recent studies have been shown that apelin protects against the ischemic-reperfused injury in the brain. We examined whether apelin had protective effects on the NMDA-induced retinal ganglion cell (RGC) death using B6.Cg-TgN(Thy1-CFP)23Jrs/J transgenic mice, which express the enhanced cyan fluorescent protein in RGCs in the retina, *in vivo*. The mice were anesthetized by ketamine and xylazine, and NMDA (40 nmol/eye) was intravitreally injected. We evaluated the effects of apelin-13, [Glp^1]-apelin-13, a potent agonist of apelin receptor, and apelin-36 on the NMDA-induced retinal ganglion cell death. NMDA-induced retinal ganglion cell loss was clearly seen 7 days after NMDA injection. Intravitreal apelin-36 (0.33 nmol/eye), but not apelin-13 (1 nmol/eye) nor [Glp^1]-apelin-13 (1 nmol/eye), simultaneously injected with NMDA significantly reduced the cell loss. The protective effect of apelin-36 was not reduced by ML221 (0.1 nmol/eye; 5-[(4-Nitrobenzoyl)oxy]-2-[(2-pyrimidinylthio) methyl]-4*H*-pyran-4-one), an apelin receptor antagonist, GF109203X (0.03 nmol/eye), a protein kinase C inhibitor, U0126 (0.2 nmol/eye), a MAPK/ERK kinase inhibitor, LY294002 (0.1 nmol/eye), a phosphoinositide 3-kinase inhibitor, Akti 1/2 (0.05 nmol/eye), an Akt inhibitor, or 4,5,6,7-tetrabromobenzotriazole (0.2 nmol/eye), a casein kinase-2 inhibitor. In addition, human apelin-36 did not affect the kainic-acid (20 nmol/eye)-induced ganglion cell death. The present study suggests that apelin-36 protects against the NMDA-induced ganglion cell death independently of the activation of apelin receptor in the murine retina *in vivo*.

1. Introduction

It has been widely known that one of the characteristics of glaucoma is degeneration of retinal ganglion cell (RGC), however the underlying mechanism is not completely understood. A large Ca^{2+} influx through N-methyl-D-aspartic acid (NMDA) receptor channels occurs after activation of the NMDA receptor (Choi, 1987, 1988). Neuronal excitotoxicity, which is considered to be one of the causes of glaucoma-induced RGC death (Kuehn et al., 2005), is predominantly triggered by this excess intracellular Ca^{2+}.

Apelin was originally found in bovine stomach tissue extracts, and identified as an endogenous ligand of apelin receptor, an orphan G protein-coupled receptor related to angiotensin AT_1 receptor (Lee et al., 2000a). A 77-amino acid length precursor peptide of apelin is known to be cleaved by angiotensin-converting enzyme 2 into active apelins, including apelin-36 (42 – 77), apelin-17 (61 – 77), and apelin-13 (65 – 77) (Lee et al., 2000b). Apelin-13 shows the highest biological potency, such as a chemotactic activity (Hosoya et al., 2000), and protective effects on cardiomyocytes (Simpkin et al., 2007). In the brain, apelins are widely expressed in neuron bodies and axons (Cheng et al., 2012). However, the role of apelin in the central nervous system has not been clarified enough (Cheng et al., 2012).

Recently, it has been demonstrated that apelin has neuroprotective effects in the brain using both *in vitro* and *in vivo* experimental systems. Pretreatment with apelin-13 or apelin-36 was reported to reduce hippocampal neuronal cell death in the HIV-induced excitotoxicity (O'Donnell et al., 2007; Cook et al., 2011). It has been shown that infection of HIV in the central nervous system leads to release of excitatory amino acid, especially glutamate, from productively infected macrophages *in vitro* (Kaul et al., 2005). Apelin-13 was reported to reduce the infarct size through the activation of Akt, ERK 1/2 (Yang et al., 2014) and adenosine monophosphate-activated protein kinase (Yang et al., 2016) in the

* Corresponding author.
E-mail address: sakamotok@pharm.kitasato-u.ac.jp (K. Sakamoto).

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mouse focal transient cerebral ischemia model, and to inhibit the inflammation induced by the middle cerebral artery occlusion in the rats (Xin et al., 2015). Intranasal apelin-13 was reported to be neuroprotective in the middle cerebral artery occlusion model (Chen et al., 2015). Apelin-36 was also reported to reduce the infarct size and cerebral edema in the middle cerebral artery occlusion model, (Khaksari et al., 2012; Gu et al., 2013), and the glutamate-induced cell death by activating casein kinase-2, which phosphorylated NR2B, an NMDA receptor subunit, at serine 1480, in the cultured cortical neurons (Cook et al., 2011).

In the retina, apelin was reported to be an angiogenic factor of the endothelial cells (Kasai et al., 2004), and suggested to play a role in progression of diabetic retinopathy to a proliferative stage (Lu et al., 2013). Although the previous reports described above showed that apelin has protective effects on excitotoxicity in the brain, there is no report to demonstrate whether apelin has protective effects on excitotoxicity in the retinal neurons. In this study, we examined whether apelin has neuroprotective effects on the NMDA-induced RGC death in the mice *in vivo*. We investigated the effects of apelin-36, apelin-13 and [Glp^1]-apelin-13, which was a pyroglutamyl form of apelin-13 and a potent agonist of apelin receptor, on the NMDA-induced RGC loss in the murine retina.

2. Materials and methods

2.1. Animals

The experimental protocols used in the present study followed the Regulations for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kitasato University. Male and female B6.Cg-TgN (Thy1-CFP) 23Jrs/J mice, in which the Thy1 promoter is linked to the enhanced cyan fluorescent protein (ECFP) reporter, were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained by brother-sister mating in the animal room of our university. We used the mice aged 8–16 weeks old. The temperature of the animal room was maintained at 25 °C with a 12 h:12 h light-dark cycle. All animals were fed and watered *ad libitum*.

2.2. Intravitreal injection

The method of intravitreal injection was described previously (Sakamoto et al., 2009, 2014). Briefly, mice were anesthetized with intraperitoneal injection of 90 mg/kg ketamine (Daiichi-Sankyo, Tokyo, Japan) and 10 mg/kg xylazine (Tokyo Kasei Kogyo, Tokyo, Japan), and subjected to intravitreal injection using a 33-gauge needle connected to a 25-μL microsyringe (MS-N25, Ito Seisakujo, Fuji, Japan). We intravitreally injected 1 μL of the drug solution or the vehicle.

2.3. Drugs used in the present study

NMDA (40 nmol/eye, Nacalai Tesque, Kyoto, Japan), apelin-13 (1.0 nmol/eye; Phoenix Pharmaceuticals, Burlingame, CA), [Glp^1]-apelin-13 (1.0 nmol/eye; Peptide Institute, Ibaraki, Japan), human apelin-36 (0.33 nmol/eye; Peptide Institute), mouse/rat apelin-36 (0.33 nmol/eye; Phoenix Pharmaceuticals), 4,5,6,7-tetrabromobenzotriazole (TBB; 0.2 nmol/eye; Tokyo Kasei Kogyo), a casein kinase-2 inhibitor, and kainic acid (20 nmol/eye; Tocris Bioscience, Avonmouth, UK) were dissolved in saline. ML221 (5-[(4-Nitrobenzoyl)oxy]-2-[(2-pyrimidinylthio)methyl]-4*H*-pyran-4-one; 0.1 nmol/eye; Tocris Bioscience), an apelin receptor antagonist, GF109203X (0.03 nmol/eye; Santa Cruz Biotechnology, Dallas, TX), a protein kinase C inhibitor, U0126 (0.2 nmol/eye; InvivoGen, San Diego, CA), a MAPK/ERK kinase inhibitor, LY294002 (0.1 nmol/eye; Biorbyt, Cambridge, UK), a phosphoinositide 3-kinase inhibitor, and Akti 1/2 (0.05 nmol/eye; Calbiochem, Darmstadt, Germany), an Akt inhibitor, were dissolved in dimethyl sulfoxide. The solution of the test drug was mixed with the solution of NMDA or kainic acid, or saline, and injected intravitreally. The doses of the drugs used in the present study have previously been shown to antagonize their targets.

2.4. Retinal ganglion cell count in B6.Cg-TgN (Thy1-CFP) 23Jrs/J mice

The counting method has been described previously (Sakamoto et al., 2014) with slight modifications. Seven days after NMDA or kainic acid injection, the eyes were enucleated and fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline for 1 h at 4 °C. Then, whole mount retinas were prepared and mounted on a slide glass (Matsunami Glass, Kishiwada, Japan) sealed with a cover glass (Matsunami Glass) and fluoromount G (Southern Biotech, Birmingham, AL). Images were obtained using a confocal laser microscope (LSM710, Carl Zeiss, Oberkochen, Germany). The number of RGC expressing ECFP was manually counted using image-processing software (Adobe Photoshop CS5, San Jose, CA) in the area that was 500 μm away from the optic disc. The area in which RGC number was counted was 0.3 mm^2.

2.5. SDS-PAGE

After the mice were sacrificed, the eyes were enucleated, and then the retinas were dissected using a dissection microscope. The retinas were homogenized with lysis buffer, comprised of 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.1% sodium dodecyl sulfate, 1% Triton® X-100, 1% sodium deoxycholate, with protease inhibitor cocktail (Nacalai Tesque) and phosphatase inhibitor cocktail (Nacalai Tesque). The homogenized samples were centrifuged at 15,000x*g* for 30 min at 4 °C and the supernatant was saved as total lysate. Total protein content in the lysate was measured with a BCA protein assay kit (Nacalai Tesque). Aliquots of the lysate containing equal quantities of protein were separated on NuPAGE® Novex® 4–12% Bis–Tris Mini Gels (Invitrogen, Carlsbad, CA) according to the instructions described by the manufacturer. The separated proteins were transferred electrophoretically to PVDF membranes (Pall, Port Washington, NY) using a NuPAGE transfer buffer (Invitrogen) with 10% methanol, blocked by Blocking One-P (Nacalai Tesque) and probed with anti-α-spectrin (nonerythroid) antibody (1:200; Millipore, Darmstadt, Germany) or anti-β-actin antibody (1:500, Santa Cruz Biotechnology) for 12 h at 4 °C. The membrane was washed three times with phosphate buffered saline with 0.05% Tween® 20 for 10 min, then probed with horseradish-peroxidase-conjugated anti-mouse IgG (1:100,000; Nacalai Tesque) for 1 h at room temperature. LAS-4000 mini (Fujifilm, Tokyo, Japan) was used to detect immunoreactions visualized with Chemi-Lumi One Super (Nacalai Tesque). The density of the positive bands was quantified using ImageJ software (Rasband, W.S., National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/).

2.6. Electroretinography

The mice were dark-adapted overnight, and anesthetized by 90 mg/kg ketamine and 10 mg/kg xylazine seven days after intravitreal injection of saline (2 μL) or human apelin-36 (0.33 nmol/eye). The method for recording scotopic electroretinograms was described previously (Sakamoto et al., 2015).

2.7. Statistical analyses

All data are represented as mean ± standard error of the mean