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The protection of rat retinal ganglion cells from ischemia/reperfusion injury by the inhibitory peptide of mitochondrial μ-calpain

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ABSTRACT

Intracellular Ca^{2+}-dependent cysteine proteases such as calpains have been suggested as critical factors in retinal ganglion cell (RGC) death. However, it is unknown whether mitochondrial calpains are involved in RGC death. The purpose of the present study was to determine whether the inhibition of mitochondrial μ-calpain activity protects against RGC death during ischemia/reperfusion (I/R) injury. This study used a well-established rat model of experimental acute glaucoma involving I/R injury. A specific peptide inhibitor of mitochondrial μ-calpain, Tat-μCL, was topically applied to rats via eye drops three times a day for 5 days after I/R. RGC death was determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. The truncation of apoptosis-inducing factor (AIF) was determined by western blot analyses. Retinal morphology was determined after staining with hematoxyline and eosin. In addition, the number of Fluoro Gold-labeled RGCs in flat-mounted retinas was used to determine the percentage of surviving RGCs after I/R injury. After 1 day of I/R, RGC death was observed in the ganglion cell layer. Treatment with Tat-μCL eye drops significantly prevented the death of RGCs and the truncation of AIF. After 5 days of I/R, RGC death decreased by approximately 40%. However, Tat-μCL significantly inhibited the decrease in the retinal sections and flat-mounted retinas. The results suggested that mitochondrial μ-calpain is associated with RGC death during I/R injury via truncation of AIF. In addition, the inhibition of mitochondrial μ-calpain activity by Tat-μCL had a neuroprotective effect against I/R-induced RGC death.

1. Introduction

In the retina, ischemia/reperfusion (I/R) injury is involved in vision-threatening diseases, including acute glaucoma [1], central or branch retinal arterial occlusion [2], diabetic retinopathy [3], and retinal detachment [4]. Although the retina consists of several different cells including the pigment epithelium, photoreceptors, bipolar, Müller, and ganglion cells, the retinal ganglion cells (RGCs) are especially vulnerable to I/R injury [5]. Although neuroprotection against RGC death would be beneficial for the treatment of several ocular diseases listed above, an effective treatment has not been developed.

Calpains are Ca^{2+}-dependent cysteine proteases that are key components in RGC death [6–8]. The 14 isoforms of calpains modulate numerous cellular functions by limited proteolysis of specific substrates [9]. However, it is unknown which calpain isoform is involved in RGC death. In the retina, mitochondrial μ-calpain (calpain 1) initiates apoptotic signals via cleavage of apoptosis-inducing factor (AIF) [10]. This process enables the truncated AIF (tAIF) to translocate from the mitochondria to the nucleus where it induces chromatin condensation and large-scale DNA fragmentation [11]. In addition, the influx of Ca^{2+} led to activation of calpains in a rat model of retinal I/R injury [12]. We therefore characterized mitochondrial μ-calpain in the present study.

We previously identified a specific peptide inhibitor of mitochondrial μ-calpain, Tat-μCL [13]. The inhibition of mitochondrial μ-

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calpain and the AIF pathway using this peptide significantly prevented the retinal degeneration in animal models of retinitis pigmentosa [13,14]. In addition, we reported that topically applied Tat-μCL reached the posterior segment of the retina and the optic nerve [15].

Because I/R-induced RGC death depends on elevation of mitochondrial Ca^{2+} concentrations [16], it is possible that mitochondrial μ-calpain is activated and initiates an apoptotic signal via cleavage of AIF during I/R-induced RGC death. In the present study, we therefore determined whether Tat-μCL protects against RGC death by preventing the activation of mitochondrial μ-calpain and the truncation of AIF.

2. Materials and methods

2.1. Animals

All experimental procedures conformed to the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic Vision Research and the Hirosaki University Guidelines for animal experiments. Sprague-Dawley (SD) rats were purchased from Clea Japan (Tokyo, Japan) and maintained under a 12-h light (50 lux illumination) and dark (<10 lux illumination) cycle.

2.2. Animal model of retinal I/R

Rats were anesthetized by intraperitoneal injection of ketamine (80–125 mg/kg) and xylazine (9–12 mg/kg). Topical anesthesia was induced using one drop of 0.02% oxybuprocaine hydrochloride. Pupillary dilatation was performed using 0.5 mg/mL tropicamide and 0.5 mg/mL phenylephrine hydrochloride. After topical anesthesia and pupillary dilatation, the anterior chamber of the right eye was cannulated using a 27-gauge needle connected to a normal saline reservoir elevated to a height of 1.2 m to increase the intraocular pressure. The duration of ischemia was 60 min. Retinal ischemia was confirmed by whitening of the iris and by fundus microscopic observation. After ischemia, the needle was withdrawn, and retinal circulation was renewed. A sham operation was performed in some rat eyes for comparison with the I/R-injured rats.

2.3. The synthesis and administration of the Tat-μCL peptide

We separately synthesized Tat-μCL (Ac-GRKKRRQRRRPPQ-PDALKSRTLR-NH_2) and its scrambled peptide (Ac-GRKKRRQRRRPPQ-ASLRLDRPTK-NH_2) using an automated peptide synthesizer (PSSM-8; Shimazu, Kyoto, Japan) as described previously [13]. The resulting peptides were purified by reverse-phase high-pressure liquid chromatography (HPLC) using a C_{18} column (Jupiter, 250 mm × 10 mm; Phenomenex, Torrance, CA, USA). The molecular mass and purity of each peptide were confirmed by matrix assisted laser desorption ionization time-of-flight mass spectrometry using the AXIMA Confidence (Shimazu, Kyoto, Japan) as described previously [17]. The purity of each synthesized peptide was >95% as estimated from the relative absorbance determined by HPLC. The synthesized Tat-μCL was dissolved in saline at a concentration of 1 mM. After reperfusion, eye drops containing 15 μL 1 mM Tat-μCL or saline (vehicle) were topically applied three times a day without anesthesia.

2.4. Immunohistochemistry

Delivery of the Tat-μCL to the retina was determined using immuno-positive reactions in the retinal sections as described previously [13]. Cryosections (5 μm thick) were rinsed in phosphate-buffered saline (PBS; 10 mM phosphate buffer, 0.14 M NaCl, pH 7.4) and blocked with 1% skim milk in PBS containing 0.05% Tween (PBS-T) for 2 h at room temperature. Sections were incubated overnight at 4 °C with mouse monoclonal anti-HIV1 tat antibody (1:200, ab63957; Abcam, Cambridge, UK) diluted in PBS-T containing 1% skim milk. Sections were washed with PBS-T and then incubated with tetramethylrhodamine isothiocyanate isomer R-conjugated rabbit anti-mouse immunoglobulin G (IgG) (1:200, R0270; DAKO, Glostrup, Denmark) overnight at 4 °C. The sections were then washed with PBS-T, and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescent images were obtained using laser scanning confocal microscopy (FV1000-D; Olympus, Tokyo, Japan).

2.5. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

To determine the retinal cell death induced by I/R, DNA cleavage was visualized *in situ* using a TUNEL assay as described previously [13]. Cryosections (5 μm) were cut superior or inferior to the plane of the equator containing the optic disc and the central portion of the eyeball, respectively. The number of TUNEL-positive cells was counted in 20 sections per eye in six eyes from each group. The TUNEL assay was performed using the In Situ Apoptosis Detection Kit (Takara, Ohtsu, Japan) according to the manufacturer's instructions. Sections were counterstained with DAPI to stain the nuclei. The immunofluorescent images were acquired using laser scanning confocal microscopy (FV1000-D).

2.6. Histological analyses

Staining with hematoxylin and eosin was performed as described previously [18]. Hematoxylin staining was performed using New Hematoxylin Type M solution (Muto Pure Chemicals, Tokyo, Japan) for 10 min. After washing under running water, eosin staining was performed using 1% Eosin Y solution (Muto Pure Chemicals) for 30 s. Sections were sequentially dehydrated with 70%, 80%, 90%, 95%, and 100% ethanol and xylene, then enclosed with MGK-S (Matsunami Glass, Osaka, Japan).

2.7. Subcellular fractionation of rat retinas

Subcellular fractionation of SD rat retinas was performed as described previously [14]. After enucleation, the eyes were washed in ice-cold PBS and dissected into halves. Retinas taken from both eyes of each rat were homogenized using a 2-ml-glass-teflon homogenizer in 500 μL 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose, and 5 mM 2-mercaptoethanol. The mitochondrial or cytosolic fractions was obtained by centrifugation. The mitochondrial fraction was suspended in 200 μL 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol, and sonicated to disrupt the outer and inner membranes.

2.8. Western blot analysis

Western blot analyses were performed to determine the level of AIF as described previously [19]. Each sample was subjected to SDS-PAGE and western blotting. The membranes were treated with a rabbit polyclonal anti-AIF antibody (1:5000, ab1998; Abcam), followed by treatment with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10000, P0448; DAKO). The immunoreactive signals were developed using an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK) and quantified using a luminescent image analyzer (LAS-3000; Fujifilm, Tokyo, Japan).