Calpain Inhibitor Protects Cells against Light-Induced Retinal Degeneration

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ABSTRACT
Calpains are activated by excessive light exposure and related to retinal degeneration. We investigated the protective effects of SNJ-1945, a calpain inhibitor, against light-induced retinal degeneration in mice. SNJ-1945 was orally administrated at doses of 100 and 200 mg/kg at 30 min before and just after light exposure. Light-induced calpain activation was evaluated by using proteolysis of α-spectrin and p35 (a neuron-specific activator for cyclin-dependent kinase 5). The effects of SNJ-1945 against light-induced retinal damage were examined by the thickness of the outer nuclear layer (ONL). Photoreceptor apoptosis was assessed by counting terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells in ONL. Retinal functions were measured in terms of a- and b-wave amplitudes by using an electroretinogram. As the mechanism of SNJ-1945, caspase-3/7 measurement was carried out. SNJ-1945 inhibited the proteolysis of α-spectrin and p35 by light exposure and presented a decrease in the numbers of TUNEL-positive cells and ONL atrophy. Furthermore, SNJ-1945 presented a decrease in a- and b-wave amplitude and caspase-3/7 activation induced by light exposure. These findings suggest that the activation of calpain plays a pivotal role in photoreceptor degeneration by light exposure, and SNJ-1945 may be a candidate for effectively treating diseases related to photoreceptor degeneration.

Introduction
When retinal photoreceptors are injured by excessive light exposure (Noell et al., 1968), retinal damage is irreversible and leads to serious visual field loss. Photoreceptor loss is the primary cause of blindness in degenerative diseases such as age-related macular degeneration and retinitis pigmentosa. However, in these diseases, there are few usable therapeutic agents. To search for a candidate compound against retinal diseases, we used a light-induced photoreceptor degeneration model and studied the mechanism of progressive disease and drug efficacy.

Calpains are calcium-activated cysteine proteases that occur during the apoptosis process (Utz and Anderson, 2000). The calpain family is represented by 15 genes in mammals and consists of two major isozymes (μ- and m-calpain) that have been well characterized (Saido et al., 1994; Evans and Turner, 2007). The calpain family is related to many human diseases, such as Alzheimer’s disease, brain ischemia, and cataracts (Huang and Wang, 2001). Furthermore, the involvement of calpain activation was reported in retinal diseases such as glaucoma, age-related macular degeneration, and retinitis pigmentosa (Paquet-Durand et al., 2007; Tamada et al., 2007). Pharmacological inhibition of calpain activation protects from retinal neuronal degeneration (Azuma and Shearer, 2008). Therefore, calpains may be a therapeutic target for treatment of retinal disorders.

A calpain inhibitor, (2S)-4-methyl-2-[N-(4-fluorophenyl) sulfonyl]-L-valyl]amino]pentanal (SJA-6017), potently inhibits calpain-1 and -2 and showed a protective effect against ischemia-reperfusion-induced retinal cell death (Sakamoto et al., 2000). However, SJA-6017 had low oral bioavailability and required high-dose administration (500 mg/kg p.o.). On the other hand, a novel calpain inhibitor, (1S)-1-(((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)carbonyl)-3-methylbutyl]carbamic acid 5-methoxy-3-oxapentyl ester (SNJ-1945) was synthesized from SJA-6017 (Shirasaki et al., 2005). SNJ-1945 has more favorable retinal bioavailability and required low-dose administration (50 mg/kg p.o.). SNJ-1945 was orally administrated at doses of 100 and 200 mg/kg at 30 min before and just after light exposure. Light-induced calpain activation was evaluated by using proteolysis of α-spectrin and p35 (a neuron-specific activator for cyclin-dependent kinase 5). The effects of SNJ-1945 against light-induced retinal damage were examined by the thickness of the outer nuclear layer (ONL). Photoreceptor apoptosis was assessed by counting terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells in ONL. Retinal functions were measured in terms of a- and b-wave amplitudes by using an electroretinogram.
pharmacokinetics, such as good retinal penetration, high oral bioavailability, and long half-life (Shirasaki et al., 2006). We have reported previously that SNJ-1945 has a protective effect against cerebral ischemia-induced neuronal cell death (Koumura et al., 2008) and N-methyl-D-aspartate-induced retinal cell death (Shimazawa et al., 2010). It is noteworthy that SNJ-1945 showed protective effects by oral administration in these models. Furthermore, Oka et al. (2007) have reported that oral administration of SNJ-1945 protects against N-methyl-N-nitrosourea (MNU)-induced photoreceptor degeneration in rats. On the other hand, Perche et al. (2009) reported that intravitreal injection of μ-Phe-hPhe-FMK, a calpain inhibitor, showed no neuroprotective effects against light damage. Therefore, we investigated whether calpain activation would participate in photoreceptor degeneration and retinal dysfunction after light exposure. In the present study, to demonstrate the protective effects of SNJ-1945, a calpain inhibitor, against photoreceptor cell death induced by light exposure, we examined histological and electrophysiological analyses and the underlying mechanism using an in vivo mouse model.

Materials and Methods

Animals. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Male albino ddY mice (Japan SLC, Inc., Hamamatsu, Japan), aged 9 to 10 weeks, were used in this study. They were kept under controlled lighting conditions (12-h light/dark cycle).

Exposure to Light. After dark adaptation for 24 h, the pupils of the mice were dilated with 1% cyclopentolate hydrochloride eye drops (Santen, Osaka, Japan) 30 min before exposure to light. Non-anesthetized mice were exposed to 8000 lux of white fluorescent light (Toshiba, Tokyo, Japan) for 3 h in cages with a reflective interior. The temperature during exposure to light was maintained at 25 ± 1.5°C. After the exposure to light, all mice were returned to darkness for 24 h and then placed in the normal light/dark cycle.

Treatment with SNJ-1945. The SNJ-1945 was a gift from Senju Pharmaceutical Co. Ltd. (Kobe, Japan), and it was suspended in distilled water containing 0.5% carboxymethyl cellulose. SNJ-1945 was administrated at 100 or 200 mg/kg p.o. or 100 mg/kg i.p. at 30 min before and just after exposure to light. The vehicle group was treated with 10 ml/kg carboxymethyl cellulose.

Electroretinogram. Electroretinograms (ERG) were recorded 5 days after light exposure (Mayo, Aichi, Japan). Mice were maintained in a completely dark room for 24 h. They were anesthetized with a mixture of ketamine (120 mg/kg i.p.); Daisichi-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg i.p.; Bayer Health Care, Tokyo, Japan). Pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen). Flash ERG was recorded in the left eyes of dark-adapted mice by placing a gold ring electrode (Mayo) in contact with the cornea and a reference electrode (Nihon Kohden, Tokyo, Japan) through the tongue. A neutral electrode (Nihon Kohden) was inserted subcutaneously near the tail. All procedures were performed in dim red light, and the mice were kept warm during the entire procedure. The amplitude of the a-wave was measured from the baseline to the maximum a-wave peak, and the b-wave was measured from the maximum a-wave peak to the maximum b-wave peak.

Histological Analysis. In mice under anesthesia produced by sodiun pentobarbital (80 mg/kg i.p.) (Nakalai Tesque, Kyoto, Japan), each eye was enucleated and kept immersed in a fixative solution containing 4% paraformaldehyde for at least 24 h at 4°C. Six paraffin-embedded sections (thickness 5 μm) cut through the optic disc of each eye were prepared in a standard manner and stained with hematoxylin and eosin. The damage induced by light exposure was then evaluated, six sections from each eye being used for the morphometric analysis described below. Light-microscope images were photographed, and the thickness of the outer nuclear layer (ONL) from the optic disc was measured at 240-μm intervals by photograph in a masked fashion by a single observer (S.I.). Data from three sections (selected randomly from the six sections) were averaged for each eye.

TUNEL Staining. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed according to the manufacturer’s protocols (In Situ Cell Death Detection Kit; Roche Diagnostics, Mannheim, Germany) to detect retinal cell death induced by exposure to light. The mice were anesthetized with pentobarbital sodium at 80 mg/kg i.p. at 48 h after exposure to light for 3 h. The eyes were enucleated, fixed overnight in 4% paraformaldehyde, and immersed for 2 days in 25% sucrose with phosphate-buffered saline (PBS). The eyes were then embedded in a supporting medium for frozen-tissue specimens (Tissue-Tek O.C.T. compound; Sakura Finetek Japan, Tokyo, Japan). Retinal sections (10 μm) were cut on a cryostat at −25°C and stored at −80°C until staining. After washing twice with PBS, sections were incubated with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 h. The sections were washed thrice times in PBS for 1 min at room temperature. After washing twice with PBS, fluorescence images were photographed, and the intensity was measured in the ONL at a distance between 480 and 720 μm from the optic disc obtained from the superior area of the retina. The intensity of TUNEL-positive cells was averaged for these superior areas.

Western Blot Analysis. In vivo, mice were euthanized using sodium pentobarbital at 80 mg/kg i.p., and their eyeballs were quickly removed. The retinas were carefully separated from the eyeballs and quickly frozen in dry ice. For protein extraction, the tissue was homogenized in cell-lysis buffer [radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO) with protease (Sigma-Aldrich) and phosphatase inhibitor cocktails (Sigma-Aldrich), and 1 mM EDTA] using a homogenizer (Phyiscoon; Microtec Co., Ltd., Chiba, Japan). The lysate was centrifuged at 12,000g for 20 min, and the supernatant was used for this study. The protein concentration was measured by comparison with a known concentration of bovine serum albumin using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). A mixture of equal parts of an aliquot of protein and sample buffer with 10% 2-mercaptoethanol was subjected to 10% SDS-polyacrylamide gel electrophoresis. The separated protein was then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA). For immunoblotting, the following primary antibodies were used: mouse anti-α-spectrin monoclonal antibody (1:2000; clone AA6; Millipore); p35/25 rabbit monoclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA); and β-actin mouse monoclonal antibody (1:4000; Sigma-Aldrich). The secondary antibody used was goat anti-rabbit horseradish peroxidase-conjugated (1:2000) or goat antimouse horseradish peroxidase-conjugated (1:2000). The immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology). The band intensity was measured using a Lumino Imaging Analyzer (Fujiﬁlm, Osaka, Japan).

Caspase-3/7 Assay. In vivo, mice were euthanized using sodium pentobarbital at 80 mg/kg i.p., and their eyeballs were quickly removed. The retinas were carefully separated from the eyeballs and quickly frozen in liquid nitrogen. For protein extraction, the tissue was homogenized in cell-lysing buffer using a homogenizer (Phyiscoon). The lysate was centrifuged at 12,000g for 20 min, and the supernatant was used for this study. The protein concentration was measured by comparison with a known concentration of bovine serum albumin using the BCA Protein Assay Kit (Pierce Biotechnology). Caspase-3/7 was measured by using Caspase-Glo 3/7 Assay (Promega, Madison, WI) according to the manufacturer’s instructions. The luminescence of each sample was measured in a plate-reading microplate.
Results

Effects of SNJ-1945 on the Activation of Calpain by Light Exposure. To investigate the activation of calpain after light exposure, proteolysis of the cytoskeletal protein α-spectrin was measured by Western blot analysis (Fig. 1, A and B). At 24 h after light exposure, the proteolyzed α-spectrin band at 150 kDa became clear, and a new band of proteolyzed α-spectrin at 145 kDa appeared. Just after light exposure, calpain tended to be activated by light injury. Treatment with SNJ-1945 (200 mg/kg p.o. or 100 mg/kg i.p.) significantly inhibited the two cleaved products (145 and 150 kDa) at 24 h after light exposure (Fig. 1, C and D).

Effects of SNJ-1945 against the Degradation of p35 by Light Exposure. To investigate calpain-induced photoreceptor degeneration, p35 (Cdk5 regulator protein) was measured in light-exposed mouse retinas. Time-dependent degradation of p35 after light exposure is shown in Fig. 2A. In nontreated normal retinas, p35 protein was expressed, and in light-exposed retinas, p35 was decreased at 48 and 72 h after light exposure; p35 was significantly reduced (Fig. 2B). Treatment with SNJ-1945 (200 mg/kg p.o. or 100 mg/kg i.p.) inhibited the degradation of p35 at 48 h after light exposure (Fig. 2, C and D).

Effects of SNJ-1945 on Light-Induced Photoreceptor Degeneration. In histological evaluation, Fig. 3, A–E shows exposure (Fig. 2, C and D). i.p.) inhibited the degradation of p35 at 48 h after light exposure; p35 was significantly reduced (Fig. 2B). Treatment with SNJ-1945 (200 mg/kg p.o. or 100 mg/kg i.p.)-treated group showed significantly less reduction of a- and b-wave amplitudes, compared with the saline-treated group (Fig. 5, B and C).

Electroretinogram. The effects of SNJ-1945 on light-induced photoreceptor degeneration were examined by electrophysiologic analyses. Both a- and b-wave amplitudes were significantly reduced at 5 days after 8000-lux white light exposure for 3 h, and a- and b-wave decreased by 85 and 83%, respectively, compared with nontreated retina at 0.98 log cd/m² (Fig. 5, B and C). At 0.98 log cd/m², the SNJ-1945 (200 mg/kg p.o. or 100 mg/kg i.p.)-treated group showed significantly less reduction of a- and b-wave amplitudes, compared with the saline-treated group (Fig. 5, B and C).

Effect of SNJ-1945 against Light-Induced Caspase-3/7 Activation. We investigated the mechanism of photoreceptor degeneration and the effect of SNJ-1945. We measured time-dependent changes in caspase-3/7 activity after light exposure. Caspase-3/7 activity was increased just after light exposure and decreased at 24 h. However, at 48 h, caspase-3/7 activity was elevated again (Fig. 6A). At 48 h after light exposure, we investi-
gated the effect of SNJ-1945 against light-induced caspase-3/7 activation. Systemic (oral or intraperitoneal) treatment with SNJ-1945 inhibited caspase-3/7 activity (Fig. 6B).

**Discussion**

In the present study, we showed the protective effects of SNJ-1945, a novel calpain inhibitor, against light-induced retinal degeneration in mice. As a result of light irradiation, retinal photoreceptors trigger apoptotic cell death (Abler et al., 1996). One of the causes is elevation of intracellular calcium concentrations and calpain activation. It has been reported that intracellular calcium influx and calpain activation were increased by light exposure, and a calcium channel blocker, flunarizine, was protective for retinal damage by
light exposure (Edward et al., 1991; Isayama et al., 1991; Donovan et al., 2001). Furthermore, in other retinal degeneration models such as the retinal degeneration 1 mouse and MNU-induced retinal degeneration, calpain activation was increased in photoreceptor cells (Paquet-Durand et al., 2006; Oka et al., 2007). In the present study, we examined calpain activation by measuring calpain-specific α-spectrin fragments at 145 kDa in mouse retina after light exposure. The 150-kDa fragments are broken by the effects of calpain and caspase-3, whereas the 145-kDa fragment is broken only by calpain (Nath et al., 1996). In the retina, α-spectrin is localized in inner and outer retina (Isayama et al., 1991), and the proteolysis of spectrin is thought to be a cause of retinal cell death. We showed that the cleaved products of α-spectrin at 145 kDa increased at 24 h after light exposure, and SNJ-1945 treatment (200 mg/kg p.o. or 100 mg/kg i.p.) decreased the proteolysis of α-spectrin. As the other substrate of calpain, p35 degradation was evaluated. The p35 protein is a regulator of cyclin-dependent kinases 5 (Cdk5), and p35 is degraded to p25 by calpain (Lee et al., 2000). Proteolytic cleavage of p35 by calpain produces p25, resulting in prolonged activation of Cdk5 with p25 and hyperphosphorylation of τ protein (Kusakawa et al., 2000). Accumulation of hyperphosphorylated τ protein causes neurofibrillary tangles and neuronal cell death. In retina, p35 was proteolyzed to p25 by several factors, such as hypoxia, ocular hypertension, photoreceptor cell death, and ganglion cell death (Tamada et al., 2005; Oka et al., 2006, 2007; Shimazawa et al., 2010). In the present study, p35 was decreased 48 h after light exposure, and SNJ-1945 treatment (200 mg/kg p.o. or 100 mg/kg i.p.) preserved p35 by calpain inhibition. These results indicate that calpain was activated by light exposure, and SNJ-1945 inhibited calpain activation with systemic administration. It is noteworthy that degradation of α-spectrin and p35 was mildly increased within a few hours after light exposure; afterward, their degradations increased rapidly. These biphasic changes were reported by Perche et al., (2009) and attributed to the fact that the endogenous calpain inhibitor calpastatin was increased with activation of μ-calpain and m-calpain. In a previous report, photoreceptor degeneration was associated with the difference in the time of μ- and m-calpain activation (Oka et al., 2007). The explanation suggested was that μ-calpain was activated first, and m-calpain was followed by activation of μ-calpain. Moreover, the results in this study were correlated with the proteolysis of α-spectrin. In the present study, it was confirmed that light exposure induced proteolysis of α-spectrin and p35 by calpain; therefore, we examined whether SNJ-1945 offers protection from light-induced retinal damage. In histological analysis, we evaluated the ONL thickness as photoreceptor atrophy. SNJ-1945 treatment (200 mg/kg p.o. or 100 mg/kg i.p.) reduced the loss of ONL, and this result suggests that calpain inhibitors have a protective effect against photic retinal damage. Moreover, the effect of SNJ-1945 was evaluated electrophysiologically by using ERG. At 5 days after light exposure, amplitudes of a- and b-waves were decreased, and SNJ-1945 administration (200 mg/kg p.o. or 100 mg/kg i.p.) inhibited the reduction in both a- and b-waves. a-Waves show the function of photoreceptor, and b-waves show the function of Müller cells and bipolar cells. The results indicated that SNJ-1945 prevented decreased visual function by light exposure. On the other hand, Perche et al. (2009) reported that another calpain inhibitor, μ-Phe-hPhe-FMK, showed no neuroprotective effects against light damage. They confirmed that μ-Phe-hPhe-FMK inhibited calpain activation during light exposure, but it showed no protective effects on photoreceptor loss, increasing apoptotic cells, or retinal dysfunc- tion. It was suggested that μ-Phe-hPhe-FMK may have a short-half-life in plasma and/or retina. On the other hand, oral administration of SNJ-1945 was reported to show good penetration and long half-life (t1/2 = 4.3 h) in rat retina (Shirasaki et al., 2006). Furthermore, Oka et al. (2007) have reported that oral administration of SNJ-1945 at 200 mg/kg p.o. may be an effective dose in photoreceptor degeneration of mice and rats. On the other hand, there was no report about pharmacokinetic information after intraperitoneal administration of SNJ-1945. However, the maximum plasma concentration (Cmax) after intraperitoneal administration of SNJ-1945 at 100 mg/kg is predicted to be higher than that after the oral administration at 100 mg/kg, and the half-life is shorter than that. Therefore, SNJ-1945 at 100 mg/kg i.p., but not at 100 mg/kg p.o., was effective in the ONL atrophy after light exposure.

Retinal diseases in human and animal models lead to apoptotic cell death (Nickells and Zack, 1996; Remé et al.,...
in light-induced retinal degeneration, apoptosis is a common final pathway (Wenzel et al., 2005). We investigated photoreceptor apoptosis by using TUNEL staining. Light-induced TUNEL-positive cells were significantly expressed at 48 h after the light exposure in ONL. SNJ-1945 (200 mg/kg p.o. or 100 mg/kg i.p.) inhibited increased expression of TUNEL-positive cells in ONL. This result indicates that light exposure activates calpain, and calpain activation triggers photoreceptor apoptosis. Furthermore, oral (200 mg/kg) and intraperitoneal (100 mg/kg) treatments with SNJ-1945 suppressed apoptotic DNA damage.

During apoptosis processes, caspase-3 has been identified as a key protease in the execution of apoptosis, whereas calpains have been implicated in neuronal death (Blomgren et al., 2001). In both inherited retinal degeneration and light-induced retinal degeneration, caspase-3 inhibitors showed a protective effect, suggesting that photoreceptor degeneration is related to caspase-3 activation (Perche et al., 2007, 2008). For these reasons, we confirmed that caspase-3/7 was activated by light injury, being significant at 48 h after light exposure. Costa et al. (2008) also reported that caspase-3 was up-regulated at 48 h after light exposure, and our result corresponded with those results. In the present study, SNJ-1945 inhibited caspase-3/7 activation 48 h after light exposure. Taken together, these results suggest that caspase-3 activation is partially mediated by calpain activation after light exposure. However, we have no data regarding specificity of SNJ-1945 against calpain. Some investigators have reported that SNJ-1945 inhibits µ- and m-calpain activities with IC50 values of 0.062 and 0.045 µM, respectively (Shirasaki et al., 2006) and that it exerts inhibitory actions on Ca2+-independent protease and cathepsin L and B (Yoshikawa et al., 2010). SNJ-1945 was synthesized from SJA-6017, a potent calpain inhibitor to improve its metabolic stability and water solubility (Shirasaki et al., 2006). SJA-6017 is well known to inhibit cathepsin B and L in addition to calpain but does not inhibit other cysteine proteases (interleukin 1β-converting enzyme), serine proteases (trypsin, chymotrypsin, thrombin, factor VIIa, factor Xa), or proteasome (Inoue et al., 2003). Furthermore, a docking study supported the idea that SNJ-1945 bound in the active site pocket of µ-calpain (Azuma and Shearer, 2008). Accordingly, SNJ-1945 is also predicted to have similar profiles with SJA-6017, but further studies will be needed.

In conclusion, we have demonstrated that SNJ-1945, a calpain inhibitor, has neuroprotective effects against light-induced retinal damage. SNJ-1945 may be a candidate compound for photoreceptor degeneration-related diseases.
SNJ-1945 Protects against Light-Induced Retinal Damage

Fig. 6. Effects of SNJ-1945 on light-induced expression of caspase-3 in the mouse retina. A, quantitative analysis of the fluorescent intensity of caspase-3 activation. Data are shown as mean ± S.E.M. (*, p < 0.05; **, p < 0.01 versus nontreated group (Normal)). B, quantitative analysis of the effect of SNJ-1945 against activation of caspase-3 at 48 h after light exposure. SNJ-1945 (200 mg/kg p.o. and 100 mg/kg i.p.) or an identical volume (10 ml/kg) of vehicle (0.5% sodium carboxymethyl cellulose) was administered at 30 min before and just after light exposure. Each column represents the mean ± S.E.M. above each column represents the number of animals. *+, p < 0.05; ††, p < 0.01 versus nontreated group (Normal); †+, p < 0.05 versus vehicle.

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References


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