Neuroprotective impact of prothymosin alpha-derived hexapeptide against retinal ischemia-reperfusion

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Abbreviations: a.a., amino acid; Ac, acetylation; Chx10, ceh-10 homeodomain-containing homolog; ERG, electroretinography; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; HE, hematoxylin and eosin; Iba-1, ionized calcium binding adaptor molecule 1; INL, inner nuclear layer; IPL, inner plexiform layer; i.p., intraperitoneal; i.v., intravenous; i.vt., intravitreal; NeuN, Neuronal Nuclei; NH₂, amination; Nle, norleucine; Nva, norvaline; ONL, outer nuclear layer; OPL, outer plexiform layer; P, pyrrolation; ProTα, prothymosin alpha; P₆, ProTα-derived 6-amino acid peptide.

Abstract

Prothymosin alpha (ProTα) has robustness roles against brain and retinal ischemia or serum-starvation stress. In the ProTα sequence, the active core 30-amino acid peptide/P₃₀ (a.a.49–78) is necessary for the original neuroprotective actions against ischemia. Moreover, the 9-amino acid peptide sequence/P₉ (a.a.52–60) in P₃₀ still shows neuroprotective activity against brain and retinal ischemia, though P₉ is less potent than P₃₀. As the previous structure-activity relationship study for ProTα may not be enough, the possibility still exists that any sequence smaller than P₉ that retains potent neuroprotective activity. When different P₉- and P₃₀-related peptides were intravitreally injected 24 h after retinal ischemia, the 6-amino acid peptide/P₆ (NEVDEE, a.a.51–56) showed potent protective effects against ischemia-induced retinal functional deficits, which are equipotent to the level of P₃₀ peptide in electroretinography (ERG) and histological damage in hematoxylin and eosin (HE) staining. Further studies using ERG and HE staining suggested that intravitreal or intravenous injection with modified P₆ peptide/P₆Q (NEVDQE) potently inhibited retinal ischemia-induced functional and histological damage. In an immunohistochemical analysis, the ischemia-induced loss of retinal ganglion, bipolar, amacrine and photoreceptor cells were inhibited by a systemic administration with P₆Q peptide 24 h after the ischemic stress. In addition, systemic post-treatment with P₆Q peptide significantly inhibited retinal ischemia-induced microglia and astrocyte activation in terms of increased ionized calcium binding adaptor molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP) intensity, respectively, as well as their morphological changes, increased number and migration. Thus, this study demonstrates the therapeutic significance of modified P₆ peptide P₆Q (NEVDQE) derived from 6-amino acid peptide (P₆) in ProTα against ischemic damage.

Keywords: ischemia, prothymosin alpha, neuroprotective peptide, microglia, astrocyte

1. Introduction

Ischemic stress in the brain and retina is closely linked to the dysfunction, damage and death of neuronal cells resulting from the activation of different injurious physiological, cellular, molecular and genetic mechanisms including necrosis and apoptosis, and the subsequent secretion of glial cell-derived cytotoxic mediators, such as cytokines and chemokines (Osborne et al., 2004; Fornage, 2009; Neroev et al., 2010; Iadecola and Anrather, 2011; D'Onofrio and Koeberle, 2013; Lipton, 2013; Patel et al., 2013; Biousse and Newman, 2014; Kawabori and Yenari, 2015). Nevertheless, several survival components, including brain-derived neurotrophic factor (BDNF) and erythropoietin (EPO), are synchronously up-regulated following cerebral and retinal ischemia and contribute to limited neuroprotection through the inhibition of apoptosis, but not necrosis, a predominant mechanism of cell death (Siren et al., 2001; Kim et al., 2004; Maiese et al., 2004; Fan et al., 2010; Bejot et al., 2011; Minhas et al., 2012; Berretta et al., 2014; Majid, 2014; Amantea et al., 2015; Larphaveesarp et al., 2015). Thus, the development of safe and effective therapeutic agents with anti-necrosis activity has become a challenging subject for rapid treatment of ischemic disorders.

Prothymosin alpha (ProT α), a nuclear protein, is implicated in multiple functions including cell survival, proliferation and immunogenicity (Jiang et al., 2003; Mosoian, 2011; Ueda et al., 2012; Cannavo et al., 2013; Emmanouilidou et al., 2013; Ioannou et al., 2013; Chang et al., 2014; Lin et al., 2015). We previously identified ProT α as an anti-necrotic factor in the conditioned medium of a serum-free primary culture of cortical neurons (Ueda et al., 2007; Ueda, 2009). Moreover, ProT α contributes to neuroprotection against brain and retinal ischemia (Fujita and Ueda, 2007; Ueda, 2008; Fujita et al., 2009; Ueda, 2009; Ueda et al., 2010; Ueda et al., 2012). Different moieties of ProT α exhibit their distinct activities *in vitro* and *in vivo*

(Cordero, 2011; Dong et al., 2012; Ioannou et al., 2012). The N-terminal amino acid (a.a.) sequence (a.a.2–29) of ProT α , corresponding to thymosin α 1, shows anti-cancer activity and also contributes to immunoprotection against bacterial, fungal and viral infections (Goldstein and Badamchian, 2004; Pierluigi et al., 2010; Camerini and Garaci, 2015; Guo et al., 2015; Wu et al., 2015). In contrast, the C-terminal fragments of human ProT α (a.a.89–109, a.a.100–109 and a.a.103–109) are implicated in the maturation of dendritic cells, stimulation of lymphocytes and neutrophils, secretion of pro-inflammatory cytokines and retardation of tumor growth (Skopeliti et al., 2009; Ioannou et al., 2013; Samara et al., 2013; Voutsas et al., 2013; Karachaliou et al., 2015). Previous studies have reported that the central domain of mouse ProT α (a.a.32–52) regulates cell-defensive mechanisms in response to oxidative stress through an interaction with kelch-like ECH-associated protein 1 (Keap1) of the Keap1-factor-erythroid-derived 2-like 2 (Nrf2) complex (Karapetian et al., 2005; Mel'nikov et al., 2007; Itoh et al., 2015). The cell survival activity of the central fragment in human ProT α (a.a.41–83) has also been mentioned in the *in vitro* Huntington disease model (Dong et al., 2012). Most recently, we demonstrated that the active core 30-amino acid peptide in ProT α (P₃₀: a.a.49–78) is necessary for neuroprotection against brain and retinal ischemia or serum-starvation stress (Halder et al., 2013a). We have also demonstrated the neuroprotective actions of the 9-amino acid peptide sequence (P₉: a.a.52–60) in the P₃₀ peptide of ProT α in brain and retinal ischemia models (Halder et al., 2013b). However, there is still a question of whether the smaller peptide sequence in ProT α has neuroprotective activity. Since the retina is more accessible than other areas of the central nervous system, and retinal ischemia mimics the physiological response of ischemic stroke (Minhas et al., 2012; D'Onofrio and Koeberle, 2013; Ishikawa et al., 2013), the aim of present study was to investigate the protective activity of ProT α -derived 6-amino acid peptides using retinal ischemia model.

2. Experimental Procedures

2.1. Animals

Male C57BL/6 J mice weighing 20–30 g were purchased from Tagawa Experimental Animals (Nagasaki, Japan). Mice were kept in a room maintained at $21 \pm 2^{\circ}\text{C}$ and $55 \pm 5\%$ relative humidity with a 12-h light/dark cycle. Mice had free access to standard laboratory diet and tap water. All procedures were approved (approval number: 1104190914) by the Nagasaki University Animal Care Committee (Nagasaki, Japan).

2.2. Synthesis of ProT α -derived peptides

ProT α -derived peptides and modified peptides used in this study were chemically synthesized and supplied as a white to off-white powder by GL Biochem (Shanghai) Ltd. (Minhang, Shanghai, China). All peptides were characterized by high-performance liquid chromatography (HPLC) and mass spectrometry (MS), and the purity of peptides was $> 95\%$. The 6-amino acid modified peptides were synthesized by norvaline (Nva) or norleucine (Nle) conjugation, acetylation (Ac), pyrrolation (P) and amination (NH_2) at the N- or C-terminus of original 6-amino acid peptide sequence (P_6 : NEVD EE , a.a.51–56) in ProT α , as well as single amino acid replacement in P_6 .

2.3. Peptide administration

Peptides were dissolved in dimethyl sulfoxide (DMSO) or sterile K^+ -free phosphate-buffered saline (PBS), and were further diluted in PBS. According to a previously described protocol for intravitreal (i.vt.) injection (Fujita et al., 2009; Halder et al., 2013a), mice were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg). Then, peptides with 0.1,

0.3, 1, 3 or 10 pmol were injected (each peptide in a volume of 1 μ l, i.vt.) into the ipsilateral eye 24 h after retinal ischemia. In addition, peptides were intravenously (i.v.) injected (10 mg/kg) 24 h after retinal ischemia in mice. Ischemic vehicles were prepared by intravitreal injection (1 μ l, i.vt.) or intravenous injection (i.v.) with 0.05% DMSO (in K⁺-free PBS) or PBS 24 h after retinal ischemia in mice. Untreated mice received no injection or ischemia.

2.4. Retinal ischemia model

Retinal ischemia was performed following a previously described protocol (Wang et al., 2002; Kumada et al., 2004). Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and pupils were fully dilated with 1% atropine sulfate (Nitten, Nagoya, Japan). The anterior chamber of the eye was cannulated with a 33-gauge needle attached to an infusion container of sterile intraocular irrigating solution (Opeguard Neo Kit 0.0184%, Senju Pharmaceutical Co., Ltd., Osaka, Japan). The infusion container was raised 176 cm high, and retinal ischemia was induced by elevating the intraocular pressure (IOP) to generate a hydrostatic pressure to 130 mm Hg for 45 min. The buffer level in the container was kept constant during the period of ischemia. Retinal ischemia was confirmed under the microscope by the whitening of the iris during the elevation of intraocular pressure. At 45 min after ischemia, the needle was withdrawn and 0.3% ofloxacin (Santen Pharmaceutical Co. Ltd., Osaka, Japan) was applied topically into the eye to prevent infection. All data were analyzed seven days after retinal ischemia.

2.5. Electroretinography

Electroretinography (ERG) was performed according to a previously described protocol (Fujita et al., 2009; Halder et al., 2013a). Briefly, mice were dark-adapted for 3–4 h. Mice were then

anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and pupils were dilated with 1% atropine sulfate (Nitten). A contact electrode (KE-S, Kyoto contact lenses, Kyoto, Japan) was placed topically on the corneal apex and a reference electrode was placed near the ischemic eye. The ground was a subdermal platinum needle electrode near the abdominal area. ERGs were produced by 20 J flash intensities. The flash stimulus source (SLS-3100, Nihon Kohden, Tokyo, Japan) illuminated the eye by diffuse reflection off the interior surface of the ganzfeld. Maximum flash luminance was measured with a detector (MEB-9104, Nihon Kohden, Tokyo, Japan). After the intensity series, an incandescent background light, sufficient to desensitize the rod system, was turned on, and ERGs produced by the standard stimulus were recorded every 2 min for 20 min. The background was then turned off, and ERGs were produced by the standard stimulus every 2 min for the first 30 min of dark adaptation. The a- and b-wave amplitudes were measured online (Neuropack m, QP-903B, Nihon Kohden, Tokyo, Japan) seven days after retinal ischemia. Using a dose-response curve, the median effective dose (ED₅₀) of peptides was calculated by considering the a-wave and b-wave values; 100 for untreated (non-ischemia) and 0 for ischemia plus vehicle (PBS)-treated mice.

2.6. Tissue preparation

Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and eyes were quickly isolated, nicked through the pupils and incubated with 4% paraformaldehyde (PFA) for 24 h at 4°C. Eyes were then transferred to 25% sucrose solution (in 0.1 M K⁺-free PBS) and kept overnight at 4°C. Eyes were fast-frozen in a cryoembedding compound and retinal sections were cut at a 10 µm thickness using a cryostat (Leica Microsystems, Wetzlar, Germany). In all experiments, retinal crosscut sections (10 µm) of whole eye, strictly within 0.2 mm from the

optic nerve, were used to minimize the stereological variation in retinal thickness.

2.7. Histology

Histological assessment, using hematoxylin and eosin (HE) staining, was conducted following a previously described protocol (Halder et al., 2013b). Briefly, retinal sections were washed with PBS, immersed in Mayer's hematoxylin solution (WAKO, Osaka, Japan) for 5 min at 25°C and then washed with tap water for 20 min. After a brief treatment with 95% ethanol, sections were immersed in eosin-alcohol solution (WAKO) for 4 min at 25°C. Sections were dehydrated through a series of ethanol solutions, xylene, and over-slipped with Permount (Fisher Scientific, Waltham, MA, USA). The analysis of the HE-stained retinal sections, including measurement of retinal thickness (central retina strictly within 0.2 mm from the optic nerve), was performed using a BZ-8000 microscope with BZ Image Measurement Software (KEYENCE, Osaka, Japan) equipped with a ×40 objective lens. The brightness and contrast of all images were adjusted in Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) to the same conditions.

2.8. Immunohistochemistry

For fluorescence immunostaining, retinal sections were washed with PBS and treated with 50% methanol (in PBS) followed by 100% methanol. Following treatment with blocking buffers (bovine serum albumin and normal goat serum with 0.1% Triton X-100 in PBS), sections were incubated overnight at 4°C with the following primary antibodies: anti-neuronal nuclei (NeuN) (1:100; mouse monoclonal IgG1, clone A60, Chemicon, Temecula, CA, USA), anti-ceh-10 homeodomain-containing homolog (Chx10) (1:300; sheep polyclonal, Exalpha Biologicals, Shirley, MA, USA), anti-syntaxin-1 (1:500; mouse monoclonal, Sigma-Aldrich), anti-ionized

calcium binding adaptor molecule 1 (Iba-1) (1:1000; rabbit polyclonal, WAKO, Osaka, Japan), and anti-glial fibrillary acidic protein (GFAP) (1:500; rabbit polyclonal, Glostrup, Dako, Denmark). Sections were incubated with Alexa Fluor 488-conjugated anti-mouse IgG, Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-sheep IgG secondary antibodies (1:300; Molecular Probes, Eugene, OR, USA). Sections were washed thoroughly with PBS and cover-slipped with Vectashield[®] Anti-fade Mounting Media with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA). Images were analyzed using a BZ-9000 microscope with BZ Image Measurement Software (KEYENCE, Osaka, Japan). The brightness and contrast of all images were adjusted in Adobe Photoshop (Adobe Systems Inc.). Total fluorescence intensity of Iba-1-stained or GFAP-stained retinal sections was measured according to a previously described procedure (Echevarria et al., 2013; Jensen, 2013; Narimatsu et al., 2014). Iba-1- and GFAP-stained retinal images were captured using a BZ-9000 microscope with BZ Image Measurement Software (KEYENCE) equipped with a $\times 40$ objective lens. Using Image J software (National Institutes of Health, Bethesda, MD, USA), the mean (total) fluorescence intensity of each image ($\times 40$ image, 1024×1360 pixels, stained with Iba-1 for microglia or GFAP for astrocytes was individually measured by drawing a rectangle of approximately 1.3×10^6 area (arbitrary unit) over the whole retinal image.

2.9. Cell counting

The counting of cells in the central retina was carried out according to a previously described protocol (Halder et al., 2013c). Following HE staining of retinal sections (central retina strictly within 0.2 mm from the optic nerve), the total number of cells in the ganglion cell layer (GCL) was counted using BZ-8000 microscope with BZ Image Measurement Software (KEYENCE)

equipped with a $\times 40$ objective lens. After fluorescence immunohistochemistry of the retinal sections, NeuN-positive ganglion cells in the GCL, Chx10-positive bipolar cells in the INL, Syntaxin-1-positive cell bodies of amacrine cells in the INL, DAPI-stained photoreceptor cells in the ONL, Iba-1-positive microglia and GFAP-positive astrocytes with clearly visible nuclei by DAPI were counted in each bright field image (central retina strictly within 0.2 mm from the optic nerve) using BZ-9000 microscope with BZ Image Measurement Software (KEYENCE) equipped with $\times 40$ objective lens.

2.10. Statistical analysis

All results are shown as means $\pm$ standard error of the mean (S.E.M.). The differences in multiple groups were compared using Tukey–Kramer multiple comparison *post-hoc* test after a one-way ANOVA. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Protective activity screening of ProT α -derived peptides against retinal ischemia

To examine whether small P₉ (a.a.52–60)-related peptides have protective activity against ischemia-induced functional damage in retina, peptides with 10 pmol each in a volume of 1 μ l were intravitreally (i.vt.) injected into the eye 24 h after retinal ischemia in mice. The protective activity was evaluated by ERG seven days after retinal ischemia. Fig. 1A shows the diagram for the peptide sequences related to P₉ or P₃₀ (a.a.49–78). As shown in Fig. 1B, the ERG response in a-wave for untreated mice was -88.5 ± 3.3 μ V ($n = 3$). Retinal ischemic stress with vehicle (PBS) markedly decreased this response to -29.8 ± 2.4 μ V ($n = 3$). Treatment with P₉ reversed ischemia-induced decrease in response to -58.7 ± 4.3 μ V ($n = 7$) (Halder et al., 2013b). Although the smaller P₉-related peptides, a.a.53–60 and a.a.54–60, showed decreased ERG responses in a-waves compared with the protective activity of a.a.52–60 (P₉), three other peptides (a.a.52–59, a.a.52–58, and a.a.52–57) showed equipotent responses in a-waves to the activity with a.a.52–60 (P₉). Similar patterns of ERG responses in b-waves were obtained using those P₉-related peptides (Fig. 1C). These findings suggest that trimming of C-terminal 3 amino acids of P₉ retains the original protective activity, while trimming of N-terminal amino acids lost it.

As the N-terminal region (a.a.52–53) of P₉ is crucial, we attempted to determine the effects of the addition of further upstream amino acids. As shown in Fig. 1B, a.a.51–59 and a.a.50–58 significantly showed higher activities compared with the activity of original P₉ (Halder et al., 2013b), and the activities of these peptides were equipotent to that of P₃₀ (Halder et al., 2013a), while the activity of a.a.49–57 declined. Similar patterns were also obtained with ERG responses in b-waves (Fig. 1C). The activity of the 7-amino acid peptide (a.a.51–57), which loses both N-terminal and C-terminal amino acids, retained the activities of a.a.51–59 and a.a.50–58,

but did not exceed them. Furthermore, the 6-amino acid peptide (a.a.51–56) retained a similar activity to that with a.a.51–57, while the activity of 5-amino acid peptide (a.a.51–55) markedly declined. All of these findings suggest that ProTα-derived 6-amino acid peptide (a.a.51–56) is the smallest peptide sequence to give activity equivalent to that of P₃₀. We referred this 6-amino acid peptide sequence (a.a.51–56) as P₆.

3.2. Protection of ischemic retina by ProTα-derived P₆ peptide

To examine the dose-dependent protective activity of the ProTα-derived 6-amino acid peptide/P₆ (a.a.51–56) against ischemic damage, P₆ peptide with 0.3, 1, 3 and 10 pmol each in a volume of 1 µl were injected (i.vt.) 24 h after retinal ischemia in mice. Histological analysis using HE staining revealed that the total retinal thickness extending from ganglion cell layer (GCL) to outer nuclear layer (ONL) in untreated mice was $191.0 \pm 3.1 \mu\text{m}$ ($n = 3$), as shown in Fig. 2A and 2B. Ischemic stress with vehicle (PBS) markedly decreased the total retinal thickness to $100.6 \pm 3.5 \mu\text{m}$ ($n = 3$) (Fig. 2A, B). Treatment with 10 pmol P₆ peptide significantly inhibited ischemia-induced decrease in total retinal thickness ($159.3 \pm 5.0 \mu\text{m}$) ($n = 3$) (Fig. 2A, B). Significant protection against retinal histological protection was observed when mice were post-treated with 3 pmol P₆ peptide, but not with 0.3 or 1 pmol (Fig. 2A, B).

ERG study revealed that a- and b-waves were markedly decreased seven days after retinal ischemia in vehicle (PBS)-treated (24 h) mice, compared with untreated mice (Fig. 2C, D). Treatment with 10 pmol P₆ peptide significantly inhibited retinal ischemia-induced decrease in a- and b-waves seven days after the ischemic stress. However, significant protection against retinal functional damage was obtained using 3 pmol P₆, but not 0.3 or 1 pmol (Fig. 2C, D).

3.3. Protective activity assessment of P₆-derived modified peptides against retinal ischemia

We previously reported that post-treatment (i.vt.) with 1 pmol ProTα completely inhibited retinal ischemia-induced functional damage, in ERG study (Fujita et al., 2009). As treatment (i.vt.) with 1 pmol ProTα-derived 6-amino acid peptide/P₆ (NEVD₆EE, a.a.51–56) failed to inhibit retinal ischemia-induced damage (shown in Fig. 2), we further examined whether modifications could increase the protective activity of P₆ against ischemic damage. Twenty-one modified peptides were prepared by Nva or Nle conjugation, Ac, P, and NH₂ at the N-terminus or C-terminus of P₆, as well as single amino acid replacement in P₆. P₆ and twenty-one modified P₆ peptides, each with 1 pmol in a volume of 1 μl, were individually injected (i.vt.) into the ipsilateral eye 24 h after retinal ischemia, and functional analysis using ERG was performed seven days after the ischemic stress. In ERG study, ischemia caused retinal functional damage in terms of decrease in a- and b-waves in vehicle (PBS)-treated mice (Fig. 3A, B). Ischemia-induced functional damage was significantly ($p < 0.05$) and maximally inhibited by treatment with 1 pmol modified P₆ peptide NEVDQE, but not with the P₆ peptide (Fig. 3A, B). Similar to NEVDQE, higher level of protection against ischemia-induced retinal functional damage was obtained by post-ischemia treatment with thirteen modified P₆ peptides (each with 1 pmol in a volume of 1 μl) indicated by red bars, but not with modified P₆ peptides indicated by black bars in Fig. 3A and 3B. This primary screening suggests that the protective activity of fourteen modified P₆ peptides is higher than that obtained using original P₆ peptide (Fig. 3A, B and E). In the second screening, primary-screened fourteen modified P₆ peptides, each with 0.1 pmol in the volume of 1 μl, were individually injected (i.vt.) into the ipsilateral eye 24 h after retinal ischemia, and functional analysis using ERG was carried out seven days after the ischemic stress. Among the fourteen modified P₆ peptides, post-ischemia treatment with five peptides NEVD₆EE-NH₂, NEVEEE,

NEVDQE, pEEVDDEE and pEEVNNEE (each with 0.1 pmol in a volume of 1 μ l, i.vt.) significantly and maximally inhibited retinal ischemia-induced decline in a- and b-waves using ERG, compared with ischemic vehicle as well as the protective activity of other modified P₆ peptides indicated by black bars (Fig. 3C, D, and E). This finding suggests that the modified P₆ peptides NEVDDEE-NH₂, NEVEEEE, NEVDQE, pEEVDDEE and pEEVNNEE were more potent than the original P₆ peptide and other modified P₆ peptides.

3.4. Systemic injection of P₆Q peptide protects retina from ischemic damage

To examine whether systemic administration with primary-screened five modified P₆ peptides NEVDDEE-NH₂, NEVEEEE, NEVDQE, pEEVDDEE and pEEVNNEE show protective activity against ischemic damage, mice were intravenously (i.v.) treated with these modified P₆ peptides (10 mg/kg) 24 h after retinal ischemia. The protective activity was evaluated by HE staining and ERG seven days after retinal ischemia. As shown in Fig. 4A and 4B, histological analysis using HE staining revealed that the total retinal thickness (GCL-ONL) in untreated mice was $185.1 \pm 3.7 \mu\text{m}$ ($n = 3$). Ischemic stress with vehicle treatment (PBS, i.v.) markedly decreased the total retinal thickness (GCL-ONL) to $83.4 \pm 5.3 \mu\text{m}$ ($n = 3$) (Fig. 4A, B). Systemic post-ischemia treatment with NEVDQE peptide significantly inhibited ischemia-induced decrease in retinal thickness ($154.1 \pm 5.8 \mu\text{m}$). However, significant protection of total retinal thickness was obtained by systemic post-ischemia injection with pEEVDDEE peptide ($117.8 \pm 3.7 \mu\text{m}$), but not NEVDDEE-NH₂ ($86.8 \pm 4.7 \mu\text{m}$), NEVEEEE ($87.4 \pm 3.6 \mu\text{m}$) or pEEVNNEE ($88.0 \pm 2.6 \mu\text{m}$) (Fig. 4A, B). Detailed histological analysis revealed that retinal ischemia-induced decrease in thickness of the INL, IPL, OPL and ONL was significantly inhibited when mice were treated with NEVDQE (Fig. 4A and C-F). Systemic administration with pEEVDDEE peptide, but not

NEVD_{EE}-NH₂, NEVE_{EE} or pEEVN_{EE} also significantly protected the retina from ischemia-induced decrease in thickness of the IPL, INL and OPL thickness, but not the ONL (Fig. 4A and C–F). Systemic injection with NEVDQ_E peptide significantly inhibited retinal ischemia-induced loss of cells in the GCL. In addition, significant protection of GCL cells following retinal ischemia was obtained by systemic treatment with pEEVD_{EE} peptide, but not NEVD_{EE}-NH₂, NEVE_{EE} or pEEVN_{EE} peptides (Fig. 4A, G). The protective activity of the NEVDQ_E peptide against ischemia-induced retinal histological damage, in terms of retinal thickness and GCL cell number, was significantly higher than that obtained using the pEEVD_{EE} peptide (Fig. 4B–G).

ERG study showed that the systemic administration with NEVDQ_E peptide (10 mg/kg, i.v.) completely inhibited retinal ischemia-induced decrease in a- and b-waves seven days after the ischemic stress, compared with ischemic vehicle (Fig. 4H, I). Significant inhibition of retinal ischemia-induced functional damage was also observed when mice were treated with pEEVD_{EE} peptide (10 mg/kg, i.v.), but not with NEVD_{EE}-NH₂, NEVE_{EE} and pEEVN_{EE} peptides (Fig. 4H, I). The protective activity of the NEVDQ_E peptide against retinal ischemia-induced functional damage was significantly higher than that obtained using the pEEVD_{EE} peptide (Fig. 4H–I). All these findings, using histological and functional assessments, suggest that P₆-derived modified peptide NEVDQ_E is more potent than other four modified P₆ peptides when administered through a systemic (i.v.) route in the presence of retinal ischemia. We termed this modified P₆ peptide NEVDQ_E as P₆Q.

3.5. P₆Q peptide-induced protection of cell types in the ischemic retina

To examine which types of retinal cells are protected against ischemia by systemic post-treatment with P₆Q peptide, P₆Q peptide was intravenously (i.v.) injected (10mg/kg) 24 h after

retinal ischemia in mice. Fluorescence immunostaining revealed that NeuN-positive ganglion cells in the GCL (Schlamp et al., 2013) were markedly lost seven days after the ischemic stress in vehicle (PBS)-treated (24 h) mice, whereas post-ischemia treatment with P₆Q completely inhibited ischemia-induced loss of ganglion cells (Fig. 5A, B). Similarly, systemic injection with the P₆Q peptide significantly inhibited retinal ischemia-induced loss of Chx10-positive bipolar cells (Samuel et al., 2011) in the INL (Fig. 5C, D); syntaxin-1-positive amacrine cells (De la Huerta et al., 2012), of which cell bodies and processes are located in the INL and IPL, respectively (Fig. 5E, F), and DAPI-stained photoreceptor cells in the ONL (Fig. 5G, H) seven days after the ischemic stress, compared with their respective ischemic vehicles (Fig. 5C–H).

3.6. P₆Q peptide inhibits retinal ischemia-induced activation of microglia and astrocytes

To examine the effects of P₆Q peptide on retinal microglia and astrocytes under ischemic conditions, mice were intravenously (i.v.) treated with P₆Q peptide (10 mg/kg) 24 h after retinal ischemia. Fluorescence immunostaining revealed that Iba-1-positive microglia were distributed in the retinal GCL and IPL of control mice (Fig. 6A), which is consistent with previous reports (Lin et al., 2012; Halder et al., 2013c; Halder et al., 2015). Retinal ischemia caused microglial activation in terms of morphological change, migration, increased population and Iba-1 intensity throughout different retinal layers seven days after the ischemic stress in vehicle-treated mice, compared with the retina of untreated mice (Fig. 6A–C). In contrast, systemic treatment (i.v.) with P₆Q peptide significantly inhibited ischemia-induced activation of retinal microglia seven days after the ischemic stress (Fig. 6A–C).

Immunohistochemical data revealed that the distribution of GFAP-positive astrocytes was mainly in the retinal GCL of untreated mice (Fig. 5D), consistent with previous studies (Lin

et al., 2012; Halder et al., 2013c; Halder et al., 2015). Following retinal ischemia, GFAP-positive astrocytes were activated in terms of morphological change, increased number and GFAP intensity throughout the GCL to ONL seven days after the ischemic stress (Fig. 5D–F). In contrast, systemic (i.v.) post-ischemia treatment with P₆Q peptide significantly inhibited retinal ischemia-induced activation of astrocytes seven days after the ischemic stress (Fig. 5D–F). Therefore, P₆Q peptide has inhibitory effects on retinal ischemia-induced activation of microglia and astrocytes.

4. Discussion

The present study can be summarized, as follows; (1) systemically-injected modified hexapeptide P₆Q (NEVDQE) potently inhibits retinal ischemia-induced histological and functional damage; and (2) P₆Q peptide significantly inhibits retinal ischemia-induced activation of microglia and astrocytes.

We previously demonstrated the potent neuroprotective action of ProTα against brain and retinal ischemia or serum-starvation stress (Fujita and Ueda, 2007; Fujita et al., 2009; Ueda et al., 2010). Recently, we reported that the active core peptide sequence P₃₀ (a.a.49–78), but not N-terminal sequence (a.a.1–48) or C-terminal sequence (a.a.79–112 and a.a.102–112), in ProTα is responsible for its full survival activity in cultured cortical neurons against serum-free stress (Halder et al., 2013a). The neuroprotective action of P₃₀ peptide has also been reported in brain and retinal ischemia models (Halder et al., 2013a). Furthermore, the 9-amino acid peptide (P₉: a.a.52–60) in P₃₀ ameliorates ischemic brain and retinal damage, though P₉ is 3 and 4 times less potent than P₃₀ in brain and retinal ischemia models, respectively (Halder et al., 2013b). In the present study, we screened for the protective activity of smaller peptide sequence related to P₉ and P₃₀ peptides in ProTα using ERG experiments with retinal ischemia model. When mice were intravitreally (i.vt.) treated with P₉- and P₃₀-related smaller peptides 24 h after retinal ischemia, the minimum 6-amino acid peptide sequence NEVDEE (P₆: a.a.51–56), but not the 5-amino acid peptide sequence NEVDE (P₅: a.a.51–55), showed potent protective activity against retinal functional damage in ERG analysis. Furthermore, intravitreal treatment with 10 pmol P₆ peptide 24 h after retinal ischemia abolished the ischemia-induced decrease in retinal thickness and cell number in different layers after histological HE staining and retinal functional deficits in the ERG analysis, though significant retinal protection was obtained by post-ischemia treatment

(i.vt.) with 3 pmol P₆, not 0.3 or 1 pmol. These findings suggest that 6-amino acid peptide sequence NEVD₆EE (P₆: a.a.51–56) in ProTα is important for protection of ischemic retina.

Next, we examined whether modification of 6-amino acid peptide (P₆: NEVD₆EE) increases its protective activity. Mice were intravitreally treated with twenty-one different modified P₆ peptides 24 h after retinal ischemia. Among these peptides, five modified P₆ peptides NEVDQ₆E (P₆Q), pEEVD₆EE, NEVD₆EE–NH₂, NEVEEEE and pEEVN₆EE showed a higher protective activity against retinal ischemic damage in the ERG analysis. We further investigated whether systemic injection with these modified P₆ peptides P₆Q (NEVDQ₆E), pEEVD₆EE, NEVD₆EE–NH₂, NEVEEEE and pEEVN₆EE protects the retina from ischemic damage. HE staining and ERG revealed that intravenous administration with P₆Q peptide (NEVDQ₆E) 24 h after retinal ischemia potently inhibited ischemia-induced retinal histological damage and functional deficits. However, a partial, but significant, retinal protection was obtained by systemic injection with the modified P₆ peptide pEEVD₆EE, but not with NEVD₆EE–NH₂, NEVEEEE or pEEVN₆EE. There are reports that intravenous delivery limits the therapeutic effects of proteins or peptides by systemic protease-mediated degradation, conformational changes and non-covalent complexation with blood components (Bruno et al., 2013; Craik et al., 2013). Since retinal ischemic stress causes the disruption of blood–retinal barrier (Kaur et al., 2008; Tong et al., 2013), systemic administrated P₆Q peptide would safely penetrate into the damaged retina through the disrupted blood-retinal barrier and bind to unknown targets for protection against retinal ischemic damage. We also performed immunohistochemical analysis to examine which retinal cell types are protected by the modified peptide P₆Q. The results showed that ischemia-induced loss of retinal ganglion, bipolar, amacrine and photoreceptor cells was significantly inhibited by systemic injection with P₆Q. In the ERG analysis, the median effective dose (ED₅₀)

of intravitreally-injected P₃₀ peptide to give 50 μ V was 2 pmol/eye for a-wave and to give 150 μ V was 1 pmol/eye for b-waves, while the values for P₉ peptide were 8 and 4 pmol/eye, respectively (Halder et al., 2013a; Halder et al., 2013b). In the present study, the ED₅₀ of P₆ peptide to give 50 μ V was approximately 3 pmol/eye a-waves and to give 150 μ V was 2 pmol/eye for b-waves of the ERG, whereas these values for P₆Q peptide were 0.04 and 0.03 pmol/eye for a- and b-waves, respectively. Therefore, the potency of P₆ is approximately 3 times higher than P₉, while the modified peptide P₆Q is approximately 42 times more potent than P₃₀, and 64 times more potent than P₆ peptide in the retinal ischemia model. The higher potency of P₆Q (NEVDQE) was obtained by replacement of a single amino acid glutamine (Q), instead of glutamate (E), at the fifth position in the 6-amino acid peptide (P₆: NEVDDE) of ProT α .

Microglia and astrocytes, the major types of glial cells in the CNS, are activated in response to brain and retinal ischemia, and they subsequently contribute to ischemic damage through up-regulation and secretion of various cytotoxic mediators, whereas a blockade of the activation of these glial cells ameliorates the ischemic damage (Wang et al., 2008; Dvorianchikova et al., 2009; Weinstein et al., 2010; Zhao and Rempe, 2010; Barreto et al., 2011; Barakat et al., 2012; dell'Omo et al., 2013; Fumagalli et al., 2015; Karlstetter et al., 2015). In the present study, retinal ischemia caused marked activation of microglia and astrocytes in terms of their protein intensities, morphological changes, increased number and migration throughout retinal cell layers, whereas activation of these glial cells were significantly inhibited by the systemic treatment with P₆Q peptide. The roles of microglia and astrocytes are complex due to their detrimental and beneficial actions in ischemia (Barreto et al., 2011; Gomes-Leal, 2012; Taylor and Sansing, 2013; Sayre et al., 2014; Hirayama et al., 2015). We previously demonstrated that ProT α protects the brain and retina from ischemic damage, through up-

regulation of brain-derived neurotrophic factor (BDNF) or erythropoietin (EPO) (Fujita and Ueda, 2007; Fujita et al., 2009; Ueda et al., 2012). Most recently, we reported that preconditioning with ProT α inhibits retinal ischemia-induced elevation of several injury factors, such as tumor necrosis factor α (TNF α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2) and monocyte chemoattractant protein-1 (MCP-1), and increased expression of some protective genes, such as interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interleukin 1 receptor antagonist (IL1RN), regulated on activation, normal T cell expressed and secreted (RANTES), suppressor of cytokine signaling 1 and 2 (SOCS1 and SOCS2, respectively) and phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (Ship1) (Halder et al., 2015). In addition, retinal ischemia-induced microglial activation is inhibited by preconditioning with ProT α (Halder et al., 2015). We do not know how P₆Q peptide functions on microglia and astrocytes to induce protective effects against ischemia, but it may be hypothesized that P₆Q blocks the production and secretion of toxic molecules including those toxic injury factors (TNF α , IL-1 β , IL-6, COX-2 and MCP-1), and facilitates the release of beneficial factors including BDNF from these glial cells in response to ischemia. Culture studies with glial cells and retinal neurons are necessary to evaluate the effects of P₆Q-produced toxic and/or protective factors. Taken together, our study demonstrates that P₆Q is a unique peptide molecule with higher protective activity against retinal ischemic damages *in vivo*. P₆Q may have additional beneficial effects against inflammation, pain and neurodegenerative disorders, whose etiologies include microglia and astrocyte activations.

In conclusion, intravitreal or systemic injection with P₆Q peptide (NEVDQE) derived from the 6-amino acid peptide sequence (P₆: a.a. 51–56) in ProT α 24 h after retinal ischemia potently protected retina from ischemia-induced histological and functional damage. Ischemia-

induced loss of retinal ganglion, bipolar, amacrine and photoreceptor cells was inhibited by systemic injection with P₆Q peptide. Moreover, systemic delivery with P₆Q peptide inhibited retinal ischemia-induced activation of microglia and astrocytes. Therefore, P₆Q peptide would be a powerful therapeutic tool for the treatment of ischemic disorders.

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Figure Legends

Fig. 1. Protective activity assessment of ProTα-derived peptides against retinal ischemic damage.

(A) The diagram shows P₉ (a.a.52–60) or P₃₀ (a.a.49–78)-related peptides in the mid-part sequence (a.a.41–81) of ProTα. (B, C) Peptides were intravitreally (i.vt.) injected (each with 10 pmol in a volume of 1 μl) into the ipsilateral eye 24 h after retinal ischemia in mice and all analyses were performed seven days after the ischemic stress. The evaluation of a-waves (B) and b-waves (C) using electroretinography (ERG) was performed seven days after ischemia. Data are mean ± S.E.M. Tukey–Kramer multiple comparison *post-hoc* test. ^{**}*p* < 0.01 versus untreated mice, [#]*p* < 0.05 versus ischemic vehicle, ^{##}*p* < 0.01 versus ischemic vehicle, [†]*p* < 0.05 versus peptides indicated by black bars. *n* = 3. The ERG responses in a-wave and b-wave for 10 pmol P₃₀- or P₉-treated (24 h) retinal ischemic mice were –80.7 μV and 263.3 μV (*n* = 5) or –58.7 μV and 161.2 μV (*n* = 7), respectively, and these effects were significantly higher (*p* < 0.05, Dunnett’s multiple comparison test) than that the respective ischemic vehicles (Halder et al., 2013a; Halder et al., 2013b). The ERG responses (a- and b-waves) in P₃₀-treated ischemic mice were significantly higher (*p* < 0.01, Tukey–Kramer multiple comparison *post-hoc* test) than that obtained using peptides indicated by black bars, while the peptides indicated by red bars were equipotent to that of P₃₀ (not significant, *p* > 0.05, Tukey–Kramer multiple comparison *post-hoc* test). [‡]*p* < 0.05 versus P₉ (Tukey–Kramer multiple comparison *post-hoc* test). *n* = 3 for a.a.53–60 and a.a.54–60. *n* = 7 for P₉.

Fig. 2. ProTα-derived 6-amino acid peptide P₆ protects retina from ischemic damage. (A–D) ProTα-derived 6-amino acid peptide (P₆: a.a. 51–56) was intravitreally injected (0.3, 1, 3 or 10 pmol each in a volume of 1 μl, i.vt) into the ipsilateral eye 24 h after retinal ischemia in mice and

all analyses were carried out seven days after the ischemic stress. (A) Hematoxylin and eosin (HE) staining of retinal sections showed the representative data. (B) Measurement of total retinal thickness extending from the ganglion cell layer (GCL) to outer nuclear layer (ONL). Three retinal section in each mouse (n = 3). (C, D) The analysis of a-waves (C) and b-waves (D) using ERG. Data are mean $\pm$ S.E.M. Tukey–Kramer multiple comparison *post-hoc* test. $^{**}p < 0.01$ versus untreated mice, $^{\#}p < 0.05$ versus ischemic vehicle, $^{##}p < 0.01$ versus ischemic vehicle. n = 3. Scale bar, 20 μ m.

Fig. 3. Screening of the protective activity of modified P₆ peptides against retinal ischemia. (A, B) Following intravitreal (i.vt.) injection with ProT α -derived 6-amino acid peptide P₆ (a.a.51–56) and twenty-one modified P₆ peptides (each with 1 pmol in a volume of 1 μ l) 24 h after retinal ischemia, the analysis of a-waves (A) and b-waves (B) using ERG were performed seven days after the ischemic stress. Data are mean $\pm$ S.E.M. Tukey–Kramer multiple comparison *post-hoc* test. $^{**}p < 0.01$ versus untreated mice, $^{##}p < 0.01$ versus ischemic vehicle. $p > 0.05$ (NS). NS, not significant. n = 3 for untreated mice, ischemic vehicle, P₆ peptide (NEVD₆EE) and modified P₆ peptide NEVD₆QE, and n = 2 for other twenty peptides. (C, D) Mice were treated with different fourteen modified P₆ peptides (each with 0.1 pmol in a volume of 1 μ l, i.vt.) 24 h after retinal ischemia. The analysis of a-waves (C) and b-waves (D) using ERG were carried out seven days after the ischemic stress. Data are mean $\pm$ S.E.M. Tukey–Kramer multiple comparison *post-hoc* test. $^{**}p < 0.01$ versus untreated mice, $^{\#}p < 0.05$ versus ischemic vehicle, $^{##}p < 0.01$ versus ischemic vehicle, $^{\dagger}p < 0.05$ versus peptides indicated by black bars. n = 4. (E) Flow chart shows the screening of peptides.

Fig. 4. Systemic injection with P₆Q peptide potently inhibits retinal ischemia-induced damage. (A–G) Modified P₆ peptides NEVDQE (P₆Q), NEVDDEE–NH₂, NEVEEEE, pEEVDDEE and pEEVNDEE peptides (10 mg/kg) were intravenously (i.v.) injected 24 h after retinal ischemia in mice, and all analyses were performed seven days after the ischemic stress. (A) HE staining of retinal sections showed the representative data. (B–F) Measurement of total retinal thickness spanning from GCL to ONL (B), and thickness of the IPL (C), INL (D), OPL (E) and ONL (F). (G) Counting of total cells in the GCL. Three retinal section in each mouse (n = 3). (H, I) The analysis of a-waves (H) and b-waves (I) using ERG. Data are mean ± S.E.M. ^{**}*p* < 0.01 versus untreated mice (Tukey–Kramer multiple comparison *post-hoc* test), [#]*p* < 0.05 versus ischemic vehicle (Tukey–Kramer multiple *post-hoc* comparison test), ^{##}*p* < 0.01 versus ischemic vehicle (Tukey–Kramer multiple comparison test), [†]*p* < 0.05 versus pEEVDDEE (Tukey–Kramer multiple comparison *post-hoc* test). n = 3. Scale bar, 20 μm.

Fig.5. Protection of retinal cell types by systemic treatment with P₆Q peptide. (A–G) Following P₆Q (NEVDQE) peptide injection (10 mg/kg, i.v.) 24 h after retinal ischemia in mice, fluorescence immunostaining of retinal sections and all other analyses were carried out seven days after the ischemic stress. (A) Immunostaining of NeuN-positive ganglion cells (NeuN, green) in the GCL using retinal sections showed the representative data. (B) Quantitative analysis of ganglion cells residing in the retinal GCL. (C) Representative data using immunostaining of Chx10-positive bipolar cells (Chx10, green) residing in the retinal INL. (D) Quantitative analysis of Chx10-positive bipolar cells. (E) Immunostaining of syntaxin-1-positive amacrine cells (syntaxin-1, green) in the retinal INL and IPL showed the representative data. Cell bodies and processes of syntaxin-1-positive amacrine cells are located in the retinal INL and IPL,

respectively. (F) Quantitative analysis of amacrine cell bodies residing in the retinal INL. (G) Representative data using DAPI staining of photoreceptor cells (DAPI, blue) in the ONL of retinal sections. (H) Quantitative analysis of DAPI-stained photoreceptor cells residing in the retinal ONL. Data are mean $\pm$ S.E.M. Tukey–Kramer multiple comparison *post-hoc* test. $^{**}p < 0.01$ versus untreated mice, $^{##}p < 0.01$ versus ischemic vehicle. Three retinal section in each mouse (n = 3). Scale bar, 20 μ m.

Fig.6. P₆Q peptide blocks retinal ischemia-induced activation of microglia and astrocytes. (A–F) P₆Q (NEVDQE) peptide was injected (10 mg/kg, i.v.) 24 h after retinal ischemia in mice and all analyses were performed seven days after the ischemic stress. (A–C) P₆Q peptide inhibits retinal ischemia-induced activation of microglia. (A) Immunostaining of Iba-1-positive microglia (Iba-1, red) showed the representative data. (B) Counting of Iba-1-positive microglia seven days after retinal ischemia. (C) Measurement of the mean (total) fluorescence intensity of retinal Iba-1-positive microglia seven days after ischemia. Data are mean $\pm$ S.E.M. Tukey–Kramer multiple comparison *post-hoc* test. $^{**}p < 0.01$ versus untreated mice, $^{##}p < 0.01$ versus ischemic vehicle. AU, arbitrary unit. n = 3. (D–F) P₆Q peptide inhibits retinal ischemia-induced activation of astrocytes. (D) Representative data using immunostaining of GFAP-positive astrocytes (GFAP, red) in retina seven days after retinal ischemia. (E) Counting of GFAP-positive astrocytes seven days after the ischemic stress. (F) Measurement of mean (total) fluorescence intensity of GFAP-positive astrocytes seven days after ischemia. Data are mean $\pm$ S.E.M. Tukey–Kramer multiple comparison *post-hoc* test. $^{**}p < 0.01$ versus untreated mice, $^{#}p < 0.05$ versus ischemic vehicle. AU, arbitrary unit. Three retinal section in each mouse (n = 3). Scale bar, 20 μ m.

A**Mouse ProTα (Mid-part: a.a. 41–81)** **B** **C** **FIG 1**

A

7 days after retinal ischemia

P₆ peptide (pmol/ μ l, i.vt.)

Untreated

Ischemic veh

0.3

1

3

10

GCL
IPL
INL
OPL
ONL **B** **C** **D**

fig 2

fig 3

A **B** **C** **D** **E** **F** **G** **H** **I**

fig 5

A**Iba-1 (Microglia)**

7 days after retinal ischemia

Untreated

Ischemic veh

P₆Q **B** **C** **D****GFAP (Astrocytes)**

7 days after retinal ischemia

Untreated

Ischemic veh

P₆Q **E** **F**

fig 6