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Therapeutic benefits of 9-amino acid peptide derived from prothymosin alpha against ischemic damages

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ABSTRACT

Prothymosin alpha (ProTα), a nuclear protein, plays multiple functions including cell survival. Most recently, we demonstrated that the active 30-amino acid peptide sequence/P_{30} (amino acids 49–78) in ProTα retains its substantial activity in neuroprotection *in vitro* and *in vivo* as well as in the inhibition of cerebral blood vessel damages by the ischemic stress in retina and brain. But, it has remained to identify the minimum peptide sequence in ProTα that retains neuroprotective activity. The present study using the experiments of alanine scanning suggested that any amino acid in 9-amino acid peptide sequence/P_9 (amino acids 52–60) of P_{30} peptide is necessary for its survival activity of cultured rat cortical neurons against the ischemic stress. In the retinal ischemia-perfusion model, intravitreous injection of P_9 24 h after ischemia significantly inhibited the cellular and functional damages at day 7. On the other hand, 2,3,5-triphenyltetrazolium chloride (TTC) staining and electroretinogram assessment showed that systemic delivery with P_9 1 h after the cerebral ischemia (1 h tMCAO) significantly blocks the ischemia-induced brain damages. In addition, systemic P_9 delivery markedly inhibited the cerebral ischemia (tMCAO)-induced disruption of blood vessels in brain. Taken together, the present study provides a therapeutic importance of 9-amino acid peptide sequence against ischemic damages.

1. Introduction

Ischemic stress in brain and retina causes common expression of cellular and functional damages, which include diverse injury-related cascades underlying necrosis and apoptosis, along with subsequent production and secretion of different cytotoxic mediators [7–10,21,31,34,40,44,51–53]. In addition to the release of cell-damaging mediators, some neuroprotective molecules, such as brain-derived neurotrophic factor, fibroblast growth factor and erythropoietin are simultaneously elevated after the onset of ischemia, and cause limited amelioration of ischemic injury through an inhibition of apoptosis, but not necrosis, a key mechanism of cell death [3,4,13,25,28,29,41,44,48]. Hence, it is essential to develop neuroprotective agents that target the mechanism of necrosis under ischemic condition.

We previously identified prothymosin alpha (ProTα) as a necrosis-inhibitory factor in the conditioned medium of serum-free primary culture of cortical neurons [11,45]. It has been clarified that ProTα inhibits ischemia-induced damages in brain and retina through blockade of necrosis and apoptosis [12,13,46,48]. Several studies established a relationship between ProTα and cell survival [1,23,27,30,47,49,50], and distinct amino acid sequences in ProTα are separately involved in this survival phenomenon [6,24,43]. Among them, the peptide sequence (amino acids 32–52) of the central domain in ProTα participates in the cell defensive mechanisms against oxidative stress through an interaction with Nrf2–Keap1 inhibitory complex [18,24,33]. The N-terminal sequence in ProTα (amino acids 2–29), corresponding to thymosin alpha 1, shows anti-cancer activity and induces immunodefensive action against viral infections [5,14,15,36], whereas C-terminal sequence (amino acids 89–109, 99–109 and 100–109) of human ProTα is involved in the induction of pro-inflammatory activity through toll-like receptor signaling and dendritic cell maturation [42,43]. Recently, the cell survival action of mid part (amino acids 41–83) in human ProTα against mutant huntingtin-caused cytotoxicity has been discussed [6]. Most recently, we reported that active core peptide sequence comprised of 30 amino acids (P_{30}: amino acids 49–78) in ProTα exerts its full survival effect in cultured cortical neurons against the ischemic stress and potently blocks the ischemia-induced cellular and functional damages in brain and retina and reverses the damage of cerebral blood vessels in the *in vivo* studies using various ischemic models [17]. However, it is interesting to be investigated which peptide sequence with

Abbreviations: ERG, electroretinogram; GCL, ganglion cell layer; H&E, hematoxylin and eosin; INL, inner nuclear layer; IPL, inner plexiform layer; i.v., intravenously; i.vt., intravitreously; ONL, outer nuclear layer; OPL, outer plexiform layer; ProTα, prothymosin alpha; tMCAO, transient middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride.

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minimum amino acids of P_{30} peptide in ProTα is responsible for neuroprotection. In the present study, we evaluated the neuroprotective effect of 9-amino acid peptide derived from ProTα against the ischemic stress.

2. Materials and methods

2.1. Animals

Male C57/BLJ mice weighing 20–25 g were purchased from Tagawa Experimental Animals (Nagasaki, Japan) and used for all the experiments. Mice were kept in a room maintained at constant temperature (21 ± 2 °C) and relative humidity (55 ± 5%) with an automatic 12 h light/dark cycle with free access to standard laboratory diet and tap water. Animal care and all experimental procedures were formally approved by Nagasaki University Animal Care and Use Committee (Animal Experiments Approval Number: 1104190914).

2.2. Determination of short peptide by alanine scanning

The procedure for the identification of neuroprotective peptide sequence P_{30} (P_{30}: amino acids 49–78) in ProTα has been described previously [17]. To design ProTα-derived shorter neuroprotective peptide, alanine scanning of P_{30} was performed to determine the contribution of specific amino acid residues that retain the original function of P_{30} peptide. Neuroprotective activity of primary cultured cortical neurons was measured at 12 h after the start of serum-free culture in the presence or absence of various peptides. Cultures and methods of the measurement of survival activity were previously described [17,45].

2.3. Peptide administration

Peptide P_9 was dissolved in 0.05% dimethyl sulfoxide (DMSO), which was diluted with 0.1 M potassium (K^+)-free phosphate buffered saline (PBS). Following the protocol of injection in the eye as described previously [17], P_9 was administered intravitreously (i.vt.) with doses of 1, 3 and 10 pmol/μl at 24 h after retinal ischemia ($n = 5$, $n = 7$ and $n = 7$, respectively). On the other hand, P_9 was injected intravenously (i.v.) with doses of 0.1, 0.3 and 1 mg/kg ($n = 5$, $n = 6$, and $n = 7$, respectively) 1 h after the cerebral ischemia (tMCAO). Vehicles were treated with equal volume of 0.05% DMSO in similar manners.

2.4. Ischemic models

Two types of *in vivo* ischemic models were used throughout the experiments. Retinal ischemia was performed following the method as described previously [13,17]. Briefly, mice were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) and pupils were fully dilated with 1% atropine sulfate drops (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 (BSS PLUS dilution buffer, Alcon, Fort Worth, TX, USA). Retinal ischemia was induced by elevating the IOP to generate a hydrostatic pressure of 130 mm Hg for 45 min by lifting the container. Following 45 min after retinal ischemic stress, the needle was withdrawn and 0.3% ofloxacin (Santen Pharmaceutical Co. Ltd., Osaka, Japan) was applied topically into the eye to avoid infection.

Another ischemic model is a transient middle cerebral artery occlusion (tMCAO) model, which was induced following the method as described previously [16]. Briefly, mice were anesthetized by 2% isoflurane (Mylan, Tokyo, Japan), and body temperature was monitored and maintained at 37 °C during surgery. After a midline neck incision, the middle cerebral artery was occluded transiently using 8-0 in size monofilament nylon surgical suture (Natsume Co. Ltd., Tokyo, Japan) coated with silicon (Xantopren, Bayer dental, Osaka, Japan) that was inserted through the left common carotid artery and advanced into the left internal carotid artery. Following 1 h tMCAO, the animals were briefly re-anesthetized with isoflurane and the monofilament was withdrawn for reperfusion studies. As the silicon-coated nylon suture also plugs the branch from middle cerebral artery to supply blood to hippocampus in mice, due to small brain size, the ischemia-induced brain damages are also observed in the hippocampus. Cerebral blood flow was monitored by laser Doppler flowmeter (ALF21, Advance Co., Tokyo, Japan) using a probe (diameter 0.5 mm) of a laser Doppler flowmeter (ALF2100, Advance Co., Tokyo, Japan) inserted into the left striatum (anterior: −0.5 mm, lateral: 1.8 mm from Bregma; depth: 4.2 mm from the skull surface) through a guide cannula.

2.5. Electroretinogram

Electroretinogram (ERG) study was performed following the protocol as previously described [13,17]. Briefly, mice were dark-adapted for 3–4 h, then anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg) and pupils were dilated with 1% atropine. A contact electrode (KE-S, Kyoto contact lenses, Kyoto, Japan) was placed topically on the corneal apex and reference electrode was placed near the ipsilateral 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 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). ERG was performed at day 7 after retinal ischemia.

2.6. Tissue processing

All *in vivo* experiments were performed using retinal and brain tissues. For retinal tissue preparation, mice were deeply anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Eye was quickly isolated, washed with saline and 4% paraformaldehyde (PFA). Eye was then nicked through pupil, post-fixed in 4% PFA for 24 h and finally transferred to 25% sucrose solution (in 0.1 M K^+-free PBS) overnight for cryoprotection. Following freeze in cryoembedding compound, retinal sections were prepared at 10 μm thickness for staining. For brain tissue preparation, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 0.1 M K^+-free PBS, followed by 4% PFA. Brain was then quickly removed, post-fixed in 4% PFA and transferred immediately to 25% sucrose solution overnight. Brain was frozen in cryoembedding compound and coronal sections were cut at 30 μm thickness for staining.

2.7. Morphological assessment of retinal damages

For hematoxylin and eosin (H&E) staining, on the other hand, frozen retinal sections were washed with 0.1 M K^+-free PBS, immerged in Mayer's hematoxylin solution (WAKO, Osaka, Japan) for 5 min at room temperature (25 °C) and then washed with tap