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Short communication

Neuroprotective DAMPs member prothymosin alpha has additional beneficial actions against cerebral ischemia-induced vascular damages

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ABSTRACT

Prothymosin alpha (ProTα) suppresses stress-induced necrosis of cultured cortical neurons. As neuroprotection alone could not explain the long-lasting protective actions against cerebral ischemia by ProTα, we further examined whether ProTα, in addition to neuroprotective effects, has other anti-ischemic activities. When recombinant mouse ProTα (rmProTα) at 0.3 mg/kg was intravenously (i.v.) given 2 h after the start of tMCAO, all mice survived for more than 14 days. In evaluation of CD31- and tomato lectin-labeling as well as IgG and Evans blue leakage, rmProTα treatment (0.1 mg/kg) largely blocked ischemia-induced vascular damages. Therefore, rmProTα has novel beneficial effects against ischemia-induced brain damage through vascular mechanisms.

Nuclear protein prothymosin alpha (ProTα) is extracellularly released from neuronal cells upon ischemic or starving stress and protects neurons in an autocrinic or paracrinic manner (1–4). Accordingly, ProTα is supposed to possess a nature of damage-associated molecular patterns (DAMPs), but it has robustness actions to keep the cellular survival against different aspects of stress (3–5). ProTα has been identified as an anti-necrosis factor from the conditioned medium of serum- and supplement-free primary culture of rat embryonic cortex (1,3,6). In the culture of cortical neurons, one of underlying molecular mechanisms was evidenced by ProTα-mediated externalization of glucose transporter 1 and 4, which had been endocytosed by ischemic or starving stress (1–3). This mechanism prevents the necrotic energy crisis due to cellular ATP loss (1,2). Additional anti-necrotic mechanism by ProTα is related to the activation of caspase 3, which cleaves poly (ADP-ribose) polymerase, resulting in the suppression of necrotic cell death by inhibiting a rapid decrease in mitochondrial ATP production (3,7,8). However, ProTα remarkably protects the ischemic brain (9). Detailed analyses revealed that ProTα *in vivo* inhibits not only necrosis, but also apoptosis by a help of endogenous brain-derived neurotrophic factor or erythropoietin (2,3,10). Similar mechanisms of necrosis inhibition and neurotrophic factor-mediated apoptosis inhibition were also observed in retinal ischemia–reperfusion model (11). Furthermore, *in vivo* protective roles of ProTα were evidenced in experiments using treatments with neutralizing ProTα antibody or ProTα antisense oligonucleotide (2,11).

In cerebral ischemia or stroke, the blockade of neuronal death would be the most important endpoint for medicinal treatments. However, neuroprotection alone is not enough for successful treatments, since neuroinflammation due to microvascular occlusion may persistently causes neuronal damages (12). As systemic administration of ProTα remarkably protects the ischemic brain for longer periods (9), but neuroprotective function alone could not explain the long-lasting protective actions against cerebral ischemia by ProTα, we hypothesize that ProTα might have additional beneficial effects against cerebral ischemia. In the present study, we attempted to examine the effects of ProTα on vascular damages by cerebral ischemia.

Male C57BL/6J 6-week-old mice weighing 20–24 g were purchased from TEXAM Corporation (Nagasaki, Japan) and used for all experiments. Mice were kept in a room maintained at temperature of 21 ± 2 °C and relative humidity of 55 ± 5% with a 12–h light/dark

Abbreviations: CBB, Coomassie Brilliant Blue; IgG, immunoglobulin G; i.p., intraperitoneal; i.v., intravenous; ProTα, prothymosin alpha; TEV, tobacco etch virus; tMCAO, transient middle cerebral artery occlusion.

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cycle. Mice had free access to standard laboratory diet and tap water. All experiments were approved by the Nagasaki University Animal Care Committee (Nagasaki, Japan).

Mouse recombinant ProTα (rmProTα), V8 serine protease (V8)-digested rmProTα and V8 were diluted in phosphate-buffered saline (PBS). Mice were intravenously (i.v.) treated with rmProTα (0.03, 0.1, or 0.3 mg/kg), V8-digested rmProTα (0.1 or 0.3 mg/kg), V8 (2.5 or 7.5 μg/kg) or PBS (vehicle) 1, 2, 3, or 4 h after the start of cerebral ischemia induced by transient middle cerebral artery occlusion (1 h tMCAO). The details of tMCAO, ProTα preparation and its proteolysis by V8 protease, western blotting, tissue preparation, histological analysis, labeling of blood vessels with *Lycopersicon esculentum* (tomato) lectin and cluster of differentiation 31 (CD31), detection of endogenous immunoglobulin G (IgG) leakage, Evans blue extravasation, and behavioral assessments were provided in Supplementary materials and methods.

Statistical analyses were performed using Tukey–Kramer multiple comparison *post-hoc* analysis after a one-way analysis of variance (ANOVA). Survival ratio was analyzed by Log-rank test after Kaplan–Meier survival plots using GraphPad Prism 6 software. Results are shown as means ± standard error of the mean (S.E.M). $p < 0.05$ was considered significant.

As shown in Supplementary Fig. 1, rmProTα was prepared by sequential methodological steps including PCR amplification of tobacco etch virus (TEV)-ProTα, insertion of TEV-ProTα into the pGEX4T1 expression vector, transformation of pGEX4T1-TEV-ProTα into BL21 *Escherichia coli* (*E. coli*), lysis of BL21 *E. coli* cells, and purification of rmProTα through glutathione sepharose column, Mono Q ion-exchange column and Centriprep YM-3 filter. Following removal of endotoxin contamination by EndTrap affinity system, rmProTα was finally dialyzed against PBS. We obtained 0.9 mg rmProTα in 3 ml of PBS from 4 l of Luria broth culture medium as the initial volume. The endotoxin level in purified rmProTα was 0.09 EU/ml (0.9 EU/kg). When sample fractions at different purification steps were run on sodium dodecyl sulphate (SDS)-polyacrylamide gel, a band for rmProTα (12.5 kDa) was detected by staining the SDS-polyacrylamide gel with Coomassie Brilliant Blue (CBB) (Fig. 1A). A band for rmProTα was disappeared in CBB-stained SDS-polyacrylamide gel when rmProTα was digested with V8 (Fig. 1B). The purity of rmProTα was also confirmed by western blotting using antibodies against N- and C-terminal sequences in ProTα (Fig. 1C and 1D, respectively).

To determine the better condition for rmProTα administration against cerebral ischemia, mice were treated with rmProTα (0.3 mg/kg, i.v.) 1, 2, 3, or 4 h after the start of tMCAO. Vehicle (PBS)-treated mice died within 7 days after tMCAO (Fig. 2A). When rmProTα was given 2 h after the start of tMCAO, all mice survived (significant, $p < 0.01$, Log-rank test) for more than 14 days (Fig. 2A). Significant effects in survival were also obtained by rmProTα administration at 1 h (significant, $p < 0.01$), but not at 3 or 4 h (not significant, $p > 0.05$) after the start of tMCAO (Fig. 2A). Previous studies reported that tMCAO-type cerebral ischemia caused motor dysfunction, which was evaluated by increase in neurological scores (13,14). We also observed that neurological scores were significantly increased 4 and 7 days after the start of tMCAO, whereas administration of rmProTα 2 h after the start of tMCAO markedly decreased neurological scores 4 and 7 days after tMCAO (Fig. 2B). Neurological scores were also significantly decreased 7 days after tMCAO when mice were treated with rmProTα 1 h, but not 3 or 4 h after the start of tMCAO (Fig. 2B). ProTα-caused increase in survival ratio and decrease in neurological scores were abolished in mice treated with V8-digested rmProTα (0.3 mg/kg, i.v.) 2 h after the start of tMCAO (Fig. 2C and 2D, respectively).

To examine the dose-dependent protective activity of rmProTα against cerebral ischemia, mice were treated (i.v.) with 0.03, 0.1, or 0.3 mg/kg rmProTα 2 h after the start of tMCAO. Administration of rmProTα at a dose of 0.3 mg/kg markedly reversed tMCAO-induced increase in neurological scores 4 and 7 days after tMCAO (Fig. 2E). Neurological scores were also significantly decreased 7 days after tMCAO when mice were treated with 0.1 mg/kg rmProTα, but not with 0.03 mg/kg (Fig. 2E). Administration of V8-digested rmProTα (0.3 mg/kg, i.v.) abolished ProTα-caused decrease in neurological scores 4 and 7 days after tMCAO (Fig. 2E). However, V8 alone did not show any effects against tMCAO-induced decrease in survival ratio (Fig. 2C) and increase in neurological scores (Fig. 2D, E).

To confirm whether rmProTα treatment 2 h after the start of tMCAO protects the brain from ischemic damages, histological analysis using Nissl staining of brain sections was performed 24 h after tMCAO. We observed that the ischemic core was clearly demarcated from the surrounding brain region (penumbra), and Nissl-stained neuronal cells were shrunken in the regions of ipsilateral striatum and somatosensory cortex 24 h after tMCAO in vehicle (PBS)-treated tMCAO mice (Supplementary Fig. 2A), consistent with previous studies with brain ischemia (15,16). In contrast, administration (i.v.) of 0.3 mg/kg rmProTα 2 h after the start of tMCAO partially, but significantly reduced infarct volume from 49.9 ± 2.2 to 35.6 ± 4.8% (n = 3) and inhibited shrunken of neuronal cells 24 h after tMCAO, as shown in Supplementary Fig. 2A and 2B.

Next, we examined whether ProTα has protective actions against ischemia-induced vascular damages. Mice were treated (i.v.) with rmProTα (0.1 mg/kg), or V8-digested rmProTα (0.1 mg/kg) 2 h after the start of tMCAO, and coronal brain sections (+0.98 mm from Bregma) were labeled with CD31 and tomato lectin, which are considered as endothelial markers of blood vessels (17,18) as described in Supplementary materials and methods. The damage of blood vessels in terms of disrupted labeling of CD31 and tomato lectin was observed in the region of somatosensory cortex 24 h after tMCAO (Fig. 3A and Supplementary Fig. 3A, B). Moreover, the length of blood vessels was significantly decreased 24 h after tMCAO (Fig. 3B). Administration of rmProTα significantly inhibited ischemia-induced damage of blood vessels 24 h after tMCAO (Fig. 3A, B and Supplementary Fig. 3A, B). ProTα-caused inhibition of cerebral vascular damages was abolished when mice were treated with V8-digested rmProTα 2 h after the start of tMCAO (Fig. 3A, B and Supplementary Fig. 3A, B).

Several recent studies reported that cerebral ischemia caused vascular disruption/permeability in the evaluation of endogenous immunoglobulin G (IgG) or Evans blue leakage in the brain of mice and rats (19–23). Based on previous reports and our present findings using CD31 and tomato lectin labeling, we further conducted quantitative analysis of endogenous IgG and Evans blue leakage following tMCAO to explore whether ProTα blocks cerebral ischemia-induced vascular disruption/permeability. Mice were treated (i.v.) with rmProTα (0.1 mg/kg), V8-digested rmProTα (0.1 mg/kg), or V8 alone 2 h after the start of tMCAO, and endogenous IgG or Evans blue leakage was assessed 24 h after tMCAO. Immunostaining revealed that IgG was markedly leaked after tMCAO, whereas administration of rmProTα significantly reduced the leakage of IgG (Fig. 3C, D). ProTα-caused inhibition of IgG leakage following tMCAO was abolished when mice were treated with V8-digested rmProTα (Fig. 3C, D). Similarly, administration of rmProTα significantly reduced tMCAO-induced leakage of Evans blue 24 h after tMCAO (Fig. 3C, E). Administration of V8-digested rmProTα abolished ProTα-caused reduction of Evans blue leakage 24 h after tMCAO (Fig. 3C, E). However, V8 alone had no effects on tMCAO-induced leakage of IgG or Evans blue 24 h after tMCAO (Fig. 3C–E).

Recombinant mouse ProTα (rmProTα) at 0.3 mg/kg (i.v.) showed potent survival effects in mice with tMCAO (1 h)-type cerebral