followed by P_{30} administration ($n = 7$), sectioned coronally with a 1-mm thickness and washed with K^+-free PBS. Brain slices were incubated in 2% TTC (Sigma-Aldrich) in 0.9% NaCl in dark place for 15–20 min at 25°C and transferred in 4% PFA overnight. Images of brain slices were then collected by scanner, and infarct volume was calculated by Image J software (NIH, Bethesda, MD, USA).

Behavioral assessments

Following P_{30} administration with doses of 0.03 and 3 nmol/5 μL (i.c.v., $n = 6$ and $n = 7$, respectively), 0.3 and 1 mg/kg (i.v., $n = 5$ and $n = 7$, respectively) at 1 h as well as 1 mg/kg (i.v.) at 3 and 6 h ($n = 5$ and $n = 7$, respectively) after cerebral ischemia (1 h tMCAO), behavioral studies were assessed through 14 days. Clinical score was evaluated from day 1 after ischemia in the following way: 0, no observable deficits; 1, failure to extend the forepaw fully; 2, circling; 3, falling to one side; 4, no spontaneous movement; 5, death. In this study, 0.5 point was added to each score when the motor dysfunction was severe for scores between 1 and 4. Survival rate was evaluated from day 1 after tMCAO and calculated by the percentage of vehicle or P_{30} post-treated mice that were alive through 14 days after ischemia.

Statistical analysis

All results are shown as means ± SEM. Two independent groups were compared using the Student's *t*-test. Multiple groups were compared using Dunnett's multiple comparison test after a one-factor ANOVA or a repeated measure ANOVA. Survival rate was compared using Logrank test after Kaplan–Meyer method. $p < 0.05$ was considered significant.

Results

Characterization of functionally active core peptide in ProTα

The functionally active core domain in rat recombinant ProTα was determined by measuring the survival activity of cultured cortical neurons in the presence of different deletion mutants of GST-fusion ProTα at 12 h after the ischemic (serum-free) stress (Fig. 1a–c). The findings revealed that the N-terminal deletion mutants ProTα (Δ1–29 and Δ1–48) as well as C-terminal deletion mutants ProTα (Δ79–112 and Δ102–112) elicit its protective effect as like as full-length ProTα against ischemic stress-induced cultured neuronal damages (Fig. 1b, c). However, the deletion mutants of ProTα devoid of central peptide sequence comprised of 30 amino acids (P_{30}: a.a. 49–78) abolished its neuroprotective activity against the ischemic stress (Fig. 1b, c). Interestingly, the core peptide sequence P_{30} (a.a. 49–78) itself exerted the full survival effect in cultured neurons against ischemic damages, an indication of neuroprotective characteristics of ProTα-derived peptide P_{30} (Fig. 1b, c).

Blockade of retinal ischemia-induced damages by ProTα-derived peptides

We reported previously that ProTα inhibits the retinal ischemia-induced functional and cellular damages (Fujita *et al.* 2009). To evaluate whether ProTα-derived peptide has protective activity against ischemic damages *in vivo*, P_{30} was injected (i.vt.) with doses of 1, 3 and 10 pmol/μL in the

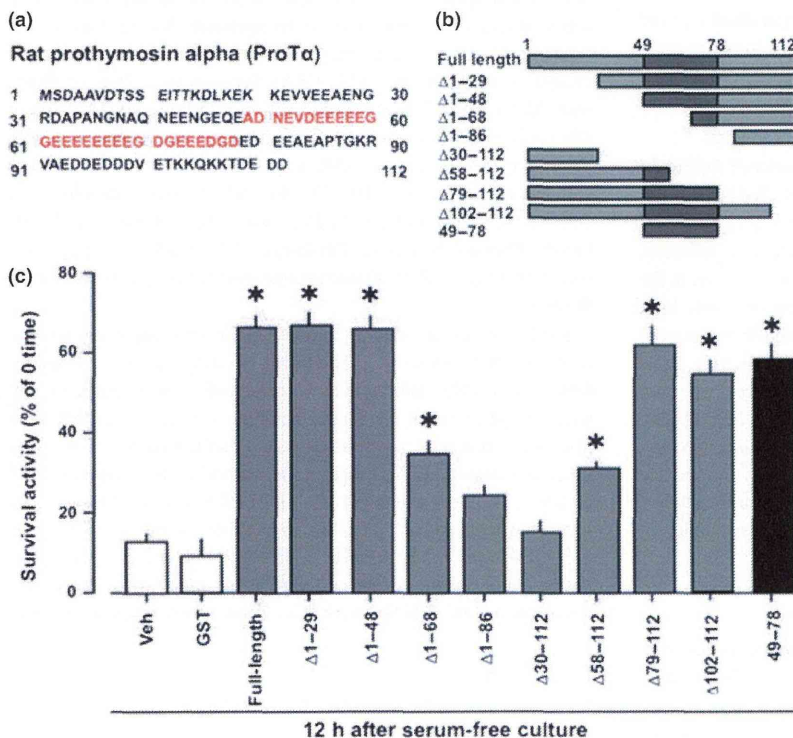

Fig. 1 The central core peptide sequence of prothymosin alpha (ProTα) is an essential domain for survival activity of ProTα against serum-free ischemic stress. (a) The amino acid sequence of rat ProTα. Red colored sequence indicates functional core domain (a.a. 49–78 referred as P_{30}) in ProTα. (b) Schematic drawings of GST-fusion of full length ProTα and its deletion mutants. (c) Identification of essential domain of ProTα for its survival activity. The primary cultured cortical neurons were incubated with GST and GST-fusion ProTα mutants under the serum-free condition. The survival activity was measured at 12 h after the start of culture. Data represent the means ± SEM. (*$p < 0.01$, vs. Veh).

ipsilateral eye at 24 h after retinal ischemia. The hematoxylin and eosin (H&E) staining data showed that the number of cells in different retinal layers as well as the retinal thickness is significantly decreased in the vehicle-treated mice at day 7 after the ischemic stress, whereas 10 pmol P_{30} maximally and significantly inhibited this cellular loss in retina and decrease in retinal thickness at day 7 (Fig. 2a, b).

In electroretinogram (ERG), the amplitude called a-wave represents the functional activity of photoreceptor cells, whereas b-wave indicates the functions of mixture of cells including bipolar, Muller, amacrine, and ganglion cells (Asi and Perlman 1992; Fujita *et al.* 2009). Following after retinal ischemia and reperfusion, the ERGs analysis showed that a- and b-wave amplitudes are significantly decreased in the vehicle-treated mice at day 7 after retinal ischemia, compared with the control (Fig. 2c, d). Following P_{30} treatment, dose-dependent increase in a- and b-wave amplitudes were observed at day 7 after the retinal ischemic stress, and 10 pmol P_{30} exerted its maximum protective effect against the ischemic damages (Fig. 2c, d). On the other hand, no significant protective effect of thymosin alpha 1 (a.a. 2–29) corresponding to N-terminal sequence of ProTα and the C-terminal peptide (a.a. 102–112) against retinal ischemic damages were observed at day 7 after ischemia (data are not shown).

P_{30}-induced cell type-specific survival against retinal ischemic damages

To examine the cell type-specific protective activity of ProTα-derived peptide in ischemic retina, P_{30} was injected (10 pmol/μL, i.vt.) in the ipsilateral eye at 24 h after retinal ischemia. The immunohistochemical analysis showed that NeuN-positive neurons (Buckingham *et al.* 2008) in the ganglion cell layer (GCL) are significantly diminished at day 7 after retinal ischemic stress, compared to the control (Fig. 3a). Following P_{30} treatment at 24 h after retinal

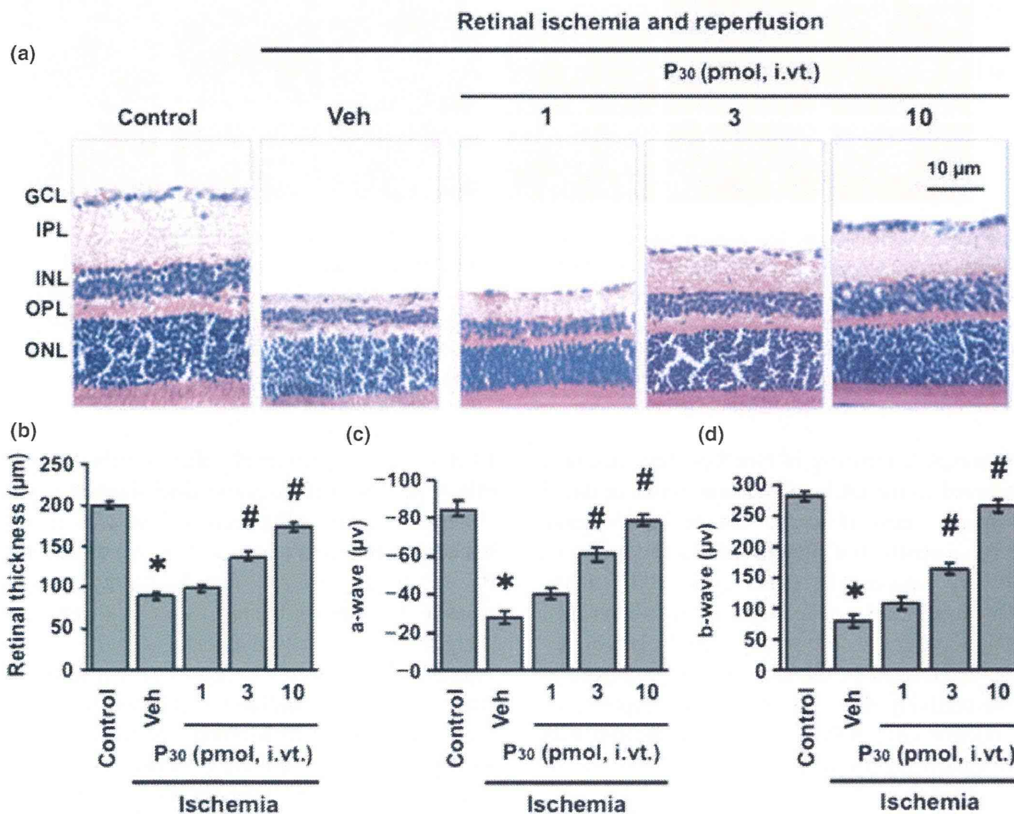

Fig. 2 Prothymosin alpha (ProTα)-derived peptide protects the retinal ischemia-induced functional damages. P_{30} is injected intravitreously (i.vt.) at the doses of 1, 3, and 10 pmol/μL in the ipsilateral eye at 24 h after retinal ischemia. Vehicle is treated with 0.05% dimethyl sulfoxide (DMSO) in a similar manner. (a–d) Protective activity of P_{30} is a dose-dependent manner. Following P_{30} injection at 24 h after retinal ischemia, (a) hematoxylin and eosin (H&E) staining of retinal section is performed at day 7 (right panel). (b–d) Measurement of retinal thickness (b) as well as the a-wave (c) and b-wave (d) amplitudes of ERG analysis are done at day 7 after retinal ischemia in P_{30} post-treated mice. Data are mean ± SEM. (*$p < 0.05$, vs. Control, $^{\#}p < 0.05$, vs. Veh) from experiments using five to seven mice for each group.

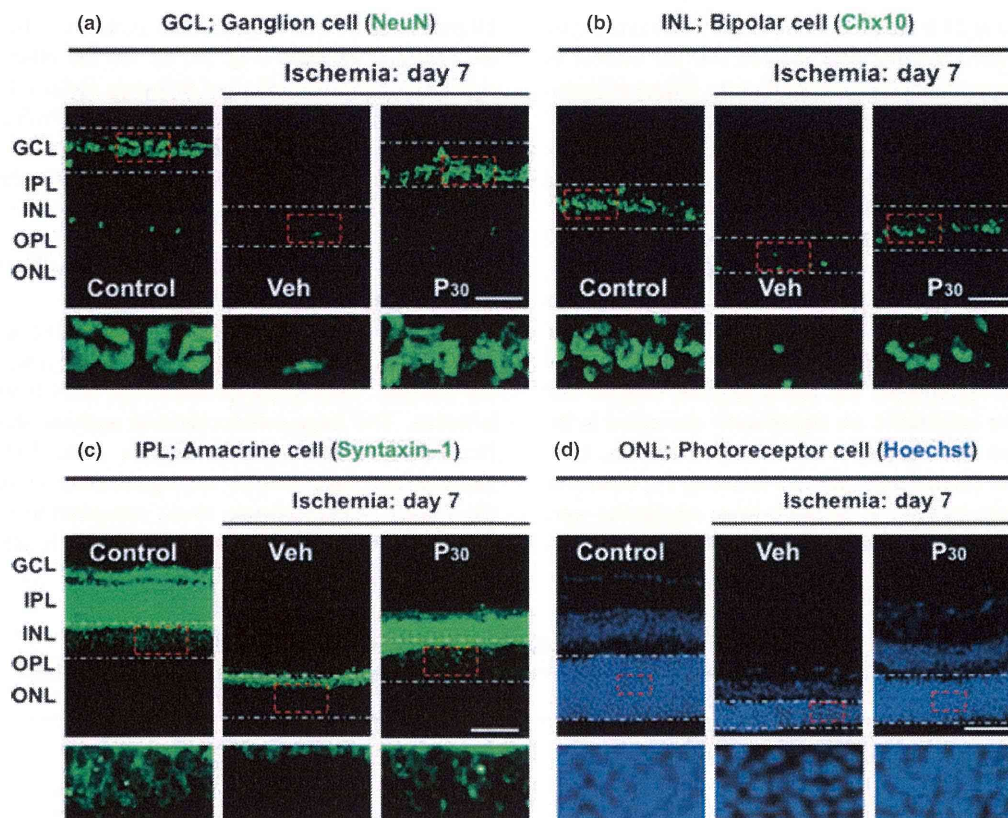

Fig. 3 Cell type-specific protection by P_{30} against retinal ischemic damages. P_{30} is injected (10 pmol/μL; i.vt.) in the ipsilateral eye at 24 h after retinal ischemic stress, and the immnunohistochemical analysis of retinal sections is performed at day 7. (a–d) The staining of ganglion cells (a) in the ganglion cell layer (GCL) (NeuN: green), bipolar cells (b) in the inner nuclear layer (INL) (Chx10: green), amacrine cells (c) (syntaxin-1: green), photoreceptor cells (d) in the outer nuclear layer (ONL) (Hoechst: blue) are done at day 7 after retinal ischemia. The higher magnification views of lower panels in (a–d) indicate the expression of retinal cell types noted by dotted rectangles (respective upper panels). Scale bars: 10 μm. Experiments were performed using five to eight mice for each group.

ischemia, the complete recovery of NeuN-positive neuronal cells was observed in the GCL of ischemic retina at day 7 after the ischemic stress (Fig. 3a). On the other hand, treatment of P_{30} partially, but significantly blocked the loss of Chx10-positive bipolar cells (Rhee *et al.* 2007) in the inner nuclear layer (INL) (Fig. 3b), syntaxin-1-positive amacrine cells (Sherry *et al.* 2006), of which the cell bodies and processes are located in the INL and inner plexiform layer (IPL), respectively (Fig. 3c), and photo-receptor cells in the outer nuclear layer (ONL) (Fig. 3d), compared with the respective controls and vehicles.

Inhibition of cerebral ischemia-induced brain damages by P_{30}

To evaluate the protective activity of P_{30} against ischemic brain damages, mice were post-treated with P_{30} in time- and dose-dependent manner following different routes of administration, and subsequent 2,3,5-triphenyl tetrazolium chloride (TTC) staining at 24 h and behavioral assessments through 14 days were performed after cerebral ischemia (1 h tMCAO). The TTC staining data showed that the infarct volume is significantly decreased at 24 h in the ischemic brain by intracerebroventicular (i.c.v.) injection of 3 nmol P_{30} at 1 h after tMCAO (Fig. 4a, b), but not by 0.03 nmol (data are not shown). We also observed that the clinical score is significantly decreased at day 1 after 1 h tMCAO in mice injected with 3 nmol P_{30} (i.c.v.) at 1 h after the ischemic stress (Fig. 4c). In addition, significant decrease in clinical score and increase in survival rate were observed through 14 days after i.c.v. delivery (1 h after ischemia) of 3 nmol P_{30}, an indication of long-lasting protective effect of P_{30} against ischemic brain damages (Fig. 4d, e).

On the other hand, P_{30} was injected intravenously (i.v.) with doses of 0.3 and 1 mg/kg at 1 h after cerebral ischemia (1 h tMCAO). Our TTC staining data revealed that the infarct volume is significantly decreased at 24 h in the ischemic brain treated with 1 mg/kg of P_{30} treatment at 1 h after the ischemic stress (Fig. 4f, g). Following

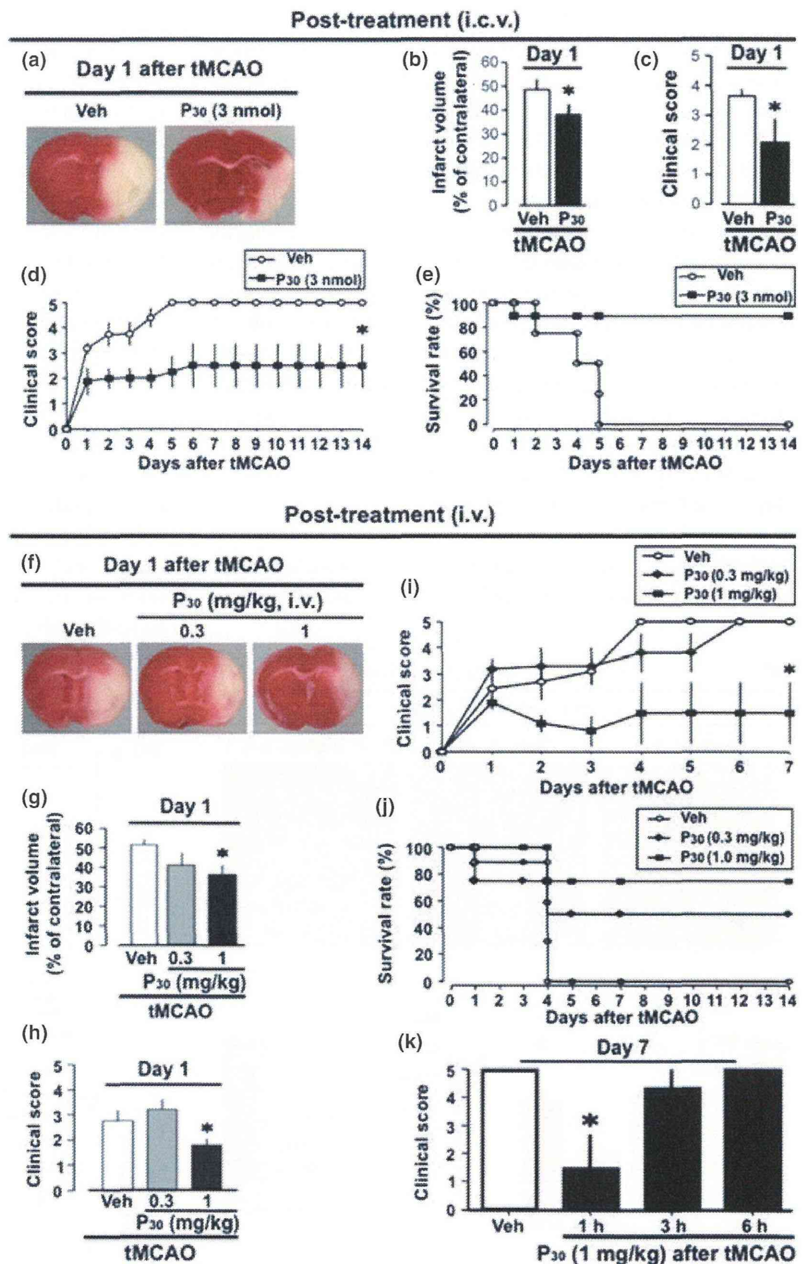

Fig. 4 P_{30} inhibits cerebral ischemia-induced brain damages. (a–e) Intracerebroventricular (i.c.v.) delivery with P_{30} protects ischemic brain damages. P_{30} is injected (3 nmol, i.c.v.) in the brain at 1 h after the cerebral ischemia [1 h transient middle cerebral artery occlusion (tMCAO)], (a–c) TTC staining (a), measurement of infarct volume (b), and clinical scores (c) are performed at day 1 after tMCAO. Data represent the means ± SEM. (*$p < 0.05$, vs. Veh). (d, e) Assessment of the clinical score (d) and survival rate (e) are done through 14 days after the tMCAO mice post-treated with P_{30}. The group of P_{30} treatment was significant compared to group of Veh treatment (*$p < 0.01$, vs. Veh). Survival rate of P_{30} treatment tended to be significant compared to Veh treatment. Experiments were performed using five to eight mice for each group. (f–k) Blockade of cerebral ischemia-induced brain damages by systemic administration of P_{30}. P_{30} is delivered intravenously (i.v.) with doses of 0.3 and 1 mg/kg at 1 h after cerebral ischemia (1 h tMCAO). (f–h) TTC staining (f), measurement of infarct volume (g) and clinical scores (h) are performed at day 1 after tMCAO. (i, j) The clinical score (i) and survival rate (j) are measured for 7 and 14 days, respectively. The group of P_{30} treatment (1 mg/kg) was significant compared to group of Veh treatment in clinical score (*$p < 0.01$, vs. Veh). Survival rate of P_{30} treatment tended to be significant compared to Veh treatment. (k) Time-course systemic injection of P_{30} (1 mg/kg, i.v.) at 1, 3, and 6 h after cerebral ischemia (1 h tMCAO). Data represent the means ± SEM. (*$p < 0.05$, vs. Veh). Experiments are performed using six to eight mice for each group.

post-treatment (i.v.) with 1 mg/kg of P_{30} at the same time point, the clinical score was significantly declined through 7 days and survival rate was maximally increased through 14 days after tMCAO, compared with the vehicle and ischemic mice treated with 0.3 mg/kg of P_{30} (Fig. 4h–j). The behavioral study also confirmed that systemic (i.v.) P_{30} delivery with the dose of 1 mg/kg at 1 h after ischemia induces it maximum protective effect at day 7 against the ischemic brain damages, compared to P_{30} treatment at 3 or 6 h after cerebral ischemia (Fig. 4k).

P_{30} inhibits the cerebral ischemia-induced blood vessel damages

In the ischemic stroke and cerebrovascular disease, vascular defect is occurred along with neuronal damages (Paul *et al.* 2001; Fujita and Ueda 2007). To investigate whether P_{30} protects the ischemia-induced blood vessels damages, P_{30} was injected (1 mg/kg; i.v.) at 1 h after cerebral ischemia (1 h tMCAO). Following blood vessel immunostaining using biotinylated tomato lectin and Alexa Fluor 488 streptavidin at 24 h after ischemia, the findings revealed that the number

blood vessels are markedly decreased in somatosensory cortex in the brain of vehicle-treated mice, compared with the control (Fig. 5a, b). In addition, the decrease in lengths of the blood vessels was observed at 24 h after tMCAO (Fig. 5a, c). This ischemia-induced loss of tomato lectin-stained blood vessels in terms of number and lengths was completely recovered in the somatosensory cortex at 24 h after the ischemic stress in mice post-treated with P_{30}, but the lengths were relatively larger than the vessels in the control brain, an indication of the protective role of P_{30} against ischemia-induced blood vessel damages (Fig. 5a–c). Similar results of the recovery of cerebral ischemia-induced blood vessels damages by P_{30} were observed in the striatum and hippocampus at 24 h after 1 h tMCAO (data are not shown).

P_{30} ameliorates the ischemic brain caused by photochemically induced thrombosis

It is well known that ischemic model because of middle cerebral artery (MCA) occlusion with photochemically induced thrombosis (PIT) is analogous to clinical condition (Tanaka *et al.* 2007). In this ischemic mouse model, there was a significant behavioral damage evaluated by clinical score (Fig. 6a). This damage was significantly attenuated by systemic post-treatment with P_{30} (1 mg/kg, i.v.) at 1 h after PIT (Fig. 6a). Following behavioral study after PIT stress, neurological assessments using TTC staining were performed at 24 h. The TTC staining data revealed that there was a marked increase in cerebral infarction observed at 24 h after PIT in vehicle-treated mice (Fig. 6b), but this cerebral brain damage in terms of infarct volume and hemisphere expansion was significantly inhibited by systemic treatment of P_{30} (Fig. 6c, d).

Discussion

This study demonstrates three major findings. First, active core peptide domain P_{30} (a.a. 49–78) derived from ProTα retains the original survival activity in cultured neuronal cells against ischemic (serum-free) stress. Second, characterizations of P_{30} actions reveal that it potently inhibits the ischemia-induced damages in retina and brain. Third, P_{30}

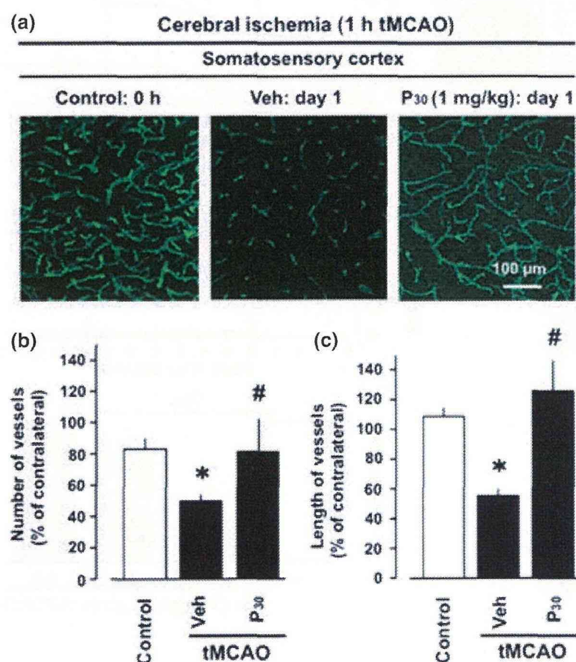

Fig. 5 P_{30} inhibits the cerebral ischemia-induced blood vessels damages. (a–c) P_{30} is injected (1 mg/kg, i.v.) at 1 h after cerebral ischemia [1 h transient middle cerebral artery occlusion (tMCAO)]. Following administration with biotinylated tomato lectin (1 mg/mL, 100 μL, i.v.) at 24 h after cerebral ischemia (1 h tMCAO), and subsequent perfusion 5 min after biotinylated tomato lectin injection, immunostaining of blood vessels by Alexa Fluor 488 streptavidin (a) as well as measurement of number (b) and length (c) of blood vessels is performed at day 1 after the ischemic stress. Data represent the means ± SEM. (* $p < 0.05$, vs. Control, # $p < 0.05$, vs. Veh) from experiments using five to seven mice for each group.

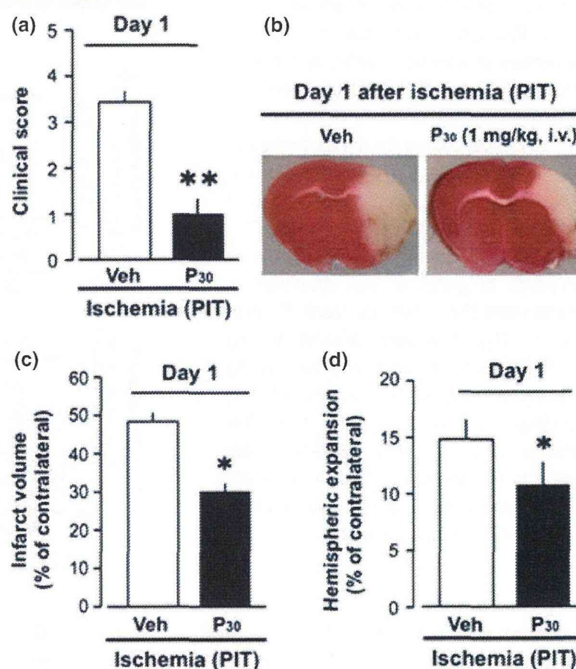

Fig. 6 P_{30} improves the ischemic brain damages caused by photochemically induced thrombosis. P_{30} is administered (1 mg/kg, i.v.) at 1 h after photochemically induced thrombosis (PIT) in mice. (a) Clinical scores at day 1 after ischemia. (b) Representative picture of TTC staining at 24 h after PIT. (c, d) Measurement of infarct volume (c) and hemispheric expansion (d) at 24 h after PIT. Data represent the means ± SEM. (* $p < 0.05$, ** $p < 0.01$, vs. Veh) from experiments using five to seven mice for each group.

induces protective action against ischemia-induced disruption of cerebral blood vessels.

Several *in vitro* studies reported about the different sequence-specific functions of ProTα, which is also involved in the mechanisms of cell survival (Jiang *et al.* 2003; Karapetian *et al.* 2005; Skopeliti *et al.* 2007; Ueda *et al.* 2007; Ueda 2009; Mosoian *et al.* 2010; Danielli *et al.* 2012; Dong *et al.* 2012). On the basis of previous information, we firstly designed *in vitro* experiments to find out the sequence-specific neuroprotective actions of ProTα using various deletion mutants of GST-ProTα in neuronal cells culture under ischemic stress. The peptides lacking sequence (a.a. 1–29), which belongs to thymosin alpha 1 (a.a. 2–29), sequence (a.a. 1–48), which mostly covers the binding region for Keap1, or C-terminal sequences (a.a. 79–112 and 102–112) completely retained the original survival activity as like ProTα. However, the significant decrease in survival effect was observed by the deficiency of parts of the central core peptide sequence comprised of 30 amino acids in ProTα (a.a. 49–78). Interestingly, this central active core peptide of ProTα referred as P_{30} (a.a. 49–78) itself exerts full survival action in neuronal cells against ischemia. Retinal ischemia causes the functional and cellular damages in different layers of retina through several destructive cascade of mechanisms, as consequence of visual impairment and blindness (Osborne *et al.* 2004). Our recent *in vivo* studies suggested that ProTα potently inhibits this ischemia-induced functional and cellular damages of retina (Fujita *et al.* 2009; Ueda *et al.* 2010). To evaluate the *in vivo* protective effect of P_{30} against ischemic damages, ischemic retina was post-treated with P_{30}. The findings using H&E staining and ERG study revealed that P_{30} significantly blocks the retinal ischemia-induced decrease in cells number of different layers and retinal thickness. In addition, immunohistochemical analysis clarified that P_{30} completely rescues the retinal ischemia-induced ganglion cell damages, along with the partial but significant blockade of the loss of bipolar, amacrine, and photoreceptor cells. Stroke following cerebral ischemia (tMCAO) or photothrombotic brain ischemia causes the neuronal damages, along with adequate disruption of cerebral blood vessels (Beck and Plate 2009; Hofmeijer and van Putten 2012; Krysl *et al.* 2012). We previously explained the protective role of ProTα against cerebral ischemia-induced brain damages (Fujita and Ueda 2007; Ueda 2009; Ueda *et al.* 2010). The present findings of TTC staining and neurological assessment suggested that intracerebroventicular (3 nmol, i.c.v.) or systemic (1 mg/kg, i.v.) treatment with P_{30} at 1 h after cerebral ischemia (1 h tMCAO) significantly blocks ischemia-induced brain damages. Following immunostaining with tomato lectin in P_{30}-treated (1 mg/kg, i.v.) ischemic mice, the complete recovery of ischemia-induced (tMCAO) cerebral blood vessels damages was observed through day 1, a consideration of P_{30} as a new angiogenic factor. In addition, systemic administration with P_{30} (1 mg/kg, i.v.) significantly ameliorated the ischemic brain caused by photochemically induced thrombosis (PIT), a representative clinical model of cerebral ischemia.

The present investigations were performed following several routes of the administration of P_{30}. According to the fact that retinal ischemia possesses high reproducibility and quantitation to understand the pathophysiological changes and signaling pathways under ischemic condition (Prasad *et al.* 2010), we used this ischemic injury as a simple model for screening of survival activity by i.vt. administration of P_{30}. We already reported that i.v. administration with full-length ProTα induces protective effect against retinal ischemia (Fujita *et al.* 2009). In brain ischemia, we firstly decided to perform i.c.v. administration of P_{30} to evaluate the improvement of ischemic injury, and successfully confirmed against ischemic brain damages. Our recent studies revealed that myc-tagged ProTα (1 mg/kg) is penetrated to the damaged area of brain at least 3 h after brain ischemia by intraperitoneal (i.p.) administration, and that systemic administration (i.p. and i.v.) of ProTα ameliorates brain ischemia-induced functional and cellular damages (Fujita and Ueda 2007). It is well known that brain ischemic stress disrupts the blood–brain barrier (BBB) (Paul *et al.* 2001; Fujita and Ueda 2007). Thus, we presume that like ProTα, systemic administrated P_{30} would penetrate to the damaged brain through the disrupted BBB. Although relationship between route of administration and penetrated amounts of P_{30} to the brain are not clear, isotope and/or fluorescence labeling might be useful method for the calculation of penetration. In the systemic administration, ProTα and P_{30} exercise the maximum improvement effect against brain ischemia in 100 μg/kg (equivalent 8.08 nmoles/kg) and 1 mg/kg (equivalent 0.30 μmoles/kg), respectively. This difference of efficacy between ProTα and P_{30} might be because of the stability of P_{30} *in vivo*, though GST-ProTα and GST-P_{30} (a.a. 49–78) showed similar survival activity in this *in vitro* study. However, the modification of amino acid and/or mutation in sequence of P_{30} may provide a better solution to improve the stability and survival activity of P_{30}. This should be the next issue to address.

Cortical neurons in serum-free primary culture rapidly die by necrosis, which is completely inhibited by ProTα (Fujita and Ueda 2003; Ueda *et al.* 2007). As ProTα also protects the retinal ischemia-induced necrosis and apoptosis through the up-regulation of BDNF and EPO, and this retinal protection is completely abolished by antisense oligodeoxynucleotide or antibody treatment against ProTα (Fujita *et al.* 2009; Ueda *et al.* 2010), it should be an interesting next subject to investigate whether the same mechanisms are involved in the P_{30}-induced functional and cellular protection against ischemic damages. Despite of being neuroprotective activity of several proteins, peptides have been detected as a new class of attractable therapeutic molecule owing to their diversity, synthesis, and higher

capability to penetrate the challenging targets (Archakov *et al.* 2003; Watt 2006; Gozes 2007; Patel *et al.* 2007; Meade *et al.* 2009). Taken together, this study confers a precise demonstration about the broad-spectrum protective activity of ProTα-derived small peptide P_{30} against ischemic damages *in vitro* and *in vivo*. Thus, it is evident that P_{30} mimics the *in vitro* and *in vivo* neuroprotective actions of ProTα. The sequence homology of P_{30} domain in ProTα among all species is highly conserved; furthermore, this sequence is completely equal in human, rat, and mouse. From these facts, it is speculated that P_{30} domain may plays important roles in robustness of ProTα against neuronal damages.

In conclusion, ProTα-derived peptide P_{30} exerted its survival actions in cultured neurons against ischemic stress. P_{30} significantly blocked the ischemia-induced functional and cellular damages in retina as well as in brain, along with inhibition of the cerebral blood vessels disruption. Therefore, detailed mechanisms underlying neuroprotection by ProTα-derived small peptide may provide a novel therapeutic approach for the treatment of ischemic damages in the central nervous system.

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