

APO1 death receptor was not induced, indicating a cell-type specificity for gene transcription and, in agreement with previous results,⁶ p21^{Waf1} was not upregulated. Furthermore, the involvement of apoptotic intrinsic pathway was shown by induction of Noxa (Figure 1d). These results indicate target genes transcriptional selectivity mediated by HIPK2 and the involvement of HIPK2 catalytic domain in this function. Therefore, to confirm the involvement of HIPK2-induced p53Ser46 phosphorylation in the transcription of KILLER/DR5 gene, we overexpressed HIPK2 and K221R vectors in combination with wtp53 and S46A-mutant expression vectors in H1299 p53/null cells. We found that KILLER/DR5 expression levels were upregulated when HIPK2 was coexpressed with wtp53, but not with p5346A mutant (Figure 1e). Similarly, K221R vector coexpressed with wtp53 failed to upregulate both KILLER/DR5 gene and the already known p53AIP1 gene. Finally, we found that HIPK2-induced caspase-3 and -8 activities were strongly impaired by inactivating KILLER/DR5 function using siRNA (Figure 1f). As expected, TUNEL assay revealed reduction of HIPK2-induced apoptosis in the absence of KILLER/DR5 (Supplementary Figure S3a and b). Therefore, we tested the involvement of Noxa (intrinsic pathway) in HIPK2-induced relative caspase-3 and -8 activities and apoptosis. We overexpressed HIPK2 in 2008 cells, following abolishment of endogenous Noxa by siRNA and found a strong reduction of caspase-3 activity, while caspase-8 activity was not affected; in agreement siRNA for Noxa function, reduced HIPK2-induced apoptosis of about 38% (Supplementary Figure S4a and S4b).

Altogether, these data demonstrate, for the first time, that exogenous HIPK2 can induce apoptosis through caspase-8 activation and that HIPK2-induced p53S46 phosphorylation may regulate the expression of KILLER/DR5 gene, involved in caspase-8/extrinsic pathway activation. However, we also show that the mitochondrial intrinsic pathway is induced by HIPK2 through Noxa, likely explaining why inhibition of caspase-8 does not completely abolish HIPK2-induced apoptosis. These are important findings because the initiator caspases, including caspase-8 and -9, appear to have some specificity for different types of upstream signals as well as

preferred downstream substrate procaspases. Moreover, components of cell apoptotic machinery are frequently altered in cancer and disruption of apoptotic pathways may be involved in tumor formation, regression, and chemoresistance. The fact that HIPK2/p53Ser46 participates in both the extrinsic and the intrinsic pathways of caspases activation supports the idea that there are multiple downstream effectors of p53 involved in cell death and strengthen the role of this specific p53Ser46 post-translational modification in selective activation of apoptotic target genes. Thus, agents that induce phosphorylation of p53 at Ser46 will favor the transcriptional repertoire of p53 towards proapoptotic genes, thereby favoring death.

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Prothymosin- α 1 prevents necrosis and apoptosis following stroke

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Dear Editor,

Prothymosin- α 1 (ProT α), a highly acidic nuclear protein of the α -thymosin family, has multiple functions both within and outside of the cell.¹ ProT α plays a cytoprotective role by inhibiting apoptosome formation,² and it has cell proliferative activity as an

extracellular signaling molecule.¹ Recently, we found that ProT α is released upon necrotic stress and protects cells from neuronal death through activation of a putative G_{i/o}-coupled receptor coupled to phospholipase C and protein kinase C β _{II}.³

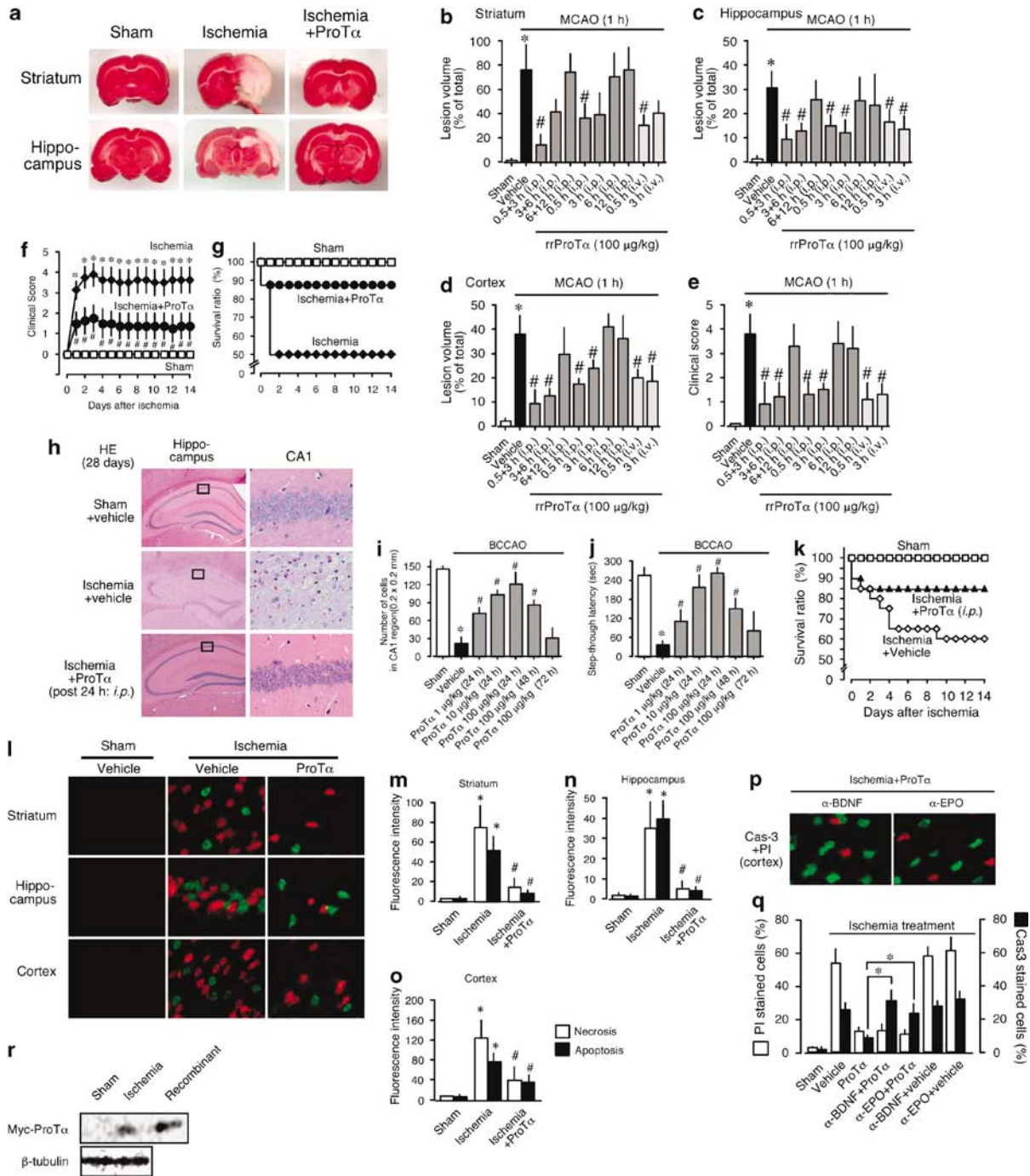


Figure 1 ProTα in brain ischemia. (a) Typical photographs of TTC staining of ischemic brain. Recombinant rat ProTα (rrProTα, 100 μg/kg; i.p.) was given to rats at 30 min and 3 h after MCAO (1 h), and TTC staining was carried out 24 h later. (b–e) ProTα-induced prevention of brain damage (b: striatum, c: hippocampus, d: cortex) and dysfunction (e) due to focal MCAO ischemia reperfusion stress. Results represent data from 5 to 10 animals. **P* < 0.05 versus sham, #*P* < 0.05 versus MCAO + vehicle. (f, g) ProTα-induced prevention of motor dysfunction (f) and lethality (g) due to MCAO stress (8–12 rats in each group). Motor dysfunction was evaluated by clinical score on each indicated day. rrProTα was given at 30 min and 3 h after MCAO. **P* < 0.05 versus sham, #*P* < 0.05 versus ischemia alone. (h) Hematoxylin and eosin (HE) staining of hippocampal sections 28 days after sublethal BCCAO (30 min). Pictures of the whole hippocampus (left panel) and the CA1 region (right panel) are shown. (i, j) ProTα-induced prevention of brain damage (i) and dysfunction (j) by global BCCAO ischemia reperfusion stress. Number of cells in the CA1 region (0.2 × 0.2 mm; square region in Figure 1h, left panel). Results represent data from 5 to 10 animals. **P* < 0.05 versus sham, #*P* < 0.05 versus BCCAO + vehicle. (k) Time course of the survival profile of mice given BCCAO with or without 24 h post-treatment with recombinant mouse ProTα (rmProTα, 100 μg/kg; i.p.). (l) ProTα-induced prevention of necrosis (propidium iodide, red) and apoptosis (activated caspase 3, green) 24 h after MCAO. (m–o) Quantitation of each cell death mode in each brain region (0.4 × 0.4 mm) was performed. **P* < 0.05 versus sham, #*P* < 0.05 versus ischemia alone. (p, q) Reversal of ProTα-induced suppression of apoptosis by α-BDNF or α-EPO IgG. α-BDNF or α-EPO IgG (1 μg each) was given into the subarachnoid space through a parietal bone, 30 min before MCAO stress. Results represent the incidence of activated caspase 3 (green) and PI staining (red). **P* < 0.05. (r) Evidence for Myc-tagged ProTα transport through the blood–brain barrier after ischemic stress. Myc-tagged ProTα at 1 mg/kg (i.p.) was given to rats 30 min after reperfusion, and proteins (30 μg) from the cortex isolated 2.5 h later were used for Western blot analysis

Following stroke or traumatic damages, both necrotic and apoptotic neuronal death cause loss of functions including memory, sensory perception and motor skills.^{4,5} Necrosis occurs during the early stages of stroke and of the affected area expands with time,⁶ whereas the apoptosis occurs with some delay, but may also have a potential role in restricting the spread of irretrievable damage.^{6,7} There have been many attempts to find compounds that inhibit apoptosis, but the protective potencies of these compounds against ischemic damage *in vivo* remain limited.^{8–11} This may be related to the possibility that rapid and expanding necrosis largely contributes to the total loss of brain neurons following ischemia. Thus, rapid treatment of stroke is currently emphasized.^{11–13} Compared with the machinery involved in apoptosis, necrosis is a more passive process, in which energy failure leads to a rupture of the plasma membrane with concomitant loss of intracellular proteins and ions.⁶ However, no compounds that inhibit necrosis are known. Here, we demonstrate that ProT α has potential therapeutic utility against the necrosis component of stroke.

In the focal ischemic model of middle cerebral artery occlusion (MCAO, 1 h) followed by reperfusion in the rat, a marked loss of triphenyltetrazolium chloride (TTC) staining, which detects the presence of mitochondrial enzymes, was observed specifically in the ipsilateral regions of the cerebral cortex, hippocampus and striatum at 24 h after reperfusion (Figures 1a–1d). Systemic administrations of recombinant rat ProT α (rrProT α , 100 μ g/kg, i.p.) at 30 min and 3 h after reperfusion, largely reversed this brain damage and suppressed ischemia-induced motor dysfunction, evaluated by a so-called clinical score,¹⁴ and lethality (Figures 1f and 1g). It should be noted that ProT α protected the brain from cell death and motor dysfunction when administered as a single injection (at 30 min or 3 h after reperfusion) or in a pair of later injections (at 3 and 6 h after reperfusion). In a sublethal global ischemic model using both common carotid artery occlusions (BCCAO, 30 min), there were marked losses of neurons in the pyramidal cell layers and dentate gyri of surviving mice at 28 days after occlusion (Figure 1(h)). A single systemic (i.p.) injection of recombinant mouse ProT α (rmProT α) at 100 μ g/kg 24 h after occlusion completely prevented brain damage, learning and memory deficits in the step-through passive avoidance task, and lethality (Figures 1i–k). Significant prevention was also observed with rmProT α at a very low dose, 1 μ g/kg (i.p.).

By cytochemical analysis, both necrosis and apoptosis were observed in damaged brain regions 24 h after MCAO, when cell death was evaluated by *in vivo* propidium iodide and activated-caspase 3 staining. Systemic rrProT α injection (i.p.) markedly inhibited both necrotic and apoptotic cell death (Figures 1l–1o).

It should be noted that ProT α inhibited both necrosis and apoptosis following ischemia. This finding contrasts with our recent study, in which ProT α was shown to inhibit necrosis by restoring the reduced membrane translocation of glucose transporters under ischemic conditions in a primary culture of cortical neurons, but to cause apoptosis through upregulation of proapoptotic Bax and Bim.³ However, complete inhibition of cell death was observed when ischemic neurons were co-treated with ProT α and antiapoptotic neurotrophins, the expression levels of which are upregulated in *in vivo* ischemic models.¹⁵ In fact, the treatment with anti-BDNF or anti-EPO

IgG (1 μ g each) into the subarachnoid space through a parietal bone, 30 min before MCAO stress, reversed ProT α -inhibited apoptosis, but not necrosis (Figures 1p and q).

A single or paired injection of ProT α through a systemic route is sufficient to completely prevent damage in brain ischemic models. As shown in Figure 1r, a significant amount of Myc-tagged rrProT α , which had been administered i.p., was detected in the cortex 3 h after MCAO stress. The neuroprotective actions of ProT α administered through systemic routes are likely to be due to the transient disruption of the blood–brain barrier in the ischemic brain.¹⁶ It is worth to note that the neuroprotective actions of ProT α were observed when injected systemically 3 h after the ischemia, a time point within the therapeutic window for the treatment of stroke with tissue plasminogen activator. The fact that neurons are the major sites of action for ProT α is also expected to be advantageous in the treatment of stroke.

The paper by Shiao *et al.*¹⁷ described that added ProT α is incorporated into the cell and nuclei to ‘enhance’ the cytokine gene transcription and increase the infarct volume through inflammatory actions, whereas ProT α mutant lacking nuclear localizing sequence (NLS) ‘inhibits’ it. As shown in the Figure 1r, Myc-ProT α incorporated into the brain does not seem to be degraded and lose C-terminal NLS. Therefore, the finding by Shiao *et al.*¹⁷ is unlikely related to the ProT α -induced cell death inhibition in this study. Furthermore, in our previous study,³ there was no significant difference in the survival activity between ProT α and its mutant lacking C-terminal NLS.

Thus, ProT α is a unique cell death regulatory molecule, in that it converts irretrievable necrotic cell death into controllable apoptosis. Because this apoptosis can be inhibited by growth factors secreted upon ischemic stress, it is expected that ProT α may have an overall neuroprotective roles in the treatment of stroke.

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Tissue transglutaminase (TG2) facilitates phosphatidylserine exposure and calpain activity in calcium-induced death of erythrocytes

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Dear Editor,

During their daily life, erythrocytes are exposed to a variety of stress situations. On average, they pass once a minute through the lung, where they are exposed to oxidative stress. More than once an hour, they travel through kidney medulla, where they face osmotic shock. Erythrocytes are deformed to squeeze through small capillaries. The loss of erythrocyte cell integrity is pathological; rupture releases hemoglobin to extracellular fluid, which may be filtered at the glomerula of the kidney, precipitate in the acid lumen of the tubules, obliterate the tubules and thus lead to renal failure. To avoid these complications, erythrocytes, as any other cells, require a mechanism allowing them to be disposed without release of intracellular components.

Erythrocytes are devoid of mitochondria and nuclei, and were considered unable to undergo apoptosis. However, recently it has been revealed that treatment of erythrocytes with the Ca^{2+} ionophore ionomycin,¹ or their exposure to oxidative or osmotic stress,² situations that mimic red blood cell aging, leads to cell shrinkage, cell membrane blebbing and phosphatidylserine (PS) exposure, all typical features of apoptosis in other cell types. As macrophages are equipped with receptors recognizing PS, erythrocytes exposing PS at their cell surface will be rapidly recognized, engulfed and degraded.³ While the requirement for Ca^{2+} entry in the induction of cell death was shown for all these three cell death forms,² only ionomycin-induced cell death was found to be dependent on activation of μ -calpain,¹ a Ca^{2+} -dependent protease.

In addition to calpain, erythrocytes express another calcium-dependent enzyme, tissue transglutaminase (TG2).⁴ In human red blood cells (RBCs), Ca^{2+} -induced TG2 activation results in crosslinking of membrane skeleton proteins, leading to an irreversible structural fixation of the plasma membrane and of cell shape.⁵ TG2 might therefore play an important role in controlling the deformability and fragility, and therefore lifespan of erythrocytes, in a number of pathophysiological situations, accompanied by an increase in free ionic intracellular Ca^{2+} concentration, such as aging, sickle cell or Köln disease. Indeed, in old RBCs, both protein levels and the *in vitro* activity of TG2 are increased.⁶ In

addition, transglutaminase-catalyzed polymers were isolated from patients with Köln disease⁴ or sickle cell anemia⁴-diseases in which the lifespan of RBCs is known to be greatly reduced. Nevertheless, the physiological role of TG2 in the erythrocyte aging process still remains unclear. To address this question, we took advantage of TG2 knockout mice.⁷

The recognition and uptake of dying erythrocytes by macrophages is a sensitive biological measure of cell death. We assessed the rate of phagocytosis of wild-type and TG2-null RBCs by wild-type macrophages, *in vitro*, to explore possible changes in the death program of TG2-null erythrocytes, including those, which might have significance in influencing the *in vivo* clearance. RBCs were induced to die by the Ca^{2+} ionophore ionomycin (1 $\mu\text{g}/\text{ml}$) for 1 h and were further incubated with wild-type peritoneal macrophages for an additional hour. As shown in Figure 1a, both wild-type and TG2-null RBCs were engulfed by macrophages, even if RBCs were not exposed to ionomycin. This uptake was different from the phagocytosis of ionomycin-treated RBCs, since it was not inhibited by annexin V or by apoptotic Jurkat cells, known competitors of apoptotic cell uptake (data not shown). In contrast, the uptake of ionomycin-treated RBCs above the background, which was fully inhibited by apoptotic Jurkat cells and annexin V, and thus represents uptake of apoptotic RBCs, was significantly reduced in the case of TG2-null RBCs, as compared to the wild types (from $31.2 \pm 3.7\%$ of wild type to $18.7 \pm 2.6\%$ of TG2-null cells, $n=5$, $P>0.05$ determined by non-paired *t*-test). These observations suggest that activation of TG2 by Ca^{2+} in dying erythrocytes facilitates recognition by macrophages.

Since the appearance of PS plays a key role in the recognition of RBCs,³ we investigated the kinetics of PS exposure on the surface of TG2-null RBCs following addition of ionomycin. The exposure of PS was delayed modestly in TG2-null erythrocytes during Ca^{2+} -induced death (Figure 1b). However, the absence of TG2 did not affect the cluster formation of PS residues (Figure 1c), which has been shown to be required for proper recognition of apoptotic cells. Since in other cell types TG2 has various biological activities,⁴ to make sure that crosslinking activity of TG2 is required for proper