Str rad: Iba-1 & ProTα

Cerebral ischemia (1 h tMCAO) and reperfusion

(a) Control; 0 h (b) Ipsi; 3 h (c) Ipsi; 24 h (d) Ipsi; 3 h + Vehicle (e) Ipsi; 3 h + Z-VAD (f) Post-natal (P1)

(g) Cerebral ischemia (1 h tMCAO) and reperfusion — 0 h, 3 h, 24 h — Striatum ipsi

(h) Cortex ipsi

(i)

Fig. 5 Microglia have no tendency to release ProTα. (a–f) Coronal brain sections are co-stained with antibodies against ProTα and Iba-1, a microglial marker. (a) Immunohistochemical data shows the expression of ProTα in Iba-1-positive microglia in the stratum radiatum of hippocampus in control brain (Iba-1, red; ProTα, green). (b) ProTα staining is still found with higher intensity in Iba-1-positive microglia in the ipsilateral stratum radiatum at 3 h after ischemic stress. (c) ProTα intensity is increased gradually in Iba-1-positive microglia through 24 h after ischemic stress. (d, e) PBS (5 µL) and Z-VAD-fmk (Z-VAD) at a dose of 1 µg/5 µL (i.c.v.) are injected in the brain 30 min before cerebral ischemia (1 h tMCAO). (d) ProTα reactivity is still found with higher intensity in Iba-1-positive microglia in the ipsilateral stratum radiatum at 3 h after ischemic stress in the PBS-pre-treated (vehicle) brain. (e) Following Z-VAD-fmk pre-treatment, intense ProTα signal is also observed in Iba-1-positive microglia in the stratum radiatum at 3 h after ischemic stress, indicates that ProTα is not released from microglia. (f) ProTα is expressed both in whole Iba-1-positive microglia in the stratum radiatum of hippocampus of post-natal (P1) brain. (g, h) ProTα is not released from micrglia in the striatum and somatosensory cortex after cerebral ischemia. ProTα signal is found with higher intensity in Iba-1-positive micrglia in the ipsilateral striatum and somatosensory cortex at 3 h after ischemic stress, compared with the ProTα signals in the control brain. ProTα intensity is gradually increased in Iba-1-positive micrglia in the ipsilateral striatum and somatosensory cortex through 24 h after ischemic stress. Insets indicate the higher magnification view of ProTα expression in microglia noted by dotted squares. (i) Quantitative analysis of ProTα-positive microglias in the somatosensory cortex. Data represent the means ± SEM.

positive astrocytes of ipsilateral stratum radiatum (data are not shown), followed by completely lost at 24 h after cerebral ischemia (Fig. 7b and d). Similar results of astroglial S100A13 release were observed in the striatum (data are not shown) and somatosensory cortex (Fig. 7e).

However, S100A13 immunoreactivity was absent in Iba-1-positive microglia in the stratum radiatum of normal adult brain (Fig. 7f). We found the similar results of S100A13 absence in the adult microglia of non-ischemic (control) brain using antigen retrieval microwave technique and proteinase K treatment (Fig. 7h and i, respectively). Our findings also suggested that S100A13 is not expressed in Iba-1-positive microglia in the stratum radiatum of post-natal (P1) brain (Fig. 7j). Interestingly, S100A13 signals were

absent in Iba-1-positive microglia in the stratum radiatum through 24 h after ischemic stress (Fig. 7g). We found the similar lack of S100A13 expression in Iba-1-positive microglia in the striatum and somatosensory cortex of non-ischemic adult and neonatal brain (data are not shown).

Discussion

ProTα, a signal peptide-deficient nuclear protein, has been identified as a unique cell death regulatory molecule in that it converts the intractable necrosis into the controllable apoptosis (Ueda and Fujita 2004; Ueda *et al.* 2007). This apoptosis is inhibited by brain-derived neurotrophic factor (Ueda 2008). In addition, ProTα potentially inhibits cerebral
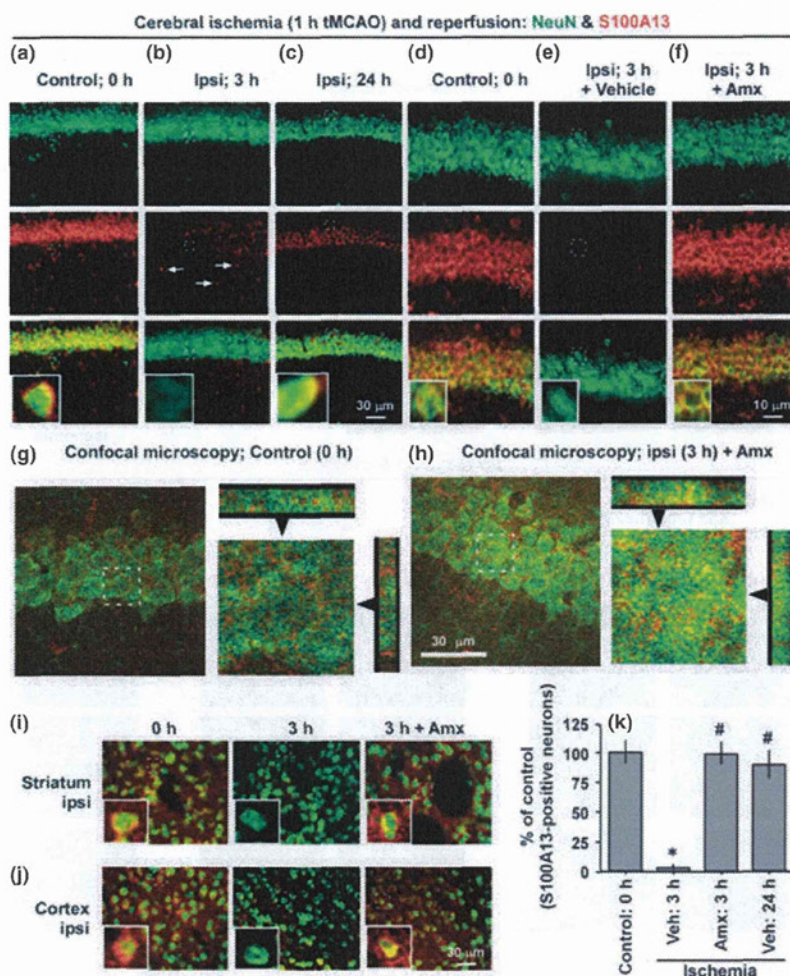
Fig. 6 Amlexanox reversibly blocks the ischemia-induced release of S100A13 from neurons. PBS (5 μL) as well as amlexanox (Amx) at a dose of 10 μg/5 μL (i.c.v.) is injected in the mice brain 30 min before cerebral ischemia (1 h MCAO). (a–j) Double fluorescence immuno-histochemical analysis of coronal brain sections is performed using antibodies against S100A13 and NeuN, a neuronal marker. (a) S100A13 is expressed in NeuN-positive CA1 pyramidal neurons of hippocampus in the control brain (NeuN, green; S100A13, red). (b) S100A13 signal is completely lost in NeuN-positive neurons in the ipsilateral CA1 pyramidal cell layer of hippocampus at 3 h after cerebral ischemia. Whereas, the dot-like signals are found in some non-neuronal cells in the stratum radiatum of hippocampus at 3 h indicated by arrows (b). (c) S100A13 is recovered with a lesser intensity in the ipsilateral NeuN-positive CA1 pyramidal neuronal cells at 24 h after ischemic stress, but the signal is completely lost in non-neuronal cells in the stratum radiatum at that time point. (d) S100A13 signal is found in NeuN-positive CA1 pyramidal neurons in the control brain. (e) S100A13 signal is completely lost at 3 h in NeuN-positive CA1 pyramidal neurons in the ipsilateral hippocampus of PBS-pre–treated (vehicle) ischemic brain. (f) Amx pre-treatment blocks S100A13 release from ipsilateral CA1 pyramidal neurons at 3 h after ischemic stress. (g, h) Conforcal microscopy observation in CA1 pyramidal neurons. A higher magnification view is indicated as doted square in panels (g) and (h), respectively. Arrowheads indicate the 3D imaged line (thickness: 10 μm), as shown in upper (x-axis) and right panels (y-axis). (i, j) Amlexanox inhibits S100A13 release from striatal and somatosensory cortical neurons. S100A13 is expressed in NeuN-positive neurons in the striatum and somatosensory cortex at 0 h as control. S100A13 signal is completely lost in NeuN-positive neurons in the striatum and cortex at 3 h after ischemia. Amx pre-treatment blocks S100A13 release from striatal and cortical neurons at 3 h after ischemic stress respectively. Insets indicate the higher magnification view of S100A13 expression neurons noted by squares. (k) Quantitative analysis of S100A13-positive neuons in the somatosensory cortex. Data represent the means ± SEM ($*,$ $^{\#}p < 0.01$, vs. the control: 0 h and the Amx: 3 h, respectively).

and retinal ischemia-induced necrosis as well as apoptosis (Fujita and Ueda 2007; Fujita *et al.* 2009). Ischemia-specific and ProTα-induced up-regulation of brain-derived neuro-trophic factor or erythropoietin is found to contribute to this

apoptosis inhibition (Fujita *et al.* 2009; Ueda 2009). Taken together the exclusive findings that the pre-treatments with antisense oligodeoxynucleotide or antibody against ProTα deteriorated the retinal ischemic damages (Fujita *et al.* 2009;
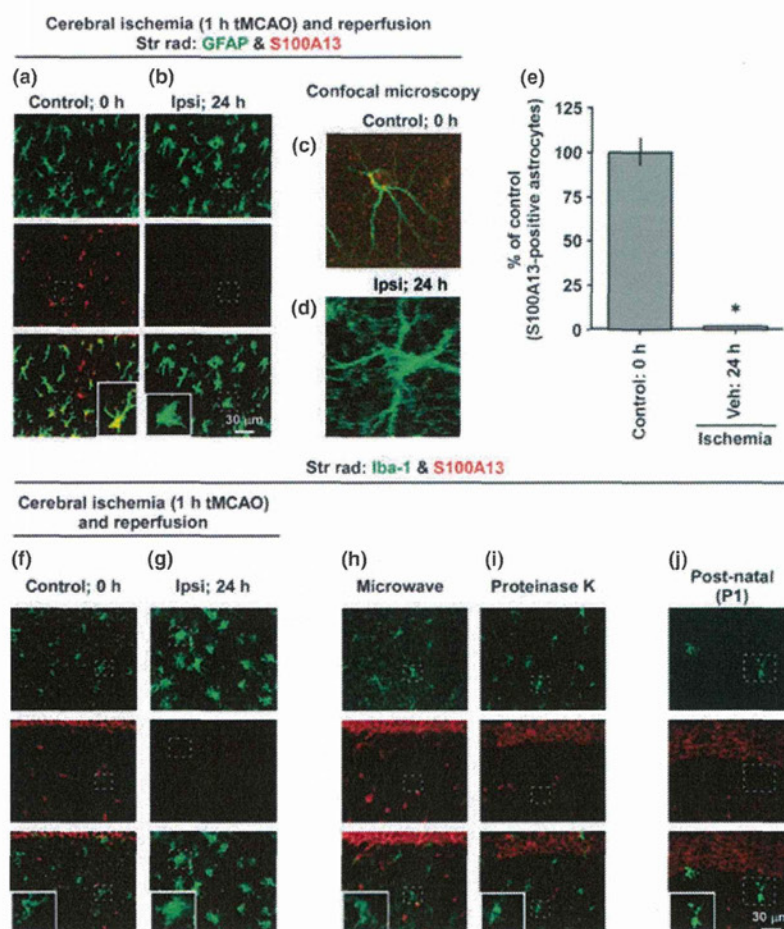
Fig. 7 Ischemia-induced release of S100A13 from astrocytes, but not from microglia. Coronal brain sections are co-stained with antibodies against S100A13, GFAP and Iba-1. (a) Double immunofluorescence staining indicates that S100A13 is expressed in GFAP-positive astrocytes in the stratum radiatum of hippocampus in the control brain (GFAP, green; S100A13, red). (b) Following cerebral ischemia and reperfusion (1 h tMCAO), S100A13 signal is lost completely at 24 h in GFAP-positive astrocytes in the stratum radiatum. (c, d) A higher magnification view of S100A13 in astrocytes. Images were collected by a conforcal microscopy. (e) Quantitative analysis of S100A13-positive astrocytes in the somatosensory cortex. Data represent the means ± SEM. (*p < 0.01, vs. the control: 0 h). (f) S100A13 reactivity is absent in Iba-1-positive microglia in the stratum radiatum of normal adult brain (Iba-1, green; S100A13, red). (g) S100A13 is not expressed in Iba-1-positive microglia in the stratum radiatum through 24 h after ischemic stress. (h, i) Antigen retrieval microwave technique (h) as well as proteinase K method (i) followed by double fluorescence immunostaining indicates that S100A13 is not expressed in Iba-1-positive microglia in the stratum radiatum of hippocampus in the adult control brain. (j) Immunostaining data show the lack of S100A13 expression in Iba-1-positive microglia in the stratum radiatum of post-natal (P1) control brain. Insets indicate the high-magnification view of S100A13 expression in astrocyte and microglia noted by dotted squares.

Ueda *et al.* 2010), it is evident that ProTα is a key neuroprotective molecule against ischemic damages.

In the present study, the novel *in vivo* findings include the followings: (i) ProTα is non-classically released along with S100A13 from neuronal cells in the CA1 pyramidal cell layer of hippocampus, striatum and somatosensory cortex of adult brain at 3 h after cerebral ischemia; (ii) amlexanox reversibly blocks this non-classical neuronal ProTα release as well as S100A13; (iii) there is no ProTα release from adult astrocytes and microglia after ischemic stress in brain, followed by

gradual up-regulation of ProTα signals in these non-neuronal cells through 24 h; (iv) caspase 3 inhibition by Z-VAD-fmk pre-treatment induces ProTα release from astrocytes as early as 3 h after cerebral ischemia, but this release is reversibly blocked by amlexanox; and (v) ischemia-induced ProTα distribution in microglia is not affected either by Z-VAD-fmk or amlexanox.

The recent *in vitro* study explained that ProTα is localized in the nuclei of cultured cortical neurons and embryonic astrocytes, and that is released from these cells into the extracellular

space upon serum-deprivation stress (Matsunaga and Ueda 2010). This study also suggested that the non-vesicular release of ProTα is initiated through the interaction with S100A13 in the serum-deprived C6 glioma cells *in vitro*. Most recently, we demonstrated that ProTα is strictly localized in nuclei of neuronal cells in adult brain, while it is found in both nuclei and cytosilic space of processes in the astrocytes and microglia (Halder and Ueda 2012). In the present study, we confirmed that ProTα is also localized both in cell body and processes in astrocytes and microglia in the neonatal mice brain, an indication of difference between the brain and culture cell experiments in terms of ProTα expression in astrocytes.

The current study suggested that ProTα is completely released from only neurons in the brain and followed by recovery the signal strictly in the neuronal nuclei after cerebral ischemia, suggesting that the active mechanism may be involved in the epigenetic regulation of ProTα gene expression in neurons of brain under stress condition. It has been reported previously that amlexanox, a potent inhibitor of S100A13, blocks the non-vesicular release of ProTα as well as S100A13 from C6 glioma cells under serum-deprivation stress *in vitro*, due to loss of interaction of S100A13 with C-terminal sequence of ProTα (Matsunaga and Ueda 2010). This *in vivo* study demonstrated that ischemia-induced neuronal ProTα release is reversibly blocked by amlexanox and the signals are diffused in the cytosol of neurons. However, S100A13 was distributed in both cell body and cytosol, and released at the same time point as like ProTα from neurons after the onset of cerebral ischemia. Interestingly, this S100A13 release was in turn blocked in neurons, as the same time point as ProTα released was inhibited, in the amlexanox pre-treated ischemic brain. Together, it can be hypothesized that ProTα is non-classically co-released with S100A13 from neuronal cells in the adult brain under ischemic stress *in vivo*.

At the later time, we characterized the phenomenon whether ProTα is released from non-neuronal cells after cerebral ischemia. We found that none of ProTα is released from astrocytes and microglia after the ischemic stress in brain. Indeed, ProTα intensity was gradually increased in astrocytes and microglia localized in the ischemic regions of brain through 24 h after ischemia. Interestingly, Z-VAD-fmk (caspase 3 inhibitor) pre-treatment induced ProTα release from astrocytes in the ischemic brain, but this release was reversibly blocked by amlexanox. However, S100A13 was expressed in astrocytes of normal brain and released partially at 3 h, followed by complete release at 24 h after brain ischemia. Several *in vitro* studies suggested that the fragmentation of ProTα is mediated by active caspase 3 at the C-terminal sequence located within the spacer region bipartite nuclear localization signal of ProTα (Rubtsov *et al.* 1997; Enkemann *et al.* 2000; Evatafieva *et al.* 2003;

Matsunaga and Ueda 2010). The presence of active caspase 3 in the nuclei of astrocytes in adult brain has also been reported *in vivo* (Duran-Vilaregut *et al.* 2010). Recently, it has been demonstrated that S100A13 interacts with C-terminal sequence of ProTα through the C-terminal 11 amino acid peptide sequence of S100A13 in Ca^{2+}-sensitive manner *in vitro*, and that the expression of Δ88–98 mutant of S100A13 selectively inhibits the stress-induced non-classical release of ProTα, but the release of S100A13 mutant itself occurs from C6 glioma cells (Matsunaga and Ueda 2010). This study explains the crucial role of C-terminal peptide sequence of ProTα for non-classical releasing itself. Most recently, we explained that nuclear ProTα level is drastically increased in the astrocytes of non-ischemic brain by Z-VAD-fmk pre-treatment, an indication of active caspase 3-mediated cleavage of C-terminal part possessing nuclear localization signal of ProTα (Halder and Ueda 2012). Therefore, we can explain the possible mechanisms in the following way: (i) astroglial ProTα in the adult brain might loose the capacity to interact with S100A13 due to the cleavage of C-terminal amino acid sequence of ProTα by activated caspase 3 so that no further release is occurred upon brain ischemia; and (ii) the full-length ProTα is redistributed from the cytosol into nuclei of astrocytes in the brain pre-treated with Z-VAD-fmk, followed by consequent release from astrocytes after cerebral ischemic stress *in vivo*. Our findings also suggested that Z-VAD-fmk as well as amlexanox has no effect on the distribution of ProTα in microglia in the ischemic brain. The present study explained the lack of S100A13 expression in microglia of non-ischemic brain, even in brain microglia under the cerebral ischemic stress. Therefore, we can explain one possible mechanism is that microglia loses its capacity to release ProTα from ischemic brain due to the absence of S100A13. However, these findings encourage us to investigate the possible intracellular roles of cytosolic ProTα in astrocytes as well as in microglia. Although there is a close interaction between neurons and non-neuronal cells, astrocytes and microglia are more resistant than neurons to most of ischemic stress (Chen and Swanson 2003; Giffard and Swanson 2005; Trendelenburg and Dirnagl 2005; Rossi *et al.* 2007; Oshiro *et al.* 2008; Lambertsen *et al.* 2009; Faustino *et al.* 2011). There is an interesting report about the ProTα-mediated cellular protection against oxidative stress through the dissociation of the intranuclear Nrf2–Keap1 complex and subsequently facilitation of oxidative stress-protecting genes expression (Karapetian *et al.* 2005). It has also been described that ProTα prevents cells from apoptosis through the inhibition of apoptosome formation (Jiang *et al.* 2003; Letsas and Frangou-Lazaridis 2006). Taken together, our findings indicate the possible *in vivo* role of cytosolic ProTα in astrocytes and microglia in the inhibition of apoptosis.

In the present study, we performed pharmacological inhibitor study against ischemic stress-induced ProTα

release. To confirm our hypothesis of intracellular and extracellular roles of ProTα, we need to perform the study using double knockdown or knockout strategies for S100A13, caspase 3, and caspase 7. As the knockdown strategy using an intracerebroventricular injection is presumed to only partially decrease in the levels of these proteins, the conclusion would not be clear. Although double or triple knockout mice would be perfect to discuss this issue, such mice are not available at present. So, detailed mechanisms would be the next subjects.

Several intracellular proteins lack of conventional signal peptides are released from varieties of cells through non-classical endoplasmic reticulum-Golgi-independent pathways under necrotic/ischemic stress (Gardella *et al.* 2002; Nickel 2005; Prudovsky *et al.* 2008; Nickel and Rabouille 2009; Matsunaga and Ueda 2010). Such a mode for necrotic/ischemic stress-induced extracellular release from neuronal nuclei seems to be similar to the case with HMGB-1, a popular member of DAMPs (Nickel 2005; Faraco *et al.* 2007; Foell *et al.* 2007; Rubartelli and Lotze 2007; Qiu *et al.* 2008). The reciprocal relation from ProTα would be found in the nature that HMGB-1 induces cytotoxic effects *in vitro* and *in vivo* (Scaffidi *et al.* 2002; Lotze and Tracey 2005; Bianchi 2007; Liu *et al.* 2007; Qui *et al.* 2008; Yang *et al.* 2010; Zitvogel *et al.* 2010). However, the pattern of ProTα release from neuronal nuclei is as similar as HMGB-1 release, but dissimilar in the case that ProTα induces robust neuroprotection (Fujita and Ueda 2007; Fujita *et al.* 2009; Ueda *et al.* 2010). Although ProTα-mediated cell survival activity against viral infection through Toll-like receptor-4 has been reported (Mosoian *et al.* 2010), the exact receptor for ProTα signaling is yet unknown. Considering the case, ProTα may be referred as a novel neuroprotective molecule of DAMPs family.

In conclusion, the present study demonstrated that neurons, but not astrocytes and microglias, is the main store of endogenous ProTα, which is released through non-classical pathway upon cerebral ischemia, due to the presence of releasing machineries in neuronal cells in brain. Therefore, the discovery cell type-specific mechanisms of ProTα signaling in the brain may provide a novel solution to protect chronic cellular damages in stroke.

Acknowledgements

Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Materials and methods.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

References

Baxevanis C. N., Thanos D., Reclos G. J., Anastasopoulos E., Tsokos G. C., Papamatheakis J. and Papamichail M. (1992) Prothymosin alpha enhances human and murine MHC class II surface antigen expression and messenger RNA accumulation. *J. Immunol.* **148**, 1979–1984.

Bianchi M. E. (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukoc. Biol.* **81**, 1–5.

Chen G. Y. and Nunez G. (2010) Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* **10**, 826–37.

Chen Y. and Swanson R. A. (2003) Astrocytes and brain injury. *J. Cereb. Blood Flow Metab.* **23**, 137–149.

Danton G. H. and Dietrich W. D. (2003) Inflammatory mechanisms after ischemia and stroke. *J. Neuropathol. Exp. Neurol.* **62**, 127–136.

Diaz-Jullien C., Perez-Estevez A., Covelo G. and Freire M. (1996) Prothymosin alpha binds histones in vitro and shows activity in nucleosome assembly assay. *Biochim. Biophys. Acta* **1296**, 219–227.

Duran-Vilaregut J., Del Valle J., Manich G., Junyent F., Camins A., Pallas M., Pelegri C. and Vilaplana J. (2010) Systemic administration of 3-nitropropionic acid points out a different role for active caspase-3 in neurons and astrocytes. *Neurochem. Int.* **56**, 443–450.

Egashira N., Hayakawa K., Mishima K., Kimura H., Iwasaki K. and Fujiwara M. (2004) Neuroprotective effect of γ-glutamylethylamide (theanine) on cerebral infarction in mice. *Neurosci. Lett.* **363**, 58–61.

Enkemann S. A., Ward R. D. and Berger S. L. (2000) Mobility within the nucleus and neighboring cytosol is a key feature of prothymosin-alpha. *J. Histochem. Cytochem.* **48**, 1341–1355.

Evstafieva A. G., Belov G. A., Rubtsov Y. P. *et al.* (2003) Apoptosis-related fragmentation, translocation, and properties of human prothymosin alpha. *Exp. Cell Res.* **284**, 211–223.

Faraco G., Fossati S., Bianchi M. E., Patrone M., Pedrazzi M., Sparatore B., Moroni F. and Chiarugi A. (2007) High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration in vitro and in vivo. *J. Neurochem.* **103**, 590–603.

Faustino J. V., Wang X., Johnson C. E., Klibanov A., Derugin N., Wendland M. F. and Vexler Z. S. (2011) Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. *J. Neurosci.* **31**, 12992–13001.

Foell D., Wittkowski H. and Roth J. (2007) Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. *Nat. Clin. Pract. Rheumatol.* **3**, 382–390.

Fujita R. and Ueda H. (2007) Prothymosin-alpha1 prevents necrosis and apoptosis following stroke. *Cell Death Differ.* **14**, 1839–1842.

Fujita R., Ueda M., Fujiwara K. and Ueda H. (2009) Prothymosin-alpha plays a defensive role in retinal ischemia through necrosis and apoptosis inhibition. *Cell Death Differ.* **16**, 349–358.

Garbin F., Eckert K., Immenschuh P., Kreuser E. D. and Maurer H. R. (1997) Prothymosin alpha 1 effects, in vitro, on the antitumor activity and cytokine production of blood monocytes from colorectal tumor patients. *Int. J. Immunopharmacol.* **19**, 323–332.

Gardella S., Andrei C., Ferrera D., Lotti L. V., Torrisi M. R., Bianchi M. E. and Rubartelli A. (2002) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep.* **3**, 995–1001.

George E. M. and Brown D. T. (2010) Prothymosin alpha is a component of a linker histone chaperone. *FEBS Lett.* **584**, 2833–2836.

Giffard R. G. and Swanson R. A. (2005) Ischemia-induced programmed cell death in astrocytes. *Glia* **50**, 299–306.

Gomez-Marquez J. (2007) Function of prothymosin alpha in chromatin decondensation and expression of thymosin beta-4 linked to angiogenesis and synaptic plasticity. *Ann. N. Y. Acad. Sci.* **1112**, 201–209.

Gomez-Marquez J. and Rodriguez P. (1998) Prothymosin alpha is a chromatin-remodelling protein in mammalian cells. *Biochem. J.* **333**, 1–3.

Halder S. K. and Ueda H. (2012) Regional distribution and cell type-specific subcellular localization of prothymosin alpha in brain. *Cell. Mol. Neurobiol.* **32**, 59–66.

Jiang X., Kim H. E., Shu H. *et al.* (2003) Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway. *Science* **299**, 223–226.

Karapetian R. N., Evstafieva A. G., Abaeva I. S. *et al.* (2005) Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes. *Mol. Cell. Biol.* **25**, 1089–1099.

Karetsou Z., Kretsovali A., Murphy C., Tsolas O. and Papamarcaki T. (2002) Prothymosin alpha interacts with the CREB-binding protein and potentiates transcription. *EMBO Rep.* **3**, 361–366.

Kono H. and Rock K. L. (2008) How dying cells alert the immune system to danger. *Nat. Rev. Immunol.* **8**, 279–288.

Lambertsen K. L., Clausen B. H., Babcock A. A. *et al.* (2009) Microglia protect neurons against ischemia by synthesis of tumor necrosis factor. *J. Neurosci.* **29**, 1319–1330.

Letsas K. P. and Frangou-Lazaridis M. (2006) Surfing on prothymosin alpha proliferation and anti-apoptotic properties. *Neoplasma* **53**, 92–96.

Liu K., Mori S., Takahashi H. K. *et al.* (2007) Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB J.* **21**, 3904–3916.

Lotze M. T. and Tracey K. J. (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol.* **5**, 331–342.

Martini P. G., Delage-Mourroux R., Kraichely D. M. and Katzenellenbogen B. S. (2000) Prothymosin alpha selectively enhances estrogen receptor transcriptional activity by interacting with a repressor of estrogen receptor activity. *Mol. Cell. Biol.* **20**, 6224–6232.

Matsumoto M., Inoue M., Hald A., Xie W. and Ueda H. (2006) Inhibition of paclitaxel-induced A-fiber hypersensitization by gabapentin. *J. Pharmacol. Exp. Ther.* **318**, 735–740.

Matsunaga H. and Ueda H. (2010) Stress-induced non-vesicular release of prothymosin-alpha initiated by an interaction with S100A13, and its blockade by caspase-3 cleavage. *Cell Death Differ.* **17**, 1760–1772.

Mosoian A., Teixeira A., High A. A., Christian R. E., Hunt D. F., Shabanowitz J., Liu X. and Klotman M. (2006) Novel function of prothymosin alpha as a potent inhibitor of human immunodeficiency virus type 1 gene expression in primary macrophages. *J. Virol.* **80**, 9200–9206.

Mosoian A., Teixeira A., Burns C. S., Sander L. E., Gusella G. L., He C., Blander J. M., Klotman P. and Klotman M. E. (2010) Prothymosin-alpha inhibits HIV-1 via Toll-like receptor 4-mediated type I interferon induction. *Proc. Natl Acad. Sci. USA* **107**, 10178–10183.

Muhammad S., Barakat W., Stoyanov S. *et al.* (2008) The HMGB1 receptor RAGE mediates ischemic brain damage. *J. Neurosci.* **28**, 12023–12031.

Nickel W. (2005) Unconventional secretory routes: direct protein export across the plasma membrane of mammalian cells. *Traffic* **6**, 607–614.

Nickel W. and Rabouille C. (2009) Mechanisms of regulated unconventional protein secretion. *Nat. Rev. Mol. Cell Biol.* **10**, 148–155.

Niizuma K., Yoshioka H., Chen H., Kim G. S., Jung J. E., Katsu M., Okami N. and Chan P. H. (2010) Mitochondrial and apoptotic neuronal death signaling pathways in cerebral ischemia. *Biochim. Biophys. Acta* **1802**, 92–99.

Oshiro S., Kawamura K., Zhang C., Sone T., Morioka M. S., Kobayashi S. and Nakajima S. (2008) Microglia and astroglia prevent oxidative stress-induced neuronal cell death: Implications for aceruloplasminemia. *Biochim. Biophys. Acta* **1782**, 109–117.

Pineiro A., Cordero O. J. and Nogueira M. (2000) Fifteen years of prothymosin alpha: contradictory past and new horizons. *Peptides* **21**, 1433–1446.

Pisetsky D. S. (2011) Cell death in the pathogenesis of immune-mediated diseases: the role of HMGB1 and DAMP-PAMP complexes. *Swiss Med. Wkly* **141**, 1–7.

Prudovsky I., Tarantini F., Landriscina M., Neivandt D., Soldi R., Kirov A., Small D., Kathir K. M., Rajalingam D. and Kumar T. K. (2008) Secretion without Golgi. *J. Cell. Biochem.* **103**, 1327–1343.

Qiu J., Nishimura M., Wang Y., Sims J. R., Qiu S., Savitz S. I., Salomone S. and Moskowitz M. A. (2008) Early release of HMGB-1 from neurons after the onset of brain ischemia. *J. Cereb. Blood Flow Metab.* **28**, 927–938.

Rossi D. J., Brady D. J. and Mohr C. (2007) Astrocyte metabolism and signaling during brain ischemia. *Nat. Neurosci.* **10**, 1377–1386.

Rubartelli A. and Lotze M. T. (2007) Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends Immunol.* **28**, 429–436.

Rubtsov Y. P., Zolotukhin A. S., Vorobjev I. A., Chichkova N. V., Pavlov N. A., Karger E. M., Evstafieva A. G., Felber B. K. and Vartapetian A. B. (1997) Mutational analysis of human prothymosin alpha reveals a bipartite nuclear localization signal. *FEBS Lett.* **413**, 135–141.

Scaffidi P., Misteli T. and Bianchi M. E. (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418**, 191–195.

Schmidt E. P. and Tuder R. M. (2010) Role of apoptosis in amplifying inflammatory responses in lung diseases. *J. Cell Death* **3**, 41–53.

Sims G. P., Rowe D. C., Rietdijk S. T., Herbst R. and Coyle A. J. (2010) HMGB1 and RAGE in inflammation and cancer. *Annu. Rev. Immunol.* **28**, 367–388.

Swanson R. A., Ying W. and Kauppinen T. M. (2004) Astrocyte influences on ischemic neuronal death. *Curr. Mol. Med.* **4**, 193–205.

Trendelenburg G. and Dirnagl U. (2005) Neuroprotective role of astrocytes in cerebral ischemia: focus on ischemic preconditioning. *Glia* **51**, 307–320.

Ueda H. (2008) Prothymosin alpha plays a key role in cell death mode-switch, a new concept for neuroprotective mechanisms in stroke. *Naunyn Schmiedebergs Arch. Pharmacol.* **377**, 315–323.

Ueda H. (2009) Prothymosin alpha and cell death mode switch, a novel target for the prevention of cerebral ischemia-induced damage. *Pharmacol. Ther.* **123**, 323–333.

Ueda H. and Fujita R. (2004) Cell death mode switch from necrosis to apoptosis in brain. *Biol. Pharm. Bull.* **27**, 950–955.

Ueda H., Fujita R., Yoshida A., Matsunaga H. and Ueda M. (2007) Identification of prothymosin-alpha1, the necrosis-apoptosis

switch molecule in cortical neuronal cultures. *J. Cell Biol.* **176**, 853–862.

Ueda H., Matsunaga H., Uchida H. and Ueda M. (2010) Prothymosin alpha as robustness molecule against ischemic stress to brain and retina. *Ann. N. Y. Acad. Sci.* **1194**, 20–26.

Yang H., Hreggvidsdottir H. S., Palmblad K. *et al.* (2010) A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc. Natl Acad. Sci. USA* **107**, 11942–11947.

Zhang J., Takahashi H. K., Liu K. *et al.* (2011) Anti-high mobility group box-1 monoclonal antibody protects the blood–brain barrier from ischemia-induced disruption in rats. *Stroke* **42**, 1420–1428.

Zhao Y. and Rampe D. A. (2010) Targeting astrocytes for stroke therapy. *Neurotherapeutics* **7**, 439–451.

Zitvogel L., Kepp O. and Kroemer G. (2010) Decoding cell death signals in inflammation and immunity. *Cell* **140**, 798–804.

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Thymosins in Health and Disease*

Prothymosin α plays multifunctional cell robustness roles in genomic, epigenetic, and nongenomic mechanisms

Hiroshi Ueda, Hayato Matsunaga, and Sebok Kumar Halder

Department of Molecular Pharmacology and Neuroscience, Nagasaki University Graduate School of Biomedical Sciences, Bunkyo-machi, Nagasaki, Japan

Address for correspondence: Hiroshi Ueda, Department of Molecular Pharmacology and Neuroscience, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki, 852-8521, Japan. ueda@nagasaki-u.ac.jp

Prothymosin α (ProTα) possesses multiple functions for cell robustness. This protein functions intracellularly to stimulate cell proliferation and differentiation through epigenetic or genomic mechanisms. ProTα also regulates the cell defensive mechanisms through an interaction with the Nrf2-Keap1 system. Under the apoptotic conditions, it inhibits apoptosome formation by binding to Apaf-1. Regarding extracellular functions, ProTα is extracellularly released from the nucleus upon necrosis-inducing ischemia stress in a manner of nonclassical release, and thereby inhibits necrosis. However, under the condition of apoptosis, the C-terminus of ProTα is cleaved off and loses binding activity to cargo protein S100A13 for nonclassical release. However, cleaved ProTα is retained in the cytosol and inhibits apoptosome formation. ProTα was recently reported to cause immunological actions through the Toll-like receptor 4. However, the authors also suggest the possible existence of additional receptors for robust cell activities against ischemia stress.

Keywords: linker histone H1; CBP/p300; estrogen receptor; Nrf2-Keap1; Apaf-1; Toll-like receptor-4

Introduction

ProTα is a highly acidic nuclear protein of the α-thymosin family and is found in the nuclei of virtually all mammalian cells.[1,2] ProTα is generally thought to be an oncoprotein that is correlated with cell proliferation by sequestering anticoactivator factor, a repressor of estrogen receptor activity, in various cells.[3,4] With regard to cell death regulation, intracellular ProTα was reported to play a cytoprotective role by inhibiting apoptosome formation in HeLa cells subjected to apoptotic stress.[5] On the other hand, ProTα has been reported to act as an extracellular signaling molecule, as observed in the activation of macrophages, natural killer cells, and lymphokine-activated killer cells, and in the production of IL-2 and TNF-α.[6] ProTα was most recently reported to exert immune responses through Toll-like receptor 4.[7,8] It should be noted that ProTα has potent neuroprotective actions through unique mechanisms by inhibiting neuronal necrosis.[9,10] A recent study revealed the machinery of nonclassical/nonvesicular release of ProTα from the nuclei upon the ischemia/cell starving stress.[11] Thus, there are accumulating findings supporting the conclusion that ProTα plays multiple roles inside and outside of the cell, particularly for cell survival and proliferation.[12]

Nuclear functions

Epigenetic mechanisms

ProTα is highly acidic (pI = 3.55) owing to its abundance of glutamic and aspartic acids (approximately 50% of the total amino acid residues) in the middle part of the protein. The cluster of acidic amino acids in this region seems to resemble a putative histone-binding domain, being consistent with the fact that there is a nuclear localization signal (NLS) at the C-terminal end (human ProTα: KR and KKQK at 87 and 101, respectively). Indeed there are reports that ProTα is highly expressed in many different types of cancer cells,[13–20] and closely related to the cell proliferation and differentiation.[13,21–23] Nuclear ProTα epigenetically stimulates
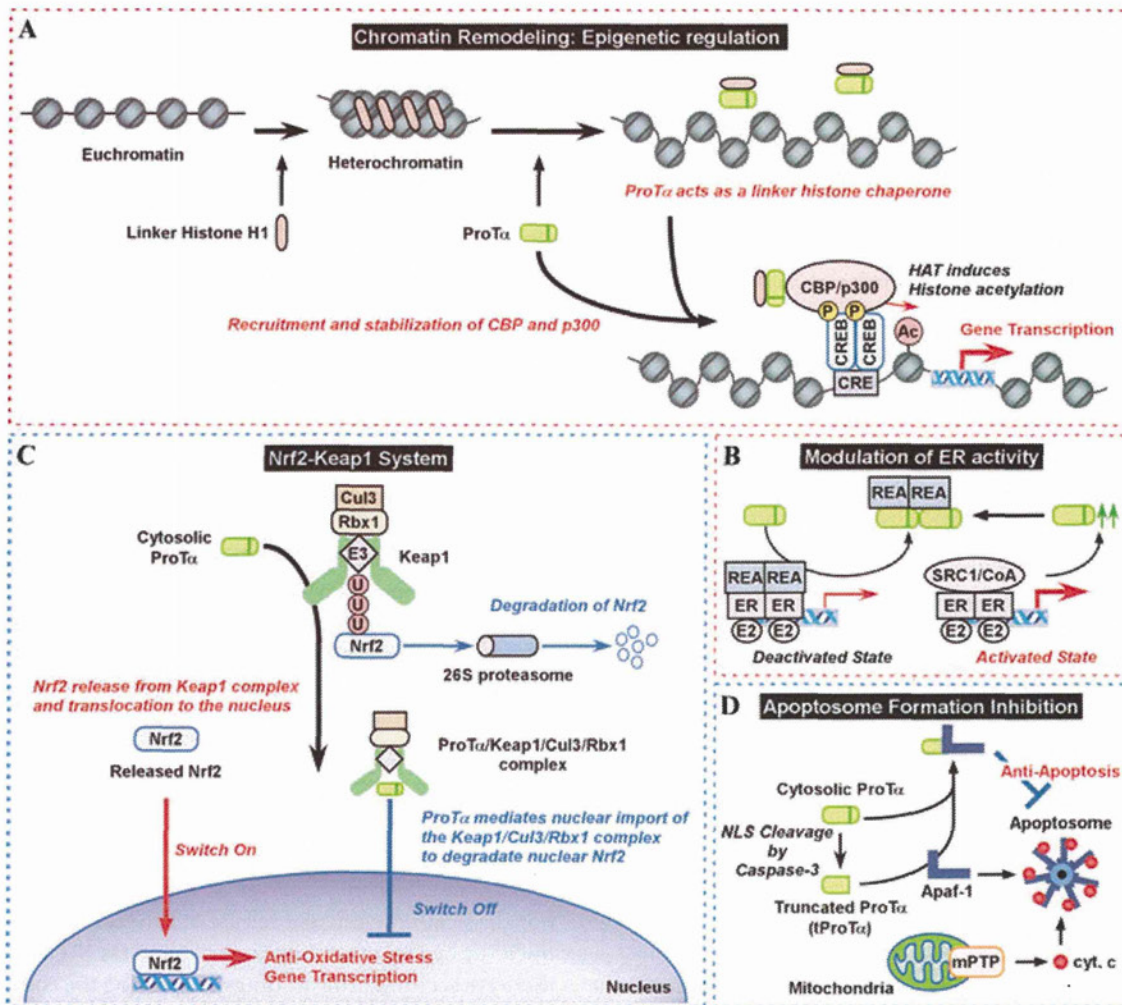
Figure 1. Intracellular multiple functions of ProTα for cell survival and proliferation through a variety of protein–protein interaction. (A) ProTα mediates chromatin remodeling and gene transcription. ProTα acts as a linker histone chaperone. Histone H1 binds to nucleosomal DNA and induces chromatin condensation. ProTα can facilitate H1 displacement from and deposition onto the chromatin template. ProTα is involved in histone acetylation by recruitment and stabilization of p300 histone acetyl transferase (HAT) and CREB-binding protein (CBP). (B) Enhancement of estrogen receptor (ER) transcriptional activity by binding to its repressor (REA: repressor estrogen receptor activity). (C) ProTα regulates the Nrf2-Keap1 system. ProTα binds to Keap1-releasing transcription factor Nrf2, which in turn upregulates antioxidative stress genes involving in apoptosis and autophagy (depicted as switch on). Meanwhile ProTα mediates nuclear import of Keap1/Cul3/Rbx1 complex leads to ubiquitination and degradation of Nrf2 (switch off). (D) ProTα-mediated inhibition of apoptosome by binding of to Apaf-1.

gene transcription by binding to histones,[12] p300 histone acetyltransferase,[24] and CREB-binding protein (CBP).[25] These findings suggests the role of ProTα in the chromatin remodeling (Fig. 1A). According to this hypothesis, ProTα detaches linker histone H1 from chromatin[26] and thus "loosens" it. ProTα also recruits CBP/p300 and stabilizes CBP/p300-CREB complex, resulting in the enhancement of CRE-regulated gene transcrip-

tion through histone acetylation and chromatin remodeling.

Modulation of estrogen receptor (ER)–mediated transcriptional activity

ProTα enhances the transcriptional activity of estrogen (E2) receptor through a removal of a repressor (estrogen receptor activity (REA)/B cell receptor-associated protein BAP37/prohibitin-2).[3,27] In the

presence of E2, REA, and coactivator SRC-1 compete for the binding to the ER. ProTα causes a dissociation of REA from ER and shifts the ER status to the SRC-1-binding, or transcription-active, form (Fig. 1B).

Cytosol functions

Switch on/off of Nrf2-Keap1 mechanisms

ProTα possessing an NLS at the C-terminus is localized in the nucleus in most cells. The nuclear import of these molecules with an NLS is mediated by importin α. As the nucleus-to-cytosol export of ProTα is presumed to occur in a passive diffusion due to its smaller size,[11] it is natural that ProTα may have some biological actions in the cytosol, as well as in the nucleus. The Nrf2 (nuclear factor erythroid 2-related factor 2)-Keap1 (Kelch-like ECH-associated protein 1) system is known to play roles in the cell adaptation to oxidative and electrophilic stress.[28–30] Nrf2 is a nuclear transcription factor that regulates expression of several defensive genes, including detoxifying enzymes, and antioxidant genes.[28–30] In the absence of stress, cytosol Nrf2 is "trapped" by Keap1, ubiquitinated by the Cul3/Rbx1-dependent E3 ubiquitin ligase, and then subsequently degraded by the 26S proteasome.[31] Recent studies revealed that ProTα binds to Keap 1 and releases Nrf2 from the Nrf2-Keap1 complex ("switch on").[32] Interestingly, ProTα also mediates nuclear import of the Keap1/Cul3/Rbx1 complex to degrade nuclear Nrf2 ("switch off").[33] Thus, it is speculated that ProTα regulates the cell-defensive roles of Nrf2 by switch on/off mechanisms (Fig. 1C).

Inhibition of apoptosome formation

When the cell is under mitochondrial stress, such as in the case with a growth factor deprivation, cytochrome c (cyt. c)—a member of soluble mitochondrial intermembrane proteins (SIMPs)—is released from mitochondria (Fig. 2A). As a result, the apoptosome, composed of cyt. c and Apaf-1, is formed and followed by a cascade of caspase activation and apoptotic DNA fragmentation.[34,35] Recent studies revealed that ProTα inhibits apoptosome formation by interaction with Apaf-1.[5,36] During apoptosis, on the other hand, ProTα loses its C-terminus containing the NLS, due to the digestion by caspase-3, and is redistributed to the cytosol.[11,37,38] As both cytosolic full-length ProTα and truncated ProTα have a similar potential of apoptosome inhi-

bition, it is interesting to speculate that ProTα has a potential as a natural inhibitor of apoptosis (Fig. 1D). This speculation is further supported by a report that ProTα inhibits apoptogenic compound–induced apoptosis by interacting with p8 (a nuclear protein-1 and candidate of metastasis-1).[39,40]

Extracellular functions

Identification of ProTα as a neuronal necrosis inhibitory factor

Based on the findings that cortical neurons at a low density rapidly die by necrosis under a serum-free or starving condition and that survival is density dependent,[10] we previously attempted to search for antinecrotic factors from the conditioned medium by using molecular weight cut-off ultrafiltration, ion-exchange filtration, SDS-PAGE separation, and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). A subsequent search of the nonredundant NCBI protein database for matching peptide mass fingerprints revealed 17 peptides consistent with the conclusion that the only active substance was acetylated ProTα.[9] After various approaches, we discovered an efficient way to obtain significant amounts of active materials that were unique to rat ProTα. Moreover, tandem MS analysis confirmed that the N-terminal of purified ProTα was an acetylated serine, in agreement with a previous report.[9]

Non-classical release mechanisms

ProTα was discovered in conditioned medium from serum-free or starving stress conditions of cultured cortical neurons. To examine the molecular basis of stress-induced ProTα release, we used C6 glioma (astrocytoma) cells because of their robustness against ischemia stress. On the analogy of the nonclassical release of FGF-1,[41,42] which lacks signal peptide sequence, ischemic stress caused a limiting extracellular release of ProTα (which also lacks signal peptide sequence). Our study revealed that the mechanisms underlying the nonclassical ProTα release from C6 glioma cells are mediated by the loss of ATP and Ca^{2+} influx through N-type voltage-dependent Ca^{2+} channel activity.[11] In this mechanism (Fig. 3A), the first step is the release of ProTα from the nucleus (passive diffusion) due to ATP loss, followed by a Ca^{2+}-dependent interaction with Ca^{2+} binding protein S100A13, a cargo protein. The release of ProTα from the nucleus upon an ischemic
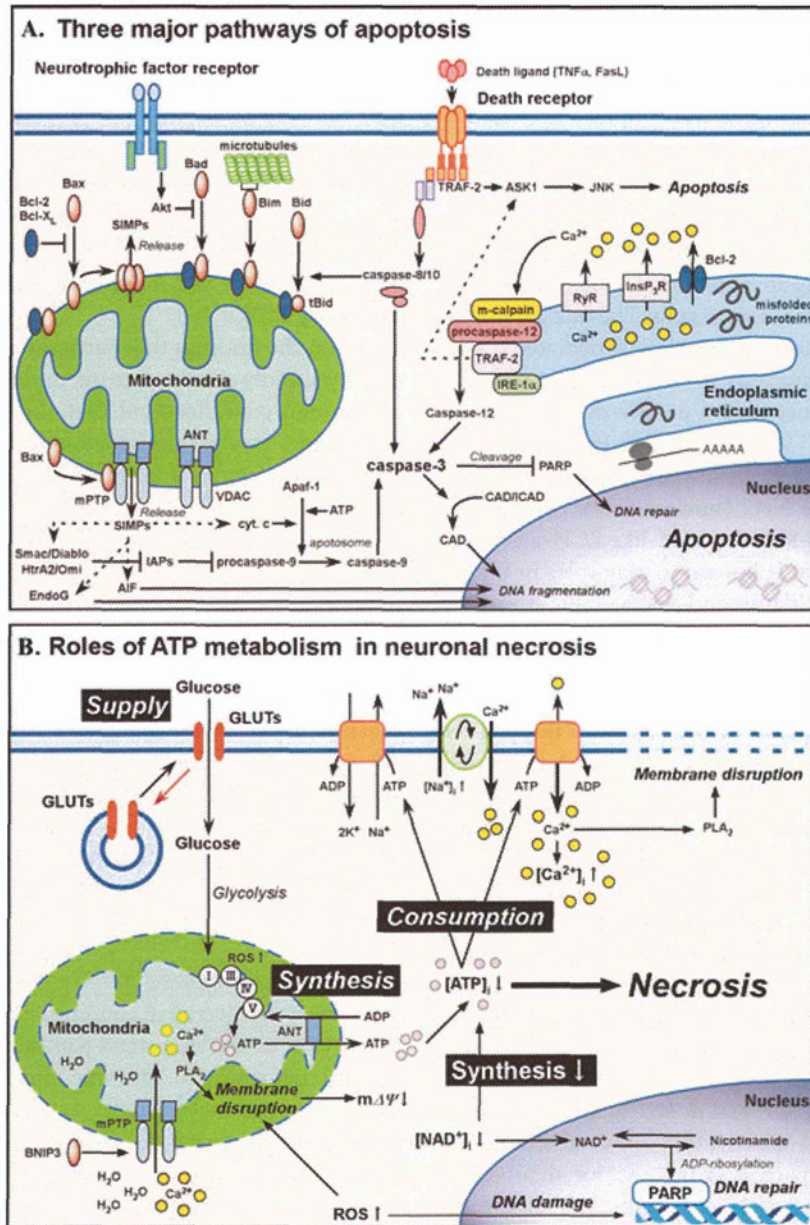
Figure 2. Schematic illustration of apoptosis and necrosis. (A) Three major pathways of apoptosis. Mitochondrial pathways are closely related to the expression of members of the Bcl-2 family of proteins. Proapoptotic Bak and Bax open mitochondrial permeability transition pores (mPTPs) to release soluble intermembrane proteins (SIMPs), including cytochrome c (cyt.c), apoptosis-inducing factor (AIF), Smac/DIABLO, EndoG, and HtrA2/Omi. Among these, cyt.c plays a major role in inducing apoptosis through activation of caspase-3 and caspase-activated DNase (CAD). Bcl-2 and Bcl-XL are major antiapoptotic proteins that inhibit the functions of these proapoptotic proteins. The other two pathways through death receptors (FAS and TNF-α receptors) or endoplasmic reticulum stress also use caspase-3 activation as the common execution pathway. (B) Roles of ATP metabolism in neuronal necrosis. Glucose transporters (GLUTs) are involved in the supply of cellular glucose (depicted as supply), a substrate for ATP production through glycolysis and oxidative phosphorylation (depicted as synthesis) in mitochondria. Some species of GLUT are constitutively localized, while others are translocated to the membrane upon cell stimulation by extracellular signals. Abundant cellular ATP molecules maintain intracellular ionic behavior (depicted as consumption). Poly (ADP-ribose) polymerase (PARP) restores the DNA damage caused by cellular stress, by using abundant NAD^+ molecules. NAD^+ reduction induces the decline of NADH-dependent ATP synthesis. A rapid decrease in the cellular ATP levels leads to necrosis.
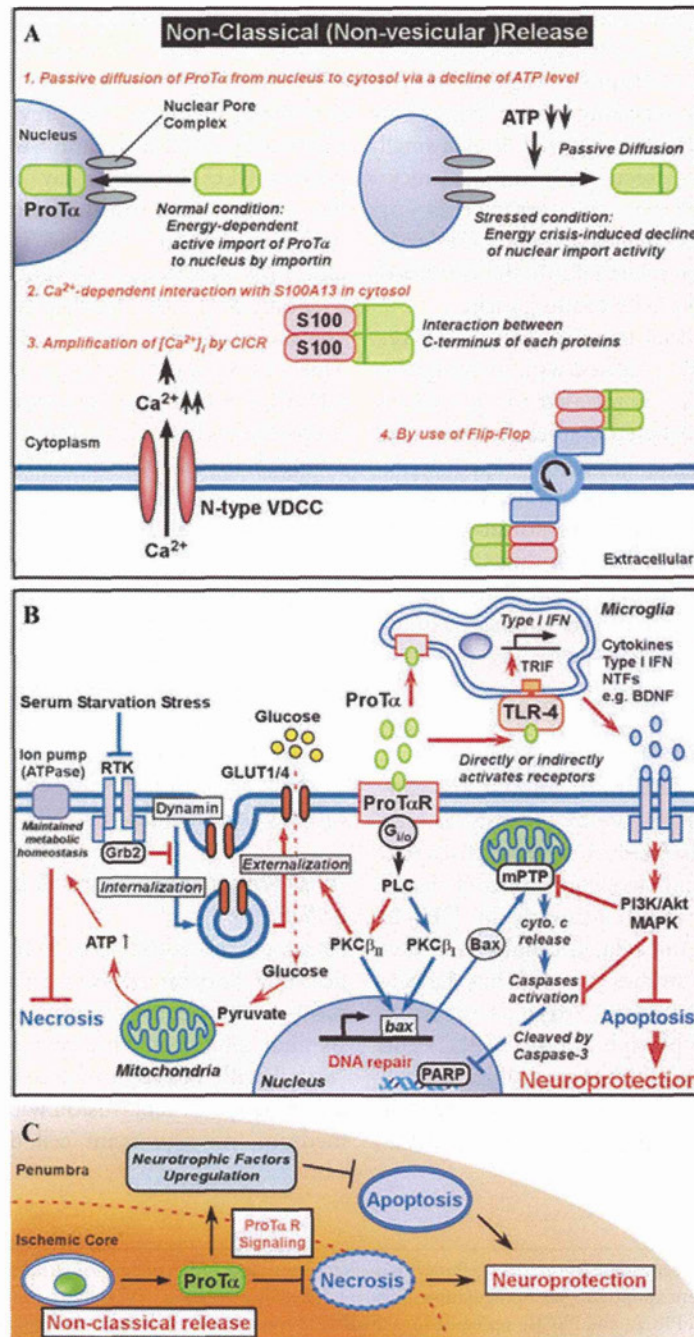
Figure 3. Schematic illustration for the extracellular release of ProTα and its neuroprotective roles in ischemic condition. (A) Necrotic stress–induced nonclassical release of ProTα. Necrosis-dependent energy crisis induces decline of nuclear import activity, subsequently ProTα, a small protein is passively diffused from the nucleus to the cytosol. Interaction between ProTα and extacellular cargo protein S100A13 is a Ca^{2+}-dependent manner through N-type voltage-dependent Ca^{2+} channel (VDCC) activity. Intracellular $[Ca^{2+}]_i$ is amplified by the mechanism of Ca^{2+}-induced Ca^{2+} release (CICR). Extracellular release of ProTα-S100A13 complex is driven by use of a flip-flop mechanism. (B) Mechanism of ProTα-induced cell death mode switch and neuroprotection. Serum-free or starvation stress leads to endocytosis of the glucose transporters GLUT1/4, which in turn causes bioenergetic catastrophe-mediated necrosis through a rapid loss of glucose supply. Addition of ProTα to ischemia-treated neurons causes translocation of GLUT1/4 to the membrane to allow sufficient glucose supply through activation of $G_{i/o}$, PLC and $PKC\beta_{II}$. ProTα-induced apoptosis occurs

condition, causing rapid decrease in cellular ATP levels, is explained by the following possible mechanism. To retain ProTα in the nucleus against passive diffusion, the repeated recycling of importin α between cytosol and nucleus is required. Ran, a small G protein, is known to execute this importin recycling process.[43,44] Therefore, the energy crisis—or cellular ATP loss—leads to a decrease in GTP levels and disables importin recycling due to the decreased level of Ran in an active GTP-binding form.

It should be noted that there was no ProTα release when the cells were treated with apoptogenic reagents, though ProTα is released to the cytosol from the nucleus.[11] Detailed studies revealed that caspase-3, activated by apoptogenic reagent treatments, cleaves the C-terminus of ProTα, which contains an NLS and a key domain responsible for the interaction with S100A13. These findings enable us to speculate that cytosolic ProTα is redistributed to the cytosol from the nucleus under apoptotic conditions and may have an antiapoptotic self-defensive function, as mentioned above.

Antinecrosis mechanisms

Under the serum-free condition without any supplements, neurons rapidly die by necrosis, as seen in the typical necrosis features, such as disrupted plasma membranes and swollen mitochondria (in TEM analysis), and rapid decreases in $[^3H]$-2-deoxyglucose uptake and related cellular ATP levels.[9] Pharmacological studies revealed that the survival activity of recombinant ProTα is mediated through activation of phospholipase C (PLC) and protein kinase C (PKC) β.[9] Quite similar changes were reproduced in the low-oxygen and low-glucose (LOG) ischemia-reperfusion culture model. Addi-

tion of ProTα reversed the rapid decrease in the cellular ATP levels of cortical neurons following LOG-ischemic stress and reperfusion with serum-containing medium.[9] We previously reported that the membrane translocation of the glucose transporters GLUT1/4 is largely inhibited in serum-free cultures of cortical neurons, which leads to necrotic cell death.[45,46] In fact, LOG-stress decreased GLUT1/4 membrane translocation, as evaluated by immunocytochemistry and Western blot analysis, and addition of ProTα reversed these changes. This cell biological change with ProTα-induced GLUT1/4 membrane translocation enabled us to successfully characterize the putative receptor signaling mechanisms.[9] The ProTα-induced GLUT1/4 translocation was blocked by the treatments with pertussis toxin and PLC and PKCβ inhibitors. More specifically, the action was abolished by PKCβ$_{II}$ antisense oligodeoxynucleotide (AS-ODN), though ProTα activates PKC isoforms, α, β$_I$, and β$_{II}$, but not γ, δ, ε, or ς. Taken together, the pharmacological studies revealed that the ProTα-induced membrane translocation of GLUT1/4, which underlies the mechanisms for necrosis inhibition, is mediated through activation of putative G$_{i/o}$-coupled receptor, PLC and PKCβ$_{II}$ (Fig. 3B).

Inhibition of rapid necrosis by caspase activation

Although the addition of ProTα delayed the cell death of cortical neurons in serum-free culture, most of the neurons completely died by apoptosis after 24 hours. However, when neurons were treated with ProTα under conditions of ischemia and subsequent reperfusion with serum-containing medium, no significant cell death was observed

later at 12 h after the start of serum-free stress. The machinery is mediated by upregulation of Bax, which in turn causes mitochondrial cyto c release and subsequent apoptosis. Bax upregulation is also mediated by activation of G$_{i/o}$, PLC, and PKC, similar to the case for necrosis. However, both PKCβ$_I$ and PKCβ$_{II}$ upregulations mediate this apoptotic mechanism. Since caspase-3–mediated PARP degradation minimizes the ATP consumption, the apoptosis induction may have a crucial role in inhibiting the rapid necrosis. In addition, since pyruvate, a substrate for ATP production in mitochondria, inhibits necrosis but does not cause apoptosis, the apoptosis machinery seems to be independent of the necrosis inhibition. Neurotrophins, such as BDNF or EPO and type I IFN, which are expected in the ischemic brain and retina, can inhibit the apoptosis machinery at a later stage. (C) *In vivo* neuroprotective role of ProTα in the ischemic brain and retina. ProTα is first released upon ischemic stress in the ischemic core. Released ProTα exerts a suppression of the necrosis of neighboring neurons, which play a role of the early stage of neuroprotection. Expression of neurotrophic factors, such as BDNF and EPO will then occur and block the apoptosis in the penumbra (late stage of neuroprotection). Although dead cell-derived cytotoxic molecules also cause late apoptosis in the penumbra, the initial blockade of necrosis may minimize the occurrence of late apoptosis. This view may be consistent with the rationale for therapies for acute ischemic stroke.

for at least 48 hours.[9] These findings indicate that serum factors prevented ProTα-induced apoptosis. Indeed, further addition of nerve growth factor (NGF), brain-derived growth factor (BDNF), basic fibroblast growth factor (bFGF), or interleukin (IL)-6—representative apoptosis inhibitors[47–51]—rescued the cell survival in serum-free culture for 48 h, while these factors alone had no effects on the survival.[9] Similar effects were observed with BIP-V5, which blocks the translocation of Bax to mitochondria.[52] BIP-V5 also selectively inhibited the ProTα-induced apoptosis.

However, mixed addition of ProTα and N-benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (zVAD-fmk), a pan-type caspase inhibitor, caused necrosis at the later stage.[9,10] This unexpected results may be explained by the view that ProTα-induced continuous cyto.c release causes a loss of ATP and necrosis induction. The reason why ProTα alone causes apoptosis without causing necrosis may be explained by the fact that caspase-3 cleaves poly-(ADP-ribose) polymerase, PARP, which heavily uses NAD, and in turn leads to a decreased ATP synthesis (Fig. 2B). In other words, apoptosis-induction in the early stage after ischemia may play a defensive role in inhibiting rapid cell death by necrosis (Fig. 3C).

In vivo neuroprotection

Systemic and local injections of ProTα markedly inhibits the histological and functional damage induced by cerebral and retinal ischemia.[53,54] Although ProTα inhibits apoptosis as well as necrosis in *in vivo* studies, the discrepancy from *in vitro* studies may be explained by the fact that antiapoptotic factors may inhibited the apoptotic machineries induced by ProTα. This speculation was successfully tested by the *in vivo* administration of antibodies against either brain-derived neurotrophic factor (BDNF) or erythropoietin (EPO), which restores apoptosis in cerebral and retinal ischemia models. Although ProTα upregulates BDNF levels in the retina only in the presence of ischemia, the underlying mechanisms remain elusive. Most recently, it was reported that ProTα causes immune responses through Toll-like receptor 4 (TLR4).[7,8] In this study, exogenous full-length ProTα, and endogenous ProTα released by CD8$^+$ T cells, may act as a signaling ligand for TLR4 and trigger the TRIF-mediated IFN-β induction and MyD88-mediated induction of proinflammatory cytokines, such as TNF-α, to suppress HIV-1 after the entry into macrophages.[7] As there are reports that stroke-induced brain damage is inhibited by a preconditioning treatment with LPS (an activator of TLR4),[55,56] this mechanism might cause the *in vivo* induction of antiapoptotic factors. There are several reports that TLRs cause induction of type-I IFN, which can be neroprotective.[56,57] TLR-4–mediated TRIF-IRF3 signaling may underlie major mechanisms for neuroprotection against stroke.[56,58] It is of interest that heterotrimeric G protein $G_{i/o}$ is important for the activation of MAPK and Akt downstream of TLR4, as well as for the full activation of IFN signaling downstream of TRIF-dependent signaling via TLR4.[59–61] The involvement of $G_{i/o}$ would be also consistent with the ProTα signaling, in terms of the antinecrosis pathway (see Fig. 3B).[9] In our preliminary studies, however, the preconditioning treatment of ProTα only partially inhibited retinal ischemia damage, while posttreatment with ProTα 24 h after the ischemia completely inhibited damage. Therefore, it is also speculated that additional receptor systems for ProTα play a more important for the retinal protection system.

Conclusions

ProTα and thymosin α1 possess a number of extracellular cytokine–like functions, including stimulating up-regulation of HA-DR, IL-2 receptor, dendritic cell maturation, chemotaxis, and possible antiviral, anticancer, and antifungal activities.[62–65] As we do not detect any similar neuroprotective actions with thymosin α1 for necrosis inhibition and neuroprotective actions, it is evident that the machineries underlying beneficial activities of both biologically active molecules are different. The most important take-home messages here is that ProTα plays key roles in the survival activity of intact cells, inhibits necrosis under the condition of neuronal necrosis, and inhibits apoptosis under the condition of apoptosis. The hypothesis is that ProTα acts as a "robustness" or cell death mode switch molecule from uncontrollable necrosis to neurotrophin-reversible apoptosis, and may provide a promising novel strategy for preventing serious damage in stroke (Fig. 3C). However, clarification of mechanisms underlying the intrinsic robust activity of ProTα must wait for the identification of ProTα-binding proteins, including

the G$_{i/o}$-coupled receptor for cell death mode switch. Further studies to examine how these candidate molecules play such multiple functions in cell death regulation are the next exciting step.

## Acknowledgments

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Haritos, A.A., J. Caldarella & B.L. Horecker. 1985. Simultaneous isolation and determination of prothymosin alpha, parathymosin alpha, thymosin beta 4, and thymosin beta 10. *Anal. Biochem.* **144:** 436–440.

2. Clinton, M., L. Graeve, H. el-Dorry, *et al.* 1991. Evidence for nuclear targeting of prothymosin and parathymosin synthesized in situ. *Proc. Natl. Acad. Sci. USA* **88:** 6608–6612.

3. Martini, P.G., R. Delage-Mourroux, D.M. Kraichely & B.S. Katzenellenbogen. 2000. Prothymosin alpha selectively enhances estrogen receptor transcriptional activity by interacting with a repressor of estrogen receptor activity. *Mol. Cell Biol.* **20:** 6224–6232.

4. Bianco, N.R. & M.M. Montano. 2002. Regulation of prothymosin alpha by estrogen receptor alpha: molecular mechanisms and relevance in estrogen-mediated breast cell growth. *Oncogene* **21:** 5233–5244.

5. Jiang, X., H.E. Kim, H. Shu, *et al.* 2003. Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway. *Science* **299:** 223–226.

6. Piñeiro, A., O.J. Cordero & M. Nogueira. 2000. Fifteen years of prothymosin alpha: contradictory past and new horizons. *Peptides* **21:** 1433–1446.

7. Mosoian, A., A. Teixeira, C.S. Burns, *et al.* 2010. Prothymosin-alpha inhibits HIV-1 via Toll-like receptor 4-mediated type I interferon induction. *Proc. Natl. Acad. Sci. USA* **107:** 10178–10183.

8. Mosoian, A. 2011. Intracellular and extracellular cytokine-like functions of prothymosin α: implications for the devel-opment of immunotherapies. *Future Med. Chem.* **3:** 1199–1208.

9. Ueda, H., R. Fujita, A. Yoshida, *et al.* 2007. Identification of prothymosin-alpha1, the necrosis-apoptosis switch molecule in cortical neuronal cultures. *J. Cell Biol.* **176:** 853–862.

10. Ueda, H. 2009. Prothymosin alpha and cell death mode switch, a novel target for the prevention of cerebral ischemia-induced damage. *Pharmacol. Ther.* **123:** 323–333.

11. Matsunaga, H. & H. Ueda. 2010. Stress-induced non-vesicular release of prothymosin-á initiated by an interaction with S100A13, and its blockade by caspase-3 cleavage. *Cell Death Differ.* **17:** 1760–1772.

12. Gómez-Márquez, J. 2007. Function of prothymosin alpha in chromatin decondensation and expression of thymosin beta-4 linked to angiogenesis and synaptic plasticity. *Ann. N.Y. Acad. Sci.* **1112:** 201–209.

13. Dominguez, F., C. Magdalena, E. Cancio, *et al.* 1993. Tissue concentrations of prothymosin alpha: a novel proliferation index of primary breast cancer. *Eur. J. Cancer* **29A:** 893–897.

14. Wu, C.G., N.A. Habib, R.R. Mitry, *et al.* 1997. Overexpression of hepatic prothymosin alpha, a novel marker for human hepatocellular carcinoma. *Br. J. Cancer* **76:** 1199–1204.

15. Magdalena, C., F. Dominguez, L. Loidi & J.L. Puente. 2000. Tumour prothymosin alpha content, a potential prognostic marker for primary breast cancer. *Br. J. Cancer* **82:** 584–590.

16. Sasaki, H., Y. Sato, S. Kondo, *et al.* 2001. Expression of the prothymosin alpha mRNA correlated with that of N-myc in neuroblastoma. *Cancer Lett.* **168:** 191–195.

17. Hapke, S., H. Kessler, B. Luber, *et al.* 2003. Ovarian cancer cell proliferation and motility is induced by engagement of integrin alpha(v)beta3/Vitronectin interaction. *Biol. Chem.* **384:** 1073–1083.

18. Shiwa, M., Y. Nishimura, R. Wakatabe, *et al.* 2003. Rapid discovery and identification of a tissue-specific tumor biomarker from 39 human cancer cell lines using the SELDI ProteinChip platform. *Biochem. Biophys. Res. Commun.* **309:** 18–25.

19. Leys, C.M., S. Nomura, B.J. LaFleur, *et al.* 2007. Expression and prognostic significance of prothymosin-alpha and ERp57 in human gastric cancer. *Surgery* **141:** 41–50.

20. Kashat, L., A. So, O. Masui, *et al.* 2010. Secretome based Identification and Characterization of Potential Biomarkers in Thyroid Cancer. *J. Proteome Res.* **9:** 5757–5769.

21. Gómez-Márquez, J., F. Segade, M. Dosil, *et al.* 1989. The expression of prothymosin alpha gene in T lymphocytes and leukemic lymphoid cells is tied to lymphocyte proliferation. *J. Biol. Chem.* **264:** 8451–8454.

22. Conteas, C.N., M.G. Mutchnick, K.C. Palmer, *et al.* 1990. Cellular levels of thymosin immunoreactive peptides are linked to proliferative events: evidence for a nuclear site of action. *Proc. Natl. Acad. Sci. USA* **87:** 3269–3273.

23. Smith, M.R., A. al-Katib, R. Mohammad, *et al.* 1993. Prothymosin alpha gene expression correlates with proliferation, not differentiation, of HL-60 cells. *Blood* **82:** 1127–1132.

24. Subramanian, C., S. Hasan, M. Rowe, *et al.* 2002. Epstein–Barr virus nuclear antigen 3C and prothymosin alpha interact with the p300 transcriptional coactivator at the CH1 and CH3/HAT domains and cooperate in regulation of transcription and histone acetylation. *J. Virol.* **76:** 4699–4708.

25. Karetsou, Z., A. Kretsovali, C. Murphy, *et al.* 2002. Prothymosin alpha interacts with the CREB-binding protein and potentiates transcription. *EMBO Rep.* **3:** 361–366.

26. Happel, N. & D. Doenecke. 2009. Histone H1 and its isoforms: contribution to chromatin structure and function. *Gene* **431:** 1–12.

27. Martini, P.G. & B.S. Katzenellenbogen. 2003. Modulation of estrogen receptor activity by selective coregulators. *J. Steroid Biochem. Mol. Biol.* **85:** 117–122.

28. Dhakshinamoorthy, S., D.J. Long & A.K. Jaiswal. 2000. Antioxidant regulation of genes encoding enzymes that detoxify xenobiotics and carcinogens. *Curr. Top Cell Regul.* **36:** 201–216.

29. Jaiswal, A.K. 2000. Regulation of genes encoding NAD(P)H:quinone oxidoreductases. *Free Radic. Biol. Med.* **29:** 254–262.

30. Kobayashi, M. & M. Yamamoto. 2006. Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv. Enzyme Regul.* **46:** 113–140.

31. McMahon, M., N. Thomas, K. Itoh, *et al.* 2006. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex. *J. Biol. Chem.* **281:** 24756–24768.

32. Karapetian, R.N., A.G. Evstafieva, I.S. Abaeva, *et al.* 2005. Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes. *Mol. Cell Biol.* **25:** 1089–1099.

33. Niture, S.K. & A.K. Jaiswal. 2009. Prothymosin-alpha mediates nuclear import of the INrf2/Cul3 Rbx1 complex to degrade nuclear Nrf2. *J. Biol. Chem.* **284:** 13856–13868.

34. Schafer, Z.T. & S. Kornbluth. 2006. The apoptosome: physiological, developmental, and pathological modes of regulation. *Dev. Cell* **10:** 549–561.

35. Reubold, T.F. & S. Eschenburg. 2012. A molecular view on signal transduction by the apoptosome. *Cell Signal* **24:** 1420–1425.

36. Qi, X., L. Wang & F. Du. 2010. Novel Small Molecules Relieve Prothymosin alpha-Mediated Inhibition of Apoptosome Formation by Blocking Its Interaction with Apaf-1. *Biochemistry* **49:** 1923–1930.

37. Evstafieva, A.G., G.A. Belov, M. Kalkum, *et al.* 2000. Prothymosin alpha fragmentation in apoptosis. *FEBS Lett.* **467:** 150–154.

38. Evstafieva, A.G., G.A. Belov, Y.P. Rubtsov, *et al.* 2003. Apoptosis-related fragmentation, translocation, and properties of human prothymosin alpha. *Exp. Cell Res.* **284:** 211–223.

39. Malicet, C., J.C. Dagorn, J.L. Neira & J.L. Iovanna. 2006. p8 and prothymosin alpha: unity is strength. *Cell Cycle* **5:** 829–830.

40. Malicet, C., V. Giroux, S. Vasseur, *et al.* 2006. Regulation of apoptosis by the p8/prothymosin alpha complex. *Proc. Natl. Acad. Sci. USA* **103:** 2671–2676.

41. Matsunaga, H. & H. Ueda. 2006. Voltage-dependent N-type Ca^{2+} channel activity regulates the interaction between FGF-1 and S100A13 for stress-induced non-vesicular release. *Cell Mol. Neurobiol.* **26:** 237–246.

42. Matsunaga, H. & H. Ueda. 2006. Evidence for serum-deprivation-induced co-release of FGF-1 and S100A13 from astrocytes. *Neurochem. Int.* **49:** 294–303.

43. Yasuda, Y., Y. Miyamoto, T. Saiwaki & Y. Yoneda. 2006. Mechanism of the stress-induced collapse of the Ran distribution. *Exp. Cell Res.* **312:** 512–520.

44. Yasuhara, N., M. Oka & Y. Yoneda. 2009. The role of the nuclear transport system in cell differentiation. *Semin Cell Dev. Biol.* **20:** 590–599.

45. Fujita, R. & H. Ueda. 2003. Protein kinase C-mediated necrosis-apoptosis switch of cortical neurons by conditioned medium factors secreted under the serum-free stress. *Cell Death Differ* **10:** 782–790.

46. Fujita, R. & H. Ueda. 2003. Protein kinase C-mediated cell death mode switch induced by high glucose. *Cell Death Differ* **10:** 1336–1347.

47. Kaplan, D.R. & F.D. Miller. 2000. Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* **10:** 381–391.

48. Ay, I., H. Sugimori & S.P. Finklestein. 2001. Intravenous basic fibroblast growth factor (bFGF) decreases DNA fragmentation and prevents downregulation of Bcl-2 expression in the ischemic brain following middle cerebral artery occlusion in rats. *Brain Res. Mol. Brain Res.* **87:** 71–80.

49. Patapoutian, A. & L.F. Reichardt. 2001. Trk receptors: mediators of neurotrophin action. *Curr. Opin. Neurobiol.* **11:** 272–280.

50. Sofroniew, M.V., C.L. Howe & W.C. Mobley. 2001. Nerve growth factor signaling, neuroprotection, and neural repair. *Annu. Rev. Neurosci.* **24:** 1217–1281.

51. Yamashita, T., K. Sawamoto, S. Suzuki, *et al.* 2005. Blockade of interleukin-6 signaling aggravates ischemic cerebral damage in mice: possible involvement of Stat3 activation in the protection of neurons. *J. Neurochem.* **94:** 459–468.

52. Yoshida, T., I. Tomioka, T. Nagahara, *et al.* 2004. Bax-inhibiting peptide derived from mouse and rat Ku70. *Biochem. Biophys. Res. Commun.* **321:** 961–966.

53. Fujita R. & H. Ueda. 2007. Prothymosin-alpha1 prevents necrosis and apoptosis following stroke. *Cell Death Differ* **14:** 1839–1842.

54. Fujita, R., M. Ueda, K. Fujiwara & H. Ueda. 2009. Prothymosin-alpha plays a defensive role in retinal ischemia through necrosis and apoptosis inhibition. *Cell Death Differ* **16:** 349–358.

55. Stevens, S.L., P.Y. Leung, K.B. Vartanian, *et al.* 2011. Multiple preconditioning paradigms converge on interferon regulatory factor-dependent signaling to promote tolerance to ischemic brain injury. *J. Neurosci.* **31:** 8456–8463.

56. Vartanian, K.B., S.L. Stevens, B.J. Marsh, *et al.* 2011. LPS preconditioning redirects TLR signaling following stroke: TRIF-IRF3 plays a seminal role in mediating tolerance to ischemic injury. *J. Neuroinflammation* **8:** 140.

57. Leung, P.Y., S.L. Stevens, A.E. Packard, *et al.* 2012. Toll-like receptor 7 preconditioning induces robust neuroprotection against stroke by a novel type I interferon-mediated mechanism. *Stroke* **43:** 1383–1389.

58. Marsh, B., S.L. Stevens, A.E. Packard, *et al.* 2009. Systemic lipopolysaccharide protects the brain from ischemic injury by

reprogramming the response of the brain to stroke: a critical role for IRF3. *J. Neurosci.* **29:** 9839–9849.

59. Fan, H., B. Zingarelli, O.M. Peck, *et al.* 2005. Lipopolysaccharide- and gram-positive bacteria-induced cellular inflammatory responses: role of heterotrimeric Galpha(i) proteins. *Am. J. Physiol. Cell Physiol.* **289:** C293–C301.

60. Cuschieri, J., J. Billgren & R.V. Maier. 2006. Phosphatidylcholine-specific phospholipase C (PC-PLC) is required for LPS-mediated macrophage activation through CD14. *J. Leukoc. Biol.* **80:** 407–414.

61. Dauphinee, S.M., V. Voelcker, Z. Tebaykina, *et al.* 2011. Heterotrimeric Gi/Go proteins modulate endothelial TLR signaling independent of the MyD88-dependent pathway. *Am. J. Physiol. Heart Circ. Physiol.* **301:** H2246–H2253.

62. Grünberg, E., K. Eckert, H.R. Maurer, *et al.* 1997. Prothymosin alpha1 effects on IL-2-induced expression of LFA-1 on lymphocytes and their adhesion to human umbilical vein endothelial cells. *J. Interferon. Cytokine Res.* **17:** 159–165.

63. Heidecke, H., K. Eckert, K. Schulze-Forster & H.R. Maurer. 1997. Prothymosin alpha 1 effects in vitro on chemotaxis, cytotoxicity and oxidative response of neutrophils from melanoma, colorectal and breast tumor patients. *Int. J. Immunopharmacol.* **19:** 413–420.

64. Moody, T.W., J. Leyton, F. Zia, *et al.* 2000. Thymosinalpha1 is chemopreventive for lung adenoma formation in A/J mice. *Cancer Lett.* **155:** 121–127.

65. Romani, L., F. Bistoni, C. Montagnoli, *et al.* 2007. Thymosin alpha1: an endogenous regulator of inflammation, immunity, and tolerance. *Ann. N.Y. Acad. Sci.* **1112:** 326–338.
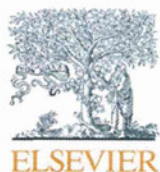
Flexible, layered biofuel cells

Takeo Miyake [a,b,*], Keigo Haneda [a], Syuhei Yoshino [a], Matsuhiko Nishizawa [a,b,*]

[a] Department of Bioengineering and Robotics, Tohoku University, 6-6-1 Aramaki Aoba, Aoba-ku, Sendai 980-8579, Japan
[b] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Tokyo 102-0075, Japan

ARTICLE INFO

ABSTRACT

Similar to conventional electrolyte batteries, biofuel cells often need to be stacked in order to boost their single cell voltage (< 1 V) up to a practical level. Here, we report a laminated stack of biofuel cells that is composed of bioanode fabrics for fructose oxidation, hydrogel sheets containing electrolyte and fuel (fructose), and O_2-diffusion biocathode fabrics. The anode and cathode fabrics were prepared by modifying fructose dehydrogenase and bilirubin oxidase, respectively, on carbon nanotubes-decorated carbon fiber fabrics. The total thickness of the single set of anode/gel/cathode sheets is just 1.1 mm. The laminated triple-layer stack produces an open-circuit voltage of 2.09 V, which is a 2.8-fold increase over that of a single set cell (0.74 V). The present layered cell (5 mm × 5 mm) produces a maximum power of 0.64 mW at 1.21 V, a level that is sufficient to drive light-emitting diodes.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Enzyme-based biofuel cells (BFCs) that generate electricity through enzymatic oxidation of biological fuels like sugars and alcohols have attracted attention as ubiquitous, safe power sources (Heller, 2004; Barton et al., 2004; Cooney et al., 2008; Willner et al., 2009). In this decade, the output current of enzymatic BFCs have been dramatically improved from μA to mA level (Sakai et al., 2009; Gao et al., 2010; Zebda et al., 2011; Miyake et al., 2011a). In contrast, the cell voltage is determined by the coupling of redox reactions at anode and cathode, and is typically limited around 1.0 V, a value that is insufficient for many practical applications; for example, a single light-emitting diode (LED) requires a voltage larger than 1.6 V. Therefore, in a similar manner to electrolyte batteries, BFCs are often stacked for boosting the output voltage (Ferrigno et al., 2002; Sakai et al., 2009; Gellett et al., 2010; Holzinger et al., in press). When stacking with series-connections, each BFC should be isolated by proper packaging to prevent short-circuits via ion-conductive fuel solutions, and these packages are then interconnected electrically with metal lead. Such requirements, however, are often troublesome from the standpoint of exploiting the BFC's simplicity and disposability.

In this manuscript, we describe a layered biofuel cell constructed by laminating enzyme-modified carbon fabric (CF) strips and hydrogel film containing electrolyte and fuel as shown in Fig. 1. The hydrogel sheets ensure ion-conduction between anode/cathode fabrics, and also serve as the fuel tank that could eliminate the necessity of packaging. A BFC sheet using a conventional agarose (Haneda et al., in press) was thick and weak due to the fragile nature of agarose. In the present work, we employ a heavy-duty "double network (DN) hydrogel", resulting in a very flexible, thinner BFC (~1 mm thickness). The pre-modification of CF with carbon nanotubes (CNTs) was effective to improve the performances of both bioanode and biocathode. The laminated stack of the improved bioelectrodes was practical for LED lighting.

2. Experimental section

2.1. Preparation of carbon fabric anodes

A 5 mm × 5 mm strip (0.3 mm thickness) of carbon fabric (CF) (TCC-3250, donated from Toho Tenax Co.) was first modified with multiwalled carbon nanotubes (CNTs) (Baytubes, donated from Bayer Material Science Co.) to increase the specific surface area (Supplementary Fig. 1). The CNTs were pretreated by heating at 400 °C for 11 h and by immersing in mixed acid (H_2SO_4+HNO_3 in a 1:3 ratio) for 5 h. The treated CNT were dispersed in water containing 0.5% Triton X-100 surfactant. A 40 μl aliquot of the 10 mg ml^{-1} CNT dispersion was dropped on a CF strip and dried in air. After degassing the CNT-modified strip by immersion in a stirred McIlvaine buffer solution for more than 1 h under vacuum

* Corresponding authors at: Tohoku University Department of Bioengineering and Robotics, 6-6-1 Aramaki Aoba, Aoba-ku, Sendai 980-8579, Japan. Tel./fax: +81 22 795 7003.
  E-mail addresses: miyake@biomems.mech.tohoku.ac.jp (T. Miyake), nishizawa@biomems.mech.tohoku.ac.jp (M. Nishizawa).
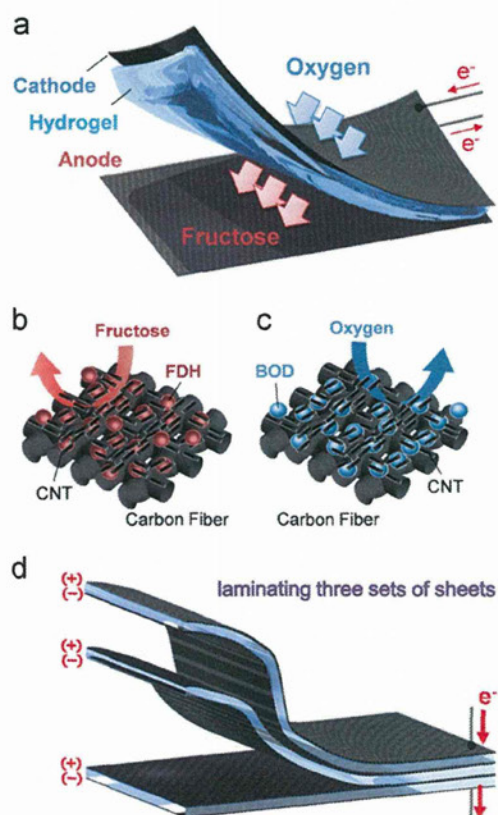
Fig. 1. (a) Schematic illustration of a biofuel cell sheet constructed by laminating enzyme-modified nanoengineered carbon fabric strips with a hydrogel film that retains electrolyte solutions and fructose fuel. (b) Schemes of fructose oxidation at the enzymatic anode. (c) Schemes of oxygen reduction at the enzymatic gas-diffusion cathode. (d) Schematic illustration of multi-lamination for boosting power.

(0.09 MPa), the CNT-modified strip shows hydrophilic property. Then, the CNT-modified CF strip was immersed in a stirred solution of D-fructose dehydrogenase (FDH) (EC1.1.99.11, 169.9 U mg^{-1}, ca. 140 kDa, from Gluconobacter, purchased from Toyobo Enzyme Co.) for FDH immobilization (Tominaga et al., 2009; Tsujimura et al., 2010; Miyake et al., 2011b). It has been reported that FDH works as an electrocatalyst for two-electron oxidation of fructose (Tominaga et al., 2007; Murata et al., 2009). The flavin-containing subunit of FDH accepts electrons from fructose, and transfers these electrons to the heme c-containing subunit that can electrically communicate with electrode. A geometric area of 0.564 cm^2 was utilized for calculation of the current density in cyclic voltammetry (CV).

2.2. Preparation of gas-diffusion carbon fabric cathodes

The preparation of the cathode basically followed the procedures used for our previous carbon particle (ketjenblack)-based BOD cathode (Miyake et al., 2011b; Haneda et al., in press). BOD is one of the multi-copper oxidases that can directly catalyze the four-electron reduction of O_2 to H_2O even without electron transfer mediators (Tsujimura et al., 2007; Wen et al., 2011). The type 1 Cu site of BOD accepts electrons from fabric electrode, and transfers these electrons to the type 2–3 cluster of BOD for O_2 reduction. A 40 μl aliquot of a 10 mg ml^{-1} CNT solution was put on a CF strip and dried in air, followed by thoroughly washing out the surfactant by soaking in an ethanol solution for more than 1 h with stirring. The surface of the CNT-modified CF electrode was

further modified with a 0.1 ml solution of 5 mg ml^{-1} bilirubin oxidase (BOD, EC 1.3.3.5, 2.5 U/mg, from Myrothecium) in vacuum oven (AVO-205N, purchased from AS ONE, 0.09 MPa, 35 C°). The strip was additionally coated with the CNT solution to make the surface hydrophobic. A surface area of 0.25 cm^2 was utilized for calculation of the current density in cyclic voltammetry (CV).

2.3. Preparation of the hydrogel films

The fructose-containing double-network (DN) hydrogel films were prepared by a three-step process(Gong, 2010; Wu and Gong, 2011): (1) single network hydrogel formation, (2) second network formation into the single network hydrogel and (3) loading of 500 mM fructose. We used stock solutions A, B and C. Solution A contains 2-acrylamido-2-methlypropane (AMPS, 1 M), N,N-methylenebisacrylamide (MBAA, 40 mM), 2-oxoglutaric acid (OA, 1 mM) and ammonium persulfate (APS, 19 mM). Solution B was a mixture of acrylamide (AAm, 4 M), OA (1 mM), NaCl (80 mM) and APS (19 mM). Solution C contains AAm (2 M), OA (1 mM) and APS (19 mM). At first, the solution A was poured into a silicone mold, and preliminarily crosslinked by UV exposure (265 nm, 8 W) for 5 h. The formed soft gel was then immersed in solution B for 14 h to prevent dramatic swelling and further irradiated with the UV lamp for 5 h to reinforce the gel in order to become a sheet. After washing with water for 24 h, the gel sheet was immersed in solution C for 14 h followed by UV irradiation (5 h) to form a DN network. Finally, the DN gel sheet was immersed in 500 mM fructose solution for 24 h.

2.4. Electrochemical measurements

The performance of the CF electrodes was analyzed by a three-electrode system (BSA, 730C electrochemical analyzer) in solution using a Ag/AgCl reference and a platinum counter electrode. The FDH-modified anodes were evaluated in stirred McIlvaine buffer (pH 5.0) containing 500 mM fructose, while the BOD-modified cathodes were used in air-saturated McIlvaine buffer (pH 5.0). The performance of a biofuel cell sheet constructed using the fructose-containing DN hydrogel film (0.5 mm thick) was evaluated from the cell voltage upon connecting with a variable external resistance between 180 Ω and 10 kΩ. The current and the power were derived from the cell voltage and the resistance. Unless otherwise indicated, the electrochemical measurements were carried out at room temperature, around 25 °C.

3. Results and discussion

3.1. Performance of FDH/CNT/CF bioanodes

Fig. 2a shows cyclic voltammograms of the FDH/CNT/CF electrodes (solid plots) at 10 mV s^{-1} in a stirred buffer solution containing 500 mM fructose. In comparison with the FDH/CF electrode prepared without CNTs (broken line plot), the increased specific surface area produced by CNT-modification obviously increased the current density by at least an order of magnitude. In fact, the measured double-layer capacitance of the CNT-modified electrodes has a 2 orders larger value (ca. 6.7 mF cm^{-2}) than that of the original CF (0.07 mF cm^{-2}). The oxidation current density depended on the concentration of the Triton X-100 surfactant used for the CNT dispersion (Haneda et al., in press). The CNT dispersion with 0.5% surfactant is capable of entirely penetrating into the CF strip (see Supplementary Fig. 1b). This uniform modification with CNT would be a reason for the enhanced anode performance. In addition, the electrode
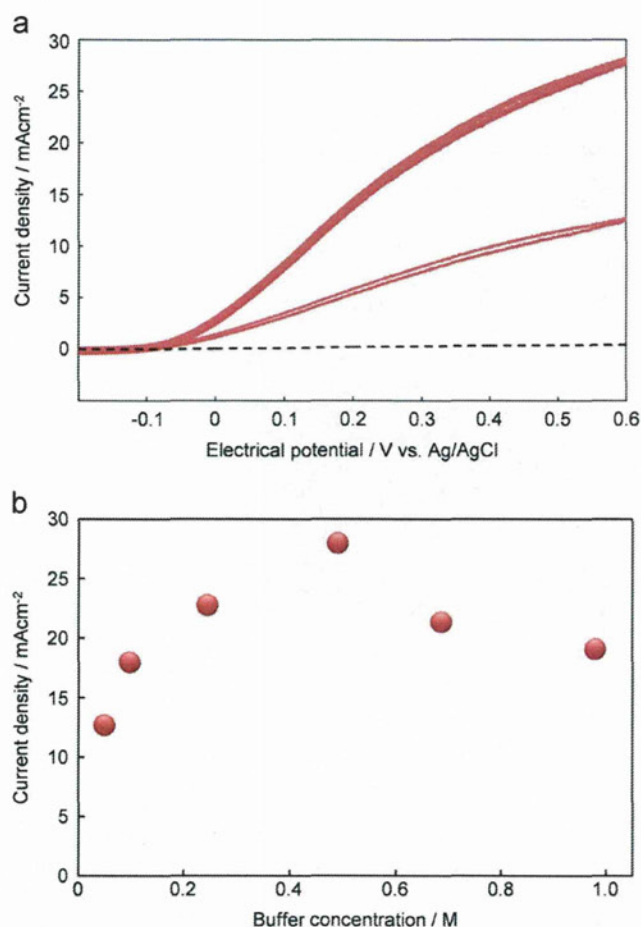
a

b

Fig. 2. (a) Cyclic voltammograms of fructose oxidation at 10 mV s^{-1} in a stirred 50 mM buffer solution (pH 5) containing 500 mM fructose. The CF strip electrodes were modified with only 10 mg ml^{-1} FDH (broken black line) or with both 10 mg ml^{-1} CNT and 10 mg ml^{-1} FDH (red line). The activity of the bioanode fabric was enhanced by optimizing the buffer concentration to 0.5 M (red bold line). (b) Current density at 0.6 V vs. Ag/AgCl of the FDH anode measured in different buffer concentration. The measurements were carried out for the same FDH anode specimen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

performance was drastically enhanced by increasing the buffer concentration in the measurement solutions from 50 mM to 0.5 M (red bold line). This is made possible by the existence of adequate buffer capacities that prevent local pH changes caused by oxidation products. Excess buffer concentration, however, lead to a low current density (see Fig. 2b). This is probably due to lowering of the enzyme activity because the enzyme is exposed by a high ionic strength solution. The maximum current of the optimized bioanode produced 15.8 mA (28.0 mA cm^{-2}) at 0.6 V using a 500 mM buffer. Even in quiescent conditions, the current reached 5.8 mA (10 mA cm^{-2}) at 0.6 V (Supplementary Fig. 2), being equivalent to that in DN hydrogel made with 500 mM McIlvaine buffer solution (pH 5.0) containing 500 mM fructose.

3.2. Performance of gas-diffusion biocathodes

Fig. 3a shows cyclic voltammograms of a BOD-modified CF cathode at 10 mV s^{-1}. The electrode strip was put on an oxygenic pH 5.0 buffer solution so as to contact the solution by the BOD-modified face (green line). The reduction current density reaches ~1.9 mA cm^{-2} (at 0 V) by utilizing an oxygen supply from the ambient air through the CF. Moreover, an additional CNT coating
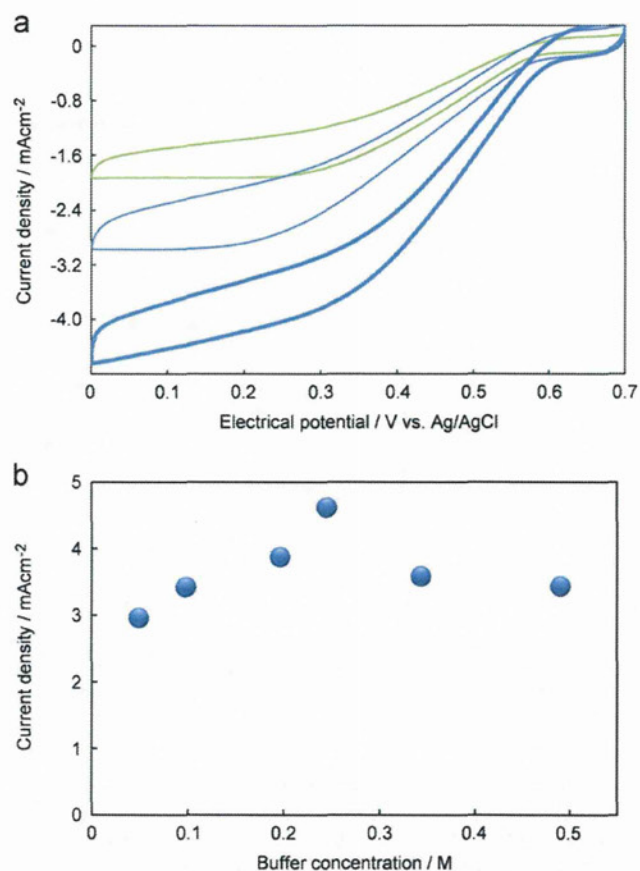
a

b

Fig. 3. (a) Cyclic voltammograms of O$_2$ reduction at a BOD/CNT-modified CF strip measured at 10 mV s^{-1} on the solution (green). The activity of the CF electrode was enhanced by further modification with CNT after the BOD immobilization (blue line) and by subsequent optimization of buffer concentration (bold blue). (b) Current density at 0 V vs. Ag/AgCl of the BOD cathode fabric with different buffer concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

onto the BOD-modified face of the CF strip enhanced the performance further to 2.9 mA cm^{-2}. Presumably, the hydrophobic nature of that coating controls excess penetration of solution into the CF electrodes (Supplementary Fig. 3) (Miyake et al., 2011b; Haneda et al., in press). In addition to optimizing the performance of the bioanode fabric, the cathodic performance can be optimized by changing the buffer concentration (Fig. 3b); the maximum current was 4.6 mA cm^{-2} at 0 V using a 250 mM buffer condition.

3.3. Performance of the laminated biofuel cell sheets

The FDH/CNT-modified CF anode and the CNT/BOD/CNT-modified gas-diffusion CF cathode were laminated to the opposite faces of a DN hydrogel sheet (0.5 mm thick) made with 250 mM McIlvaine buffer solution (pH 5.0) containing 500 mM fructose. The enzyme-modified hydrophilic anode appeared to become moistened by blotting of the solution from the hydrogel layer. On the other hand, the O$_2$ reduction at the hydrophobic cathode proceeded at the three-phase boundary of the hydrogel–electrode interface. Fig. 4a shows typical examples of the cell performance. The open-circuit voltage of the cell was 0.74 V, which is similar to the difference between the potentials at which fructose oxidation and oxygen reduction start to occur in cyclic voltammograms (−0.14 V and 0.60 V in Figs. 2a and 3, respectively). The maximum power density reached 0.95 mW cm^{-2} at 0.36 V, which was determined by the BOD-cathode fabric because of its