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Cellular prion protein directly interacts with and enhances lactate dehydrogenase expression under hypoxic conditions

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ABSTRACT

Although a physiological function of the cellular prion protein (PrP^c) is still not fully clarified a PrP^c-mediated neuroprotection against hypoxic/ischemic insult is intriguing. After ischemic stroke prion knockout mice ($Prnp^{0/0}$) display significantly greater lesions as compared to wild-type (WT) mice. Earlier reports suggested an interaction between the glycolytic enzyme lactate dehydrogenase (LDH) and PrP^c. Since hypoxic environment enhances LDH expression levels and compels neurons to rely on lactate as an additional oxidative substrate for energy metabolism, we examined possible differences in LDH protein expression in WT and $Prnp^{0/0}$ knockout models under normoxic/hypoxic conditions in vitro and in vivo, as well as in a HEK293 cell line.

While no differences are observed under normoxic conditions, LDH expression is markedly increased after 60-min and 90-min of hypoxia in WT vs. $Prnp^{0/0}$ primary cortical neurons with concurrent less hypoxia-induced damage in the former group. Likewise, cerebral ischemia significantly increases LDH levels in WT vs. $Prnp^{0/0}$ mice with accompanying smaller lesions in the WT group. HEK293 cells overexpressing PrP^c show significantly higher LDH expression/activity following 90-min of hypoxia as compared to control cells. Moreover, a cytoplasmic co-localization of LDH and PrP^c was recorded under both normoxic and hypoxic conditions. Interestingly, an expression of monocarboxylate transporter 1, responsible for cellular lactate uptake, increases with PrP^c-overexpression under normoxic conditions. Our data suggest LDH as a direct PrP^c interactor with possible physiological relevance under low oxygen conditions.

1. Introduction

The physiological function of the cellular prion protein (PrP^c) remains unclear, although numerous studies point to its neuroprotective role (Walz et al., 1999; McLennan et al., 2004; Weise et al., 2004). PrP^c is a multifunctional protein involved in counteraction of oxidative stress (Brown et al., 1997; Wong et al., 2001), modulation of cell death (Kuwahara et al., 1999; Bounhar et al., 2001) and activation of several signal transduction pathways known to promote neuronal survival (Mouillet-Richard et al., 2000; Zanata et al., 2002). Interestingly, ablation of the cellular prion protein gene (Prnp) is not crucial for viability (Bueler et al., 1992; Kuwahara et al., 1999). However, under circumstances of increased physiological demands, such as brain seizures or ischemia the presence of PrP^c becomes decisive (Walz et al., 1999; Weise et al., 2004). Hence, cerebral PrP^c is up-regulated early in response to focal cerebral ischemia and prion protein knockout ($Prnp^{0/0}$) mice display significantly greater infarct volumes as compared to wild-type (WT) mice following both permanent and transient ischemia (McLennan et al., 2004; Mitteregger et al., 2007). Even though it has been suggested that PrP^c exerts neuroprotective effects via phosphatidylinositol 3-kinase (PI3K)/Akt (Weise et al., 2006) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway (Shyu et al., 2005b; Spudich et al., 2005), molecular mechanisms underlying PrP^c mediated neuroprotection after ischemic brain injury require further characterization.

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Several earlier reports demonstrated beneficial effects of LDH product, lactate, on post-hypoxic/post-ischemic neuronal tissue (Schurr et al., 1988, 1997, 2001). The astrocyte–neuron lactate shuttle hypothesis postulates that lactate is an essential component of metabolic crosstalk between astrocytes and neurons enabling neuronal recovery under circumstances of high energy demand such as hypoxia/ischemia (Pellerin and Magistretti, 1994). Furthermore, administration of lactate directly after middle cerebral artery occlusion leads to a significant decrease in lesion size and an improvement in neurologic outcome in mice (Berthet et al., 2009).

A previous study demonstrated PrP^c involvement in the regulation of glutamate-dependent lactate transport of cultured astrocytes (Kleene et al., 2007). We reported a marked PrP^c-induced up-regulation of the LDH isoform A (LDH-A) after introduction of PRNP gene into $Prnp^{0/0}$ cells (Ramljak et al., 2008). Meanwhile, interactome analyses identified the LDH-A isoform not only as a PrP^c interaction partner but also as an interactor of Doppel and Shadoo, two mammalian PrP^c paralogues (Watts et al., 2009), indicating a potential physiologically relevant association of both proteins.

A functional relation between PrP^c and LDH could be particularly intriguing in view of the beneficial effects cerebral lactate might exert on neuronal recovery of WT as compared to $Prnp^{0/0}$ mice after hypoxia/ischemia. In the present study, we verified LDH expression levels in primary cortical neurons derived from WT and $Prnp^{0/0}$ mice under both normoxic and hypoxic conditions. In addition, we scrutinized LDH expression in WT and $Prnp^{0/0}$ mice subjected to transient focal cerebral ischemia. Finally, we employed a HEK293 cell line as a cell model and examined regulation of LDH protein levels/activity in PrP^c-overexpressing vs. control HEK293 cells (expressing endogenous levels of PrP^c) under normoxic and hypoxic conditions. Furthermore, we investigated a possibility of direct interaction between LDH and PrP^c by means of immunoprecipitation and co-localization experiments.

2. Material and methods

2.1. Preparation and maintenance of primary cortical neurons and hypoxia–re-oxygenation treatment

Pregnant WT and $Prnp^{0/0}$ mice were anaesthetized on day 14 of gestation using 2-bromo-2-chloro-1,1,1-trifluoroethane (Sigma-Aldrich, Taufkirchen, Germany) and then sacrificed by cervical dislocation. The genetic background of WT and $Prnp^{0/0}$ mice is described in detail in the section Transgenic mice. Briefly, brains from day 14 embryos were removed and stripped of meninges. Afterwards, cortex tissue was isolated and mechanically dissociated by several pipetting passages after a 10-min treatment with trypsin/EDTA (Biochrom, Berlin, Germany) at 37 °C. Cortical cells were then centrifuged, counted and finally plated on poly-D-lysine (10 μM)-coated glass cover-slips in culture wells at a density of 1×10^6 cells/well (9.6 cm^2). Cell cultures were first grown in DMEM (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich), supplemented with 10% FCS (Biochrom), 20 mM KCl, N-2 (1:100, Gibco/Invitrogen, Karlsruhe, Germany), B-27 (1:50, Gibco/Invitrogen) supplemented with antioxidants, and 0.1% P/S (penicillin–streptomycin) (Gibco/Invitrogen). Four days after preparation, preexisting medium was replaced by fresh prepared DMEM, containing 10% FCS, B-27, N-2 and the antimitotics uridine (U) and 5-Fuoro-2′-deoxyuridine (FdU) (Sigma-Aldrich) in order to reduce astrocyte proliferation.

Hypoxia/re-oxygenation experiments were conducted after 7 days in vitro. Cells were placed in a hypoxic chamber (Labotect, incubator C42, Goettingen, Germany) at 37 °C, 5% CO_2, 95% humidity and 1% O_2 for various incubation periods (30, 60 and 90 min) followed by 12 h of re-oxygenation under standard conditions (37 °C, 5% CO_2 and 95% humidity). For Western blot analysis, cells were washed in phosphate-buffered saline (PBS), scraped on ice in a cell lysis buffer, containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100 (Roth, Karlsruhe, Germany), protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany).

2.2. Transgenic mice

All experimental procedures were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals and approved by local authorities. Adult, male WT and $Prnp^{0/0}$ mice weighing 22–27 g were used in the study. Both WT and $Prnp^{0/0}$ mice were of mixed (129/Sv × C57BL/6) genetic background. $Prnp^{0/0}$ mice were generated as described earlier (Bueler et al., 1992).

After mice have been sacrificed, brains were taken either as a whole or were dissociated on ice into four brain regions: hippocampus, cortex, cerebellum and olfactory bulb. Subsequently, whole brains/brain regions were cut, complemented with lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 (Roth) and cocktail of protease inhibitors (Roche) and homogenized. Brain lysate samples were rotated for 15 min and centrifuged at 4 °C at 13,000 ×*g*. Supernatants were transferred in separate tubes and stored at −80 °C.

2.3. Induction of transient focal cerebral ischemia

Animals were anaesthetised with 1%–1.5% isofluran (30% O_2, remainder N_2O). Rectal temperature was maintained at 36.5–37 °C employing a feedback controlled heating system. In order to assess cerebral blood flow, laser-Doppler flow (LDF) was recorded during all experiments using a 0.5 mm fiberoptic probe (Perimed, Stockholm, Sweden) attached to the skull overlying the core region of the middle cerebral artery (MCA) territory (2 mm posterior, 6 mm lateral from bregma). Focal cerebral ischemia was induced by transient occlusion (60 min) of the MCA using the intraluminal filament technique (Weise et al., 2004). Following a midline neck incision the left common and external carotid artery were isolated and ligated. After placing a microvascular clip (Aesculap, Tuttlingen, Germany) on the internal carotid artery, an 8–0 silicon resin (Xantopren, Deuker, Germany) coated nylon monofilament (Ethilon; diameter 180 to 200 μm; Ethicon, Germany) was introduced through an incision into the distal part of the common carotid artery and, after clip removal, advanced 9 mm distal from the carotid bifurcation for MCA occlusion. The monofilament was withdrawn after 60 min of ischemia to allow reperfusion of the MCA. LDF recording continued for 15 min to monitor appropriate reperfusion.

2.4. Visualization of infarcted tissue in WT as compared to $Prnp^{0/0}$ mice

Twenty-four hours after induction of transient ischemia WT and $Prnp^{0/0}$ mice were euthanized by an overdose of isofluran and their brains removed. Five coronal equidistant slices were cut (thickness: 2 mm) and immersed in 2% 2,3,5-triphenyl-tetrazolium chloride solution for 15 min in order to visualize the infarcted tissue.

2.5. Two-dimensional gel electrophoresis (2-DE)

For 2-DE mouse brain homogenates were concentrated and desalinated, using a Microcon™YM-3 centrifugal filter (Millipore, Eschborn, Germany), according to supplier's recommendations. Protein samples were diluted with rehydration buffer (7 M urea, 2 M thiourea, 15 mM dithiothreitol (DTT), 4% CHAPS, 2% ampholytes) for first-dimension isoelectric focusing (IEF). IEF on a 7 cm immobilized pH gradient (IPG) strip (pH 3–10, linear) was performed by applying 40 μg of proteins per strip. Focusing of the proteins was initiated at 200 V for 2 h, followed by ramping at 500 V for 2 h, and final focusing at 4000 V for 5 h for a total of 20,000 Vh. After IEF separation, proteins immobilized on the IPG strip were reduced in the buffer containing 6 M urea, 2% sodium dodecyl sulphate (SDS), 30% glycerol, 2% DTT, and 0.375 M Tris–HCl (pH 8.8)

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