Deletion of Cellular Prion Protein Results in Reduced Akt Activation, Enhanced Postischemic Caspase-3 Activation, and Exacerbation of Ischemic Brain Injury

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Background and Purpose—The physiological function of cellular prion protein (PrPc) is not yet understood. Recent findings suggest that PrPc may have neuroprotective properties, and its absence increases susceptibility to neuronal injury. The purpose of this study was to elucidate the role of PrPc in ischemic brain injury in vivo.

Methods—PrP knockout (Prnp0/0) and Prnp+/+ wild-type (WT) mice were subjected to 60-minute transient or permanent focal cerebral ischemia followed by infarct volume analysis 24 hours after lesion. To identify effects of PrPc deletion on mechanisms regulating ischemic cell death, expression analysis of several proapoptotic and antiapoptotic proteins was performed at 6 and 24 hours after transient ischemia and in nonischemic controls using Western blot or immunohistochemistry.

Results—Prnp0/0 mice displayed significantly increased infarct volumes after both transient or permanent ischemia when compared with WT animals (70.2 ± 23 versus 13.3 ± 4 mm³; 119.8 ± 24 versus 86.4 ± 25 mm³). Expression of phospho-Akt (Ser473) was significantly reduced in Prnp0/0 compared with WT animals both early after ischemia and in sham controls. Furthermore, postischemic caspase-3 activation was significantly enhanced in the basal ganglia and the parietal cortex of Prnp0/0 mice. In contrast, expression of total Akt, Bax, and Bcl-2 did not differ between both groups.

Conclusions—These results demonstrate that PrPc deletion impairs the antiapoptotic phosphatidylinositol 3-kinase/Akt pathway by resulting in reduced postischemic phospho-Akt expression, followed by enhanced postischemic caspase-3 activation, and aggravated neuronal injury after transient and permanent cerebral ischemia. (Stroke. 2006;37:1296-1300.)

Key Words: caspases  ■ cerebral ischemia  ■ PrPc proteins  ■ signal transduction

The pathological isoform of the prion protein (PrPSc) mediates transmissible spongiform encephalopathies like Creutzfeldt–Jakob disease. In contrast, the physiological function of the cellular prion protein (PrPc) is still unknown. Recent findings suggest that PrPc has neuroprotective properties and that its deletion increases susceptibility to neuronal injury in vitro and in vivo.1–4 However, molecular mechanisms underlying PrPc-mediated neuroprotection are still poorly understood and complex.5 Various in vitro studies have shown that in addition to its interaction with synaptic proteins (synapsin Ib and Grb2) and cell adhesion molecules,6,7 PrPc can also mediate the activation of several signal transduction pathways, including protein kinase A, Fyn, phosphatidylinositol 3-kinase (PI3K)/Akt, and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) known to promote neuronal survival.8–11 In addition, cytosolic PrPc has been demonstrated to interact with proteins of the Bcl-2 family.12 Other studies have suggested that PrPc mediates neuroprotection by counteracting oxidative stress. Activities of key antioxidant enzymes (Cu/Zn superoxide dismutase and glutathione reductase) seem to correlate with PrPc levels,13,14 and markers of oxidative stress are increased in brains of Prnp0/0 mice.15 Furthermore, cell lines from Prnp0/0 mice are more susceptible to copper or H2O2 induced toxicity,16,17 and PrPc expression is upregulated after focal cerebral ischemia in vivo depending on lesion severity.18 Recently, it has been suggested that PrPc deletion results in aggravation of neuronal injury after mild focal cerebral ischemia,4 possibly mediated by increased ERK1/2 and STAT-1 activation.19 However, molecular mechanisms underlying exacerbation of ischemic brain injury after PrPc deletion require further characterization. Furthermore, the relevance of PrPc deletion for ischemic injury after severe permanent cerebral ischemia has not been established yet.

Therefore, we analyzed the extent of ischemic brain injury in Prnp0/0 and wild-type (WT) mice after both transient and...
permanent focal cerebral ischemia. To elucidate effects of PrP deletion on molecular mechanisms involved in regulating ischemia-induced cell death, we performed an expression analysis of various proapoptotic and antiapoptotic proteins including Akt, phospho-Akt, Bax, Bcl-2, and activated caspase-3 in Prnp<sup>0/0</sup> and WT mice at different time points after transient ischemia and under nonischemic conditions.

**Materials and Methods**

**Transgenic Mice and Genotype Analysis**

Adult male PrP<sup>+</sup> knockdown (Prnp<sup>0/0</sup>) mice and PrP<sup>−/−</sup> WT mice weighing 22 to 27 g were used. All Prnp<sup>0/0</sup> and Prnp<sup>−/−</sup> mice used in this study had the same genetic background (129/Sv(ev)×C57BL/6J) and were homozygous descendants of Fl generation Prnp<sup>0/0</sup> breeding pairs, which were generated as described previously.20,21 Prnp<sup>0/0</sup> and WT mice (original animals gifts from C. Weissmann, University of Zurich, Switzerland) were propagated over multiple generations and recurrently tested for Prnp genotype.

All mice used in this study were genotyped using a published polymerase chain reaction (PCR) protocol using 3 primers (RK1: TCGACTAAATATCTGGAC; RK2: GCCTGACCAACGGAAATGC; and RK3: GCATCAGCGATGGATGACAT) to identify either the nontransgenic PrP gene in WT mice (combining RK1+RK2) or the disrupted PrP gene in Prnp<sup>0/0</sup> mice (RK1+RK3): amplification of a 730-bp fragment containing the Neo cassette.21 DNA was extracted from tail biopsies of all mice used. PCR mixture contained 200 μM each dNTP, 0.4 μM/L RK1, 0.4 μM/L RK2, 0.4 μM/L RK3, 1.25 U of Taq DNA Polymerase (Promega), 5 mM TrisHCl, pH 8.0, 10 mM/L NaCl, and 0.9 mM/L MgCl<sub>2</sub>. PCR was performed as follows: 1 cycle at 95°C, 120 seconds followed by 35 cycles: starting with 95°C, 60 seconds for denaturation; followed by 62°C, 60 seconds for annealing and completed by 72°C, 60 seconds for polynucleotide extension. PCR was completed by a final step at 72°C, 300 seconds.

Animals were kept under diurnal conditions and allowed free access to food and water.

**Induction of Focal Cerebral Ischemia**

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. Animals were anesthetized with 1% to 1.5% isofluran (30% O<sub>2</sub>, remainder N<sub>2</sub>O). Rectal temperature was maintained at 36.5°C to 37°C using a feedback-controlled heating system. For assessment of cerebral blood flow (CBF), laser Doppler flow was recorded during all ischemic steps and staining with a goat anti-rabbit, Cy-3–conjugated secondary antibody (1:400; Dianova; 1 hour at RT), sections were incubated with the rabbit polyclonal anti-activated caspase-3 antibody (1:1000 in 2% NGS, 0.3% Triton X; overnight at RT; CM-1; Becton Dickinson). After several washing steps and staining with a goat anti-rabbit, Cy-3–conjugated secondary antibody (1:400; Dianova; 1 hour at RT), sections were stained with 4′,6-diamidino-2-phenylindole (nuclear staining).

For activated caspase-3/NeuN double staining, sections were incubated with the rabbit polyclonal, anti-activated caspase-3 15b antibody (CM1, see above) and a mouse monoclonal anti-NeuN antibody (1:500; Chemicon International) overnight at 4°C, followed by staining with a goat anti-rabbit Cy-3–conjugated (see above) and a goat anti-mouse fluorescein isothiocyanate–conjugated secondary antibody (1:100; Sigma-Aldrich; 1 hour at RT). Subsequently, sections were analyzed for activated caspase-3/NeuN colocalization.

**Western Blot Analysis**

Prnp<sup>0/0</sup> and WT mice were euthanized by an overdose of isoflurane at 6 and 24 hours (n=4 per group and time point) after onset of transient cerebral ischemia. Sham-operated Prnp<sup>0/0</sup> and WT mice (n=4 per group) were used as nonischemic controls. Brains were removed and shock-frozen. Left (ischemic) hemispheres of individual mice were complemented with lysis buffer (50 mM/L Tris, pH 8.0, 150 mM/L NaCl, 1% Triton X-100, and protease inhibitors), homogenized, centrifuged, and supernatants used for SDS-PAGE.

For Western blot analysis, equal amounts of protein (40 μg) were diluted in 6× sample buffer, boiled, and loaded onto 10%–15% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes, which were immersed in blocking solution (5% milk, 0.1% Tween 20 in TBS) for 1 hour at room temperature (RT) and incubated with different mouse monoclonal (anti-Akt Ser473/anti-Akt: 1:1000; Santa Cruz Biotechnology; anti–β-tubulin [loading control]: 1:5000; Sigma-Aldrich) or rabbit polyclonal (anti–phospho-Akt (Ser473)/anti-Akt: 1:1000; Cell Signaling; anti-Bax: 1:1000; Santa Cruz Biotechnology) antibodies in 5% BSA/Tris-buffered saline with Tween-20 (TBST) (18 hours; 4°C). Membranes were incubated with a peroxidase-coupled, goat anti-mouse or anti-rabbit secondary antibody (1:2000 in 1% milk/TBST; Santa Cruz Biotechnology) antibody (1 hour at RT), immersed in enhanced chemiluminescence (ECL) solution, and exposed to ECL-Hyperfilm (Amersham).

The intensity of each Akt and phospho-Akt band was measured by densitometry (ScanMaker4; Microtek International) and normalized to intensities of corresponding β-tubulin bands (internal control) by calculating densitometry ratios Akt/β-tubulin and phospho-Akt/β-tubulin.

**Activated Caspase-3 Immunohistochemistry**

Prnp<sup>0/0</sup> and WT mice were intraperitoneally injected with chloralhydrate (420 mg/kg body weight) and transcardially perfused with 4% paraformaldehyde at 6 and 24 hours after onset of ischemia (n=3 per group/time point). Brains were removed, postfixed in paraformaldehyde, embedded in paraffin, and 2-μm coronal sections were prepared. Sections were deparaffinized, boiled in 0.2% citrate buffer, incubated with blocking solution (0.3% Triton X-100; 10% normal goat serum (NGS) in PBS; 1 hour at RT), and a rabbit polyclonal anti-activated caspase-3 antibody (1:1000 in 2% NGS, 0.3% Triton X; overnight at RT; CM-1; Becton Dickinson). After several washing steps and staining with a goat anti-rabbit, Cy-3–conjugated secondary antibody (1:400; Dianova; 1 hour at RT), sections were stained with 4′,6-diamidino-2-phenylindole (nuclear staining).

For activated caspase-3/NeuN double staining, sections were incubated with the rabbit polyclonal, anti-activated caspase-3 15b antibody (CM1, see above) and a mouse monoclonal anti-NeuN antibody (1:500; Chemicon International) overnight at 4°C, followed by staining with a goat anti-rabbit Cy-3–conjugated (see above) and a goat anti-mouse fluorescein isothiocyanate–conjugated secondary antibody (1:100; Sigma-Aldrich; 1 hour at RT). Subsequently, sections were analyzed for activated caspase-3/NeuN colocalization.

**Quantitative Analysis of Activated Caspase-3 Expression**

Cell counts of activated caspase-3–positive cells were performed in ischemic basal ganglia (4 visual fields per section; total area 1.6 mm<sup>2</sup>, including medial and lateral striatum) and parietal cortex (2 visual fields per section; total area 0.8 mm<sup>2</sup>) of Prnp<sup>0/0</sup> and WT mice 24 hours after induction of transient ischemia (n=3 per group). To standardize cell counts, areas to be analyzed within the 2 brain regions were defined by stereotactic coordinates. Four corresponding
sections per animal and region were analyzed. Data are given as number of activated caspase-3–positive cells per mm².

### Statistical Analysis

All values are given as mean±SEM. Normal distribution of the data were tested and confirmed using the Kolmogorov–Smirnov Test. Statistical analysis was performed using the 2-sided Student t test (infarct volume analysis, analysis of activated caspase-3–positive cells; statistical significance: P values <0.05) or 1-way ANOVA followed by the Tukey HSD (honestly significant difference) test (Western Blot analysis of phospho-Akt/Akt).

### Results

#### Infarct Volumes Are Increased in Prnp<sup>0/0</sup> Mice

Quantitative analysis showed significantly increased infarct volumes in Prnp<sup>0/0</sup> compared with WT mice after both 60-minute transient (70.2±23 mm³ versus 13.3±4 mm³) and permanent (119.8±24 mm³ versus 86.4±25 mm³) focal cerebral ischemia (Figures 1 and 2). After transient ischemia, infarctions in WT mice were restricted to small parts of the striatum, whereas in Prnp<sup>0/0</sup> mice, lesions included substantial parts of the basal ganglia (especially the striatum) and parts of the parietal cortex (Figure 2C and 2D).

Reduction of CBF and the extent of reperfusion (after transient ischemia) were not significantly different between Prnp<sup>0/0</sup> and WT animals (data not shown).

#### Reduced Phospho-Akt Expression in Prnp<sup>0/0</sup> Mice

Quantitative Western blot analysis of phosphorylated (ie, activated) Akt and total Akt expression revealed significant phospho-Akt upregulation 6 hours after transient ischemia compared with nonischemic controls in WT but not Prnp<sup>0/0</sup> mice. Moreover, phospho-Akt expression in nonischemic controls and especially 6 hours after induction of ischemia was significantly reduced in Prnp<sup>0/0</sup> mice (P<0.05). *Significant increase in p-Akt expression in nonischemic WT controls compared with Prnp<sup>0/0</sup> controls (P<0.05).

#### Figure 1. Infarct volume analysis in Prnp<sup>0/0</sup> and WT mice 24 hours after induction of transient (60-minute) or permanent cerebral ischemia. Infarct volumes are given as mean±SEM in mm³. Note significantly increased infarct volumes in Prnp<sup>0/0</sup> compared with WT mice after both transient (n=6 per group) and permanent (n=8 per group) ischemia. *Significant difference between Prnp<sup>0/0</sup> and WT mice (P<0.05).

#### Figure 2. 2,3,5-triphenyl-tetrazoliumchloride staining of ischemic infarcts in Prnp<sup>0/0</sup> and WT mice after transient (60-minute) or permanent ischemia. Representative samples are given. Infarct areas are identified by a lack of 2,3,5-triphenyl-tetrazoliumchloride staining in left hemispheres. A and C, Prnp<sup>0/0</sup> mice after permanent (A) and transient (C) ischemia. B and D, WT animals after permanent (B) and transient (D) ischemia. Note increased infarct volumes in Prnp<sup>0/0</sup> compared with WT mice. Arrows point at areas of infarction in the basal ganglia (Prnp<sup>0/0</sup> and WT mice) and cortical areas (found in Prnp<sup>0/0</sup> mice only) after transient ischemia.

#### Figure 3. A, Western blot analysis of cerebral phospho-Akt (p-Akt) and total Akt (Akt) expression in Prnp<sup>0/0</sup> and WT animals after transient (60-minute) ischemia and under nonischemic conditions. Note reduced phospho-Akt expression in Prnp<sup>0/0</sup> compared with WT mice, especially 6 hours after ischemia and in nonischemic animals (con) but no differences in total Akt levels between Prnp<sup>0/0</sup> and WT animals. β-tubulin: loading control for p-Akt. B, Densitometric quantification of phospho-Akt Western blot analysis. Data were normalized against β-tubulin and are given as ratio p-Akt/β-tubulin±SEM. #Significant increase in p-Akt expression in WT mice 6 hours after ischemia compared with 24 hours after ischemia, WT controls and 6 hours after ischemia in Prnp<sup>0/0</sup> mice (P<0.05). *Significant increase in p-Akt expression in nonischemic WT controls compared with Prnp<sup>0/0</sup> controls (P<0.05).
contrast, total Akt expression did not significantly differ between Prnp0/0 and WT animals, both after ischemia and in nonischemic controls (Figure 3A; densitometric data not shown). This suggests impaired Akt activation in Prnp0/0 mice with reduced phospho-Akt expression early after ischemic brain injury and in nonischemic mice.

Enhanced Postischemic Neuronal Caspase-3 Activation in Prnp0/0 Mice
Quantitative analysis of antiactivated caspase-3 immunohistochemistry revealed a significant increase in the number of activated caspase-3–positive cells in the basal ganglia (BG) and in the parietal cortex (Cor) of Prnp0/0 compared with WT mice. Double staining with NeuN (green) revealed strict colocalization (yellow) of activated caspase-3 (aCas3) and NeuN. Bar=50 μm.

Figure 4. Immunohistochemical analysis of cerebral activated caspase-3 expression in Prnp0/0 and WT mice 24 hours after transient (60-minute) ischemia. Note increased number of activated caspase-3–expressing cells (orange) in the basal ganglia (BG) and in the parietal cortex (Cor) of Prnp0/0 compared with WT mice. Double staining with NeuN (green) revealed strict colocalization (yellow) of activated caspase-3 (aCas3) and NeuN. Bar=50 μm.

Expression of Bax and Bcl-2 Does Not Differ Between Prnp0/0 and WT Mice
Western blot analysis of Bax and Bcl-2 expression at 6 and 24 hours after onset of transient ischemia and in nonischemic controls did not reveal relevant differences between Prnp0/0 and WT mice (Figure 6). This argues against a relevant interaction of PrPc with Bax and Bcl-2 expression after cerebral ischemia in vivo.

Figure 5. Quantitative analysis of activated caspase-3 (aCas3)–positive cells in Prnp0/0 and WT mice 24 hours after transient (60-minute) ischemia. Numbers of aCas3–positive cells per mm² are given as mean±SEM. Note significantly increased number of aCas3–positive cells in basal ganglia and parietal cortex of Prnp0/0 compared with WT mice. *P<0.05.

Discussion
Although the exact physiological function of PrPC is still elusive, there is accumulating evidence for its association with molecular mechanisms and signaling pathways that mediate cell survival after neuronal injury. PrPC deletion has been linked to increased susceptibility to neuronal injury in vitro and traumatic and mild ischemic brain injury in vivo. In vitro studies have suggested that neuroprotective actions of PrPC may be mediated by different mechanisms, including activation of neuroprotective signal transduction pathways, direct anti-Bax function, and regulation of antioxidant enzyme activities. However, molecular mechanisms underlying PrPC-dependent susceptibility to ischemic brain injury in vivo and the influence of lesion severity on PrPC-mediated neuroprotection require further investigation. The present study demonstrates that PrPC deletion results in exacerbation of ischemic brain injury after transient and permanent focal ischemia and identified reduced Akt activation and enhanced caspase-3 activation in Prnp0/0 mice as possible underlying mechanisms.

The antiapoptotic PI3K/Akt/phospho-Akt pathway mediates neuronal survival after cerebral ischemia. Blocking this pathway increases activation of caspase-3, a crucial mediator of apoptosis. Furthermore, it has been demonstrated that transient early postischemic upregulation of

Figure 6. Western blot analysis of cerebral Bcl-2 and Bax expression in Prnp0/0 and WT animals after transient (60-minute) ischemia. There are no relevant differences in Bcl-2 and Bax expression between Prnp0/0 and WT mice in nonischemic animals (con) or at 6 or 24 hours after transient ischemia.
phospho-Akt occurs in the cortex but not the ischemic core and is associated with reduced TUNEL staining after transient ischemia. We therefore hypothesize that reduced phospho-Akt levels in Prnp0/0 mice early after stroke lead to enhanced postischemic apoptosis reflected in larger infarcts. This hypothesis is supported by our observation that the number of activated caspase-3 expressing neurons is significantly higher in Prnp0/0 than in WT animals, especially in the parietal cortex. Because total Akt levels do not differ between Prnp0/0 and WT animals, lower phospho-Akt expression in Prnp0/0 mice is attributable to reduced Akt phosphorylation. This is in line with a recent study demonstrating lower PI3K activity, an upstream regulator of Akt phosphorylation, in neuronal cells from mice lacking PrP compared with WT cells.

In contrast to another study reporting increased basal cerebral expression of Bax and Bcl-2 in Prnp0/0 mice, we did not detect relevant differences in Bax and Bcl-2 expression between Prnp0/0 and WT animals in nonischemic brains or after transient ischemia. In our view, this argues against a relevant role for Bax and Bcl-2 in PrP-dependent susceptibility to cerebral ischemia. Short transient and permanent focal cerebral ischemia differ with regard to the molecular mechanisms involved in mediating postischemic cell death. Whereas permanent ischemia (by proximal MCA occlusion) leads to large infarcts with neuronal damage primarily mediated by excitotoxicity and necrosis, short transient ischemia results primarily in apoptotic cell death, especially in the penumbra. Considering these differences, we tried to establish whether PrP depletion is relevant for infant development after both transient and permanent ischemia. Although PrP depletion led to significantly increased infarct volumes after transient and permanent ischemia, differences were more pronounced after transient ischemia. This may be attributable to the predominant role of neuronal apoptosis and the importance of the PI3K/Akt pathway after transient ischemia as opposed to a lesser significance after permanent ischemia. However, additional pathways (eg, MAPK/ERK) or molecular events mediating necrotic cell death (eg, poly(ADP-ribose)polymerase [PARP] activation) may also contribute to enhanced ischemic brain injury in Prnp0/0 mice.

In summary, our results demonstrate that PrP depletion impairs the anti-apoptotic PI3K/Akt pathway by leading to reduced postischemic phospho-Akt expression, followed by enhanced postischemic caspase-3 activation, and aggravated neuronal injury after both transient and permanent cerebral ischemia in vivo.

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References
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