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Differential Contribution of Superoxide Dismutase Activity by Prion Protein *in Vivo*

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Normal prion protein (PrP^C) is a copper binding protein and may play a role in cellular resistance to oxidative stress. Recently, copper-bound recombinant PrP^C has been shown to exhibit superoxide dismutase (SOD)-like activity. However, as PrP^C affinity for copper is low in comparison to other cupro-proteins, the question remains as to whether PrP^C could contribute SOD activity *in vivo*. To unravel this enigma, we compared the SOD activity in lysates extracted from different regions of the brain from wild-type mice before and after the depletion of PrP^C. We found that removal of PrP^C from the brain lysates reduced the levels of total SOD activity. The level of contribution to the total SOD activity was correlated to the level of PrP expressed and to the predominant form of PrP present in the specific brain region. Collectively, these results provide strong evidence that PrP^C differentially contributes to the total SOD activity *in vivo*.

Key Words: **prion; superoxide dismutase; copper; oxidative stress; cerebellum; hippocampus; N-terminal.**

According to the "protein only" hypothesis, prion diseases arise when a helical-rich, normal cellular glycoprotein, the prion protein (PrP^C), undergoes conformational conversion to an altered isoform, PrP^{Sc}, which is rich in β-sheet and is protease-resistant and infectious (1). Normal prion protein, PrP^C has been demonstrated to be a copper binding protein (2, 3), and increasing evidences have shown correlation between the level of prion protein expression and tolerance to oxidative stress (4–6). Although earlier studies comparing cerebellar cells between prion knock-out ($Prnp^{-/-}$) and wild-type ($Prnp^{+/+}$) mice indicate lower superoxide dismutase (SOD) activity in the former (4, 7), a recent study did not (8). Recently, one of us showed that copper-bound recombinant prion protein and total brain-derived prion protein could exhibit SOD-like activity *in vitro* (9, 10). The question remained, however, as of whether PrP^C can directly contribute to the total SOD activity *in vivo* because PrP^C affinity for copper is low compared to other cupro-proteins (3, 8, 11).

To shed some light on this enigma, we compared total SOD activity from lysates extracted from different regions of the brain from only wild-type ($Prnp^{+/+}$) mice before and after the depletion of PrP^C. This approach eliminates any foreign biological reactions that might occur in comparing between prion knock-out and wild-type mice. We depleted PrP^C in the lysate by mixing it with a population of immunoabsorbant Sepharose beads immobilized with anti-PrP monoclonal antibody. We found that when PrP^C was depleted, there was a corresponding reduction in the levels of total SOD activity. This effect is due to the removal of PrP^C as the levels of the two main SOD contributing enzymes, the cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD, were not affected after the depletion process. Furthermore, the reduction of SOD activity was more significant in both the hippocampus and cortex compared to the cerebellum. This differential reduction is due to the level of PrP^C expressed and the presence of distinct population of PrP in that region. These results clearly demonstrate a role for PrP^C in differentially contributing to the total SOD activity *in vivo*.

MATERIALS AND METHODS

Unless described, all enzymes and chemicals were purchased from Roche Diagnostics and Sigma respectively.

Mice. The animals used in this report were 12-week-old female BALB/c mice and were purchased from Charles River Laboratory (Wilmington, MA).

Immunoprecipitation. Total protein was extracted from the cerebellum, hippocampus and cortex regions of the brain by gentle

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homogenization in an extraction buffer (PBS, 1% Nonidet P-40 supplemented with Complete EDTA-free protease inhibitor according to manufacturer's recommendation). After incubation on ice, the homogenate was centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant was taken as the extract. Protein concentration was determined at 280 nm on a Beckman DU-50 UV/Vis spectrophotometer to ensure identical amounts of protein were used. Immunoprecipitation was carried out by equilibrating brain extracts according to the protein content and reacted with either anti-PrP Mab 8H4 (12)-coupled Sepharose 4B (Pharmacia) or Protein A–Sepharose 4B (Pharmacia) at 4°C overnight. Unbound material was collected for analysis after pelleting the Sepharose-beads by centrifugation.

Western blotting. Identical amount of brain extracts were boiled in loading buffer (13) and electrophoresed on polyacrylamide (PAGE) gel (Novex) in the presence of 2% SDS and 100 mM DTT. After separation, the proteins were electrotransferred to nitrocellulose (Bio-Rad), and immunoblotted as described (10). Mn-SOD and Cu/Zn-SOD were detected using sheep polyclonal antibodies (Calbiochem), and mouse prion protein was detected using both the C-terminal Mab 8H4 (residues 140–190) (12) and the N-terminal Mab 8B4 (residues 34–45) (Li *et al.*, manuscript submitted). Bound antibodies were detected by incubating the membrane with either a HRP-conjugated donkey anti-mouse IgG (Chemicon) or a HRP-conjugated donkey anti-sheep IgG secondary antibody (Chemicon). Visualization of the protein was carried out using the chemiluminescence blotting substrate (POD) (Roche Diagnostic).

SOD activity assay. A nitro-blue tetrazolium (NBT)-based spectroscopic assay previously described (10) was used for the quantification of total SOD activity in this report. This method was based on the monitoring of SOD-mediated inhibition of formazan production at 560 nm. Briefly, total protein extracts at 20 μg was added to a reaction containing 0.1 mM xanthine, 0.5 mM NBT, 0.1 mM EDTA in PBS buffer, and measurement of the reaction was carried out for 2 min after adding 10 mU/ml xanthine oxidase (Sigma), a radical producer, to the mixture. The SOD activity was expressed as percentage inhibition of formazan production after adding the protein extracts; 100% formazan production refer to the reduction of nitro-blue tetrazolium (NBT) by xanthine oxidase over a 2-min period in the absence of protein extracts. The spectroscopic assay was performed in duplicate on a Beckman DU-50 UV/Vis spectrophotometer.

RESULTS AND DISCUSSION

Although much has been learned about the normal cellular prion protein (PrP^C) and its role in the pathogenesis of prion diseases, its physiological function(s) remained unclear (1). Numerous studies have shown that neurons from prion protein (PrP^C) knock-out mice ($Prnp^{-/-}$) are more susceptible to oxidative stress (4, 6) and that reintroduction of PrP^C into $Prnp^{-/-}$ derived cell lines by gene transfection can prevent neuronal cell death (14). Earlier studies comparing cerebellar cells between prion knock-out and wild-type mice indicate lower superoxide dismutase (SOD) activity in the former (4). However, this was not observed in a recent study (8). Recently, one of us demonstrated that copper-bound recombinant PrP^C and total brain-derived immunoprecipitated prion protein could exhibit SOD-like activity (9, 10). This activity is copper-dependent, but unlike Cu/Zn-SOD, it is not sensitive to KCN (9). Nevertheless, as PrP^C affinity for copper is low compared to other cuproenzymes, the question remained as of whether PrP^C can contribute SOD activity *in vivo* (3, 8, 11). In the studies reported here, we will attempt to shed some light on this enigma.

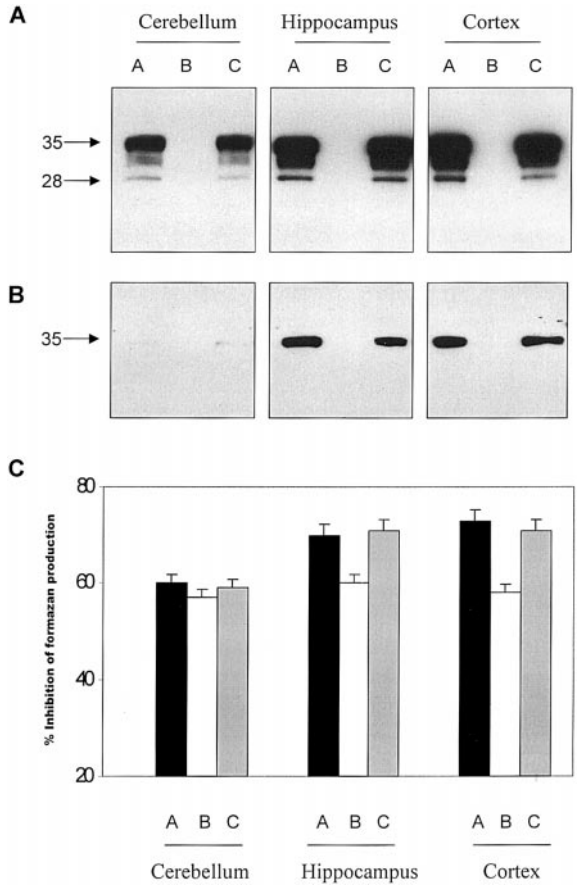

FIG. 1. Superoxide dismutase (SOD) activity associated with the level of prion protein expression in the cerebellum, hippocampus, and cortex of the mouse brain. Lane A is lysate without treatment; lane B is supernatant extracted after incubation with anti-PrP 8H4-coupled Sepharose 4B; and while lane C is supernatant extracted after incubating with protein A–Sepharose 4B. All samples are resolved and immunoblotted on the same gel for A and B. Immunoblotting of prion protein using (A) anti-PrP 8H4 (12) or (B) anti-PrP 8B4 from 20 μg of total protein lysates loaded on each lane after resolving on a 16% SDS–PAGE in the presence of 0.1 mM DDT. Molecular weight markers in kilodaltons are indicated on the left. (C) Nitro-blue tetrazolium (NBT)-based spectroscopic assay for the measurement of total SOD activity described previously (10) on lysates from the different brain regions examined. The means ± SD of assays performed in duplicate are shown, and each reaction uses 20 μg of total protein (Student t tests, $P<0.05$).

Since a previous study has showed PrP^C to be highly expressed in the cerebellum, hippocampus and cortex regions of the brain (15), we decided to prepare lysates extracted from these three brain regions. Using a previously described anti-PrP monoclonal antibody (Mab) 8H4 (12), we observed higher levels of PrP^C expression in both hippocampus and cortex compared to cerebellum (Fig. 1A). Interestingly, we noted a direct correlation between the levels of PrP^C expression and the levels of total SOD activity (Figs. 1A and C). Both

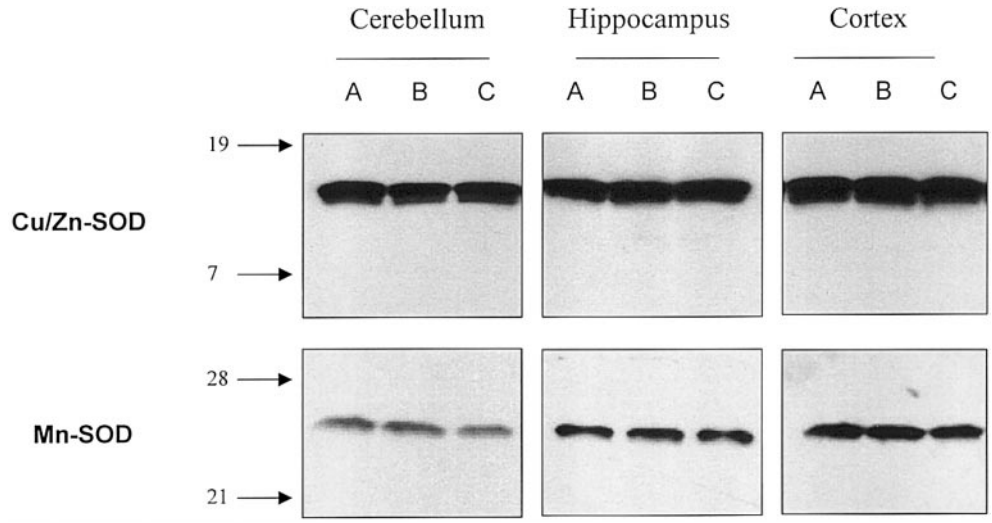

FIG. 2. Immunodetection of both cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD from lysates extracted from the cerebellum, hippocampus, and cortex of the mouse brain. Lane A refers to lysates without treatment; lane B is supernatant extracted after incubating lysates with anti-PrP 8H4-coupled Sepharose 4B; and lane C refers to supernatant extracted after incubating lysates with protein A–Sepharose 4B. For detecting Cu/Zn-SOD, 20 μg of total protein was loaded per lane, and for detecting Mn-SOD 150 μg of total protein was loaded per lane, and they were electrophoresed on a 16% SDS–PAGE in the presence of 0.1 mM DDT before immunodetection. Molecular weight markers in kilodalton (kDa) are shown on the left.

hippocampus and cortex with their higher PrP^C expression have a higher SOD activity compared to the cerebellum that has lower PrP^C expression.

To ascertain that this correlation is a direct effect of PrP, we compared SOD activity before and after depletion of PrP^C in the brain lysates by immunoabsorbant. The efficiency of the depletion process was determined by immunoblotting the lysates with both a C-terminal anti-PrP Mab 8H4 (Fig. 1A) and a N-terminal anti-PrP Mab 8B4 (Fig. 1B) after they have been absorbed with either anti-PrP Mab 8H4-coupled Sepharose beads or protein A–Sepharose beads acting as a control. As shown in Figs. 1A and 1B, we did not detect any observable presence of PrP^C after the lysate has been absorbed with Mab 8H4-coupled Sepharose (Figs. 1A and 1B, lanes B), while no observable loss of PrP was detected after absorption with the control protein A–Sepharose (Figs. 1A and 1B, lanes C).

Using a previously described nitro blue tetrazolium (NBT)-based spectroscopic assay (10), we measured total SOD activity from samples extracted from each individual brain regions examined, with or without depleting PrP^C. As shown in Fig. 1C, after depleting with anti-PrP 8H4-coupled Sepharose, a reproducible reduction in the SOD activity was detected in all brain regions examined (Fig. 1C, lanes B) (Student t test, $P < 0.05$). On the contrary, no reduction in SOD activity was observed on lysates absorbed with the control protein A–Sepharose (Fig. 1C, lanes C). These results indicate that incubation with Sepharose beads coupled with an irrelevant protein did not alter the level of SOD activity. Of interest is the greater reduction of total SOD activity in both hippocampus (10% $\pm$ 0.3) and cortex (15% $\pm$ 0.5) compared to the cerebellum (3% $\pm$ 0.1). This difference could be a result of the higher PrP^C expression level at both sites, or alternatively, it could due to the presence of a different population of PrP^C in the cerebellum. To test the later hypothesis, we immunoblotted the brain regions using anti-PrP Mab 8B4. This antibody recognize residues 34–45 at the extreme N-terminal of mature PrP (Li *et al.*, manuscript submitted). The antibody faintly detects PrP at the cerebellum, while significant levels of PrP were detected in both the hippocampus and cortex (Fig. 1B). This may explains the differential contribution of SOD by different PrP populations observed between the cerebellum on one hand, and hippocampus and cortex on the other (Fig. 1C). Recently, we and other have showed that this N-terminal region has a modulating effect on the conformation adopted by the C-terminal of PrP (16) (Li *et al.*, manuscript submitted), and that the SOD-like antibody exhibited by PrP^C is partly dependent on the conformation adopted by the C-terminal region (17).

To further eliminate the possibility that the reduction in SOD activity after depleting PrP^C is not due to the removal of both cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD, the two main SOD contributing enzymes *in vivo*, we examined the levels of this two enzymes before and after absorption with Sepharose beads. As shown in Fig. 2, the levels of these two SOD enzymes were not affected after the depletion process. This further support the above notion that the observed SOD activity reduction is due to the depletion of PrP^C, and that no SOD enzymes have been retained by

either the Mab 8H4-coupled Sepharose nor the control protein A–Sepharose.

The data presented in this report clearly implicate the ability of PrP to contribute to the total SOD activity *in vivo* despite its low affinity for copper and been surrounded by other cuproenzymes (8, 11). To overcome this limitation *in vivo,* it is possible that PrP^{C}, like increasingly numbers of cupro-proteins (18), has its own copper chaperone to facilitate the binding process. Of special interests is our observation that the reduced contribution of SOD activity in the cerebellum is related to a distinct population of PrP with part of the N-terminal undetectable. In contrast, populations of PrP with this region intact, as those detected in both the hippocampus and cortex, contribute higher level of SOD activity.

Although various studies (6, 9, 10) have implicated a role for PrP in cellular resistance to oxidative stress such as contributing SOD activity *in vivo* as demonstrated in this report, it is possible that PrP could has other roles (19, 20). Indeed, increasing evidence indicates that proteins can perform different functions at different cellular locations or under different physiological conditions (21, 22). Nevertheless, the assignment of an antioxidant ability to PrP^{C} *in vivo* and the recognition of a distinct population of PrP in certain brain region are important in understanding the normal physiological and possibly the pathological contribution by PrP^{Sc}.

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