Brain Copper Content and Cuproenzyme Activity Do Not Vary with Prion Protein Expression Level*

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Darrel J. Waggoner‡, Bettina Drisaldi‡, Thomas B. Bartnikas‡, Ruby Leah B. Casareno‡, Joseph R. Prohaska‡, Jonathan D. Gitlin‡, and David A. Harris**

From the §Edward Mallinckrodt Department of Pediatrics and §Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the §Department of Biochemistry and Molecular Biology, University of Minnesota, Duluth, Minnesota 55812

Prion diseases are neurodegenerative disorders that result from conformational transformation of a normal cell surface glycoprotein, PrP<sup>C</sup>, into a pathogenic isoform, PrP<sup>Sc</sup>. Although the normal physiological function of PrP<sup>C</sup> has remained enigmatic, the recent observation that the protein binds copper ions with micromolar affinity suggests a possible role in brain copper metabolism. In this study, we have used mice that express 0, 1, and 10 times the normal level of PrP to assess the effect of PrP expression level on the amount of brain copper and on the properties of two brain cuproenzymes. Using mass spectrometry, we find that the amount of copper in subcellular fractions from brain is similar in all three lines of mice. In addition, the enzymatic activities of Cu-Zn superoxide dismutase and cytochrome c oxidase in brain extracts are similar in these groups of animals, as is the incorporation of <sup>64</sup>Cu into Cu-Zn superoxide dismutase both in cultured cerebellar neurons and in vivo. Our results differ from those of another set of published studies, and they require a re-evaluation of the role of PrP<sup>C</sup> in copper metabolism.

The prion protein (PrP) exists in two alternative isoforms, which differ in three-dimensional structure and biological properties (1, 2). PrP<sup>Sc</sup>, the primary component of prion particles, is rich in β-sheets and is protease-resistant and infectious.

PrP<sup>C</sup> is a normal cellular glycoprotein that has minimal β-sheet structure and is protease-sensitive. Prion diseases arise from the posttranslational conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup> within the central nervous system. Although a great deal is known about the role of PrP<sup>Sc</sup> in the disease process, the normal physiological function of PrP<sup>C</sup> has remained elusive.

Several key observations have recently suggested a possible role for PrP<sup>C</sup> in copper metabolism. First, it has been found that purified recombinant PrP, as well as synthetic PrP peptides, bind copper ions with low micromolar affinity via a series of four histidine-containing peptide repeats that reside in the N-terminal half of the protein (3–6). Binding is specific for copper over other transition metals and is pH-dependent, with affinity falling sharply below pH 6 (4, 7). Interestingly, copper binding causes conformational changes in the octapeptide repeats, raising the possibility that the metal may trigger some functional alteration in PrP<sup>C</sup> (3, 8). A second major observation is the report by one group that there is a 10–15-fold reduction in the content of copper, but not of several other metals, in brain membranes from PrP-null mice compared with wild-type controls (6). This striking result suggests that PrP<sup>C</sup> could be a major copper-binding protein in brain. Finally, the same group has reported that the enzymatic activity and copper loading of Cu-Zn superoxide dismutase (SOD1) are 50% of normal in brain and cultured cerebellar neurons from PrP-null mice and that SOD1 activity and copper loading are elevated in mice that overexpress PrP (9, 10). This observation suggests that PrP may play some role in delivery of copper to cuproenzymes such as SOD1.

We undertook here to re-examine two of the critical pieces of evidence in favor of a role for PrP<sup>C</sup> in copper metabolism. Recently, a specific chaperone required for copper delivery to mammalian SOD1 has been identified and characterized (11, 12), and therefore we have evaluated the influence of PrP expression on the enzymatic activity and copper loading of SOD1, as well as on the activity of a second cuproenzyme, cytochrome c oxidase (COX). We also carried out measurements using mass spectrometry of ionic copper in the brains of mice that express different amounts of PrP.

**EXPERIMENTAL PROCEDURES**

Mice—Prn-p<sup>0/0</sup> mice (13) and Tga20 mice (14) were obtained from Dr. Charles Weissmann. Prn-p<sup>0/0</sup> mice were created on a C57BL/6J × 129 background, and Tga20 mice were created by introduction of a wild-type mouse PrP transgene into Prn-p<sup>0/0</sup> mice, so that these two lines have similar genetic backgrounds. Wild-type mice were either C57BL/6J × 129 or CD-1 Swiss; results were identical with both strains. Mice used for the experiments shown in Fig. 1 and Table I were 3–6 months of age.

Cerebellar Cell Cultures—Cultures were prepared from the cerebella of mice at postnatal day 7 as described (15) and were used 6 –7 days after plating. Virtually all of the neurons in these cultures are granule cells, and non-neuronal contamination was 1–2%.

Brain Fractionation and Mass Spectrometry—All glassware and plasticware was soaked overnight in 10% nitric acid and then rinsed extensively in deionized water (≥18 megohms) to minimize metal ion contamination. Brains were homogenized in 10 volumes of deionized water using a Teflon glass homogenizer and were then centrifuged at 1,000 × g for 10 min to produce a P1 pellet. The supernatant was then centrifuged at 12,000 × g for 20 min to produce a P2 pellet. The supernatant was then centrifuged at 100,000 × g for 60 min to produce a P3 pellet and an S3 supernatant. A portion of the P2 pellet was washed by homogenization in deionized water followed by centrifugation at 12,000 × g for 20 min to produce a P2* pellet. This subcellular fractionation scheme is similar to ones used for separation of synapto-
Brain PrP, Copper Content, and Cuproenzyme Activity

TABLE I
Copper content of subcellular fractions of brain determined by ICP-MS

| Fraction | Prn-p0/0 | Wild type | Tga20 |
|----------|----------|-----------|-------|
| P1 (1,000 × g) | 1.36 ± 0.64 | 1.12 ± 0.39 | 1.18 ± 0.58 |
| P2 (12,000 × g), unwashed | 1.27 ± 0.37 | 1.50 ± 0.48 | 1.26 ± 0.15 |
| P2+ (12,000 × g), washed | 0.74 ± 0.55 | 0.65 ± 0.56 | 0.58 ± 0.35 |
| P3 (100,000 × g) | 0.41 ± 0.06 | 0.49 ± 0.17 | 0.36 ± 0.02 |
| S3 (100,000 × g) | 1.93 ± 0.99 | 2.56 ± 2.02 | 2.06 ± 0.90 |

Total (P1 + P2 + P3 + S3) | 4.42 ± 2.02 | 5.45 ± 2.03 | 4.33 ± 1.62 |

Values are given as μg of copper per g of brain, wet weight. Values for each fraction are the mean ± S.D. of measurements derived from two (wild type) or three (Prn-p0/0, Tga20) brains. Values for total copper from Prn-p0/0 and Tga20 mice (last line) include an additional measurement on an unfractionated brain sample.

RESULTS

Brain Copper Content—We first measured the total amount of copper in brain fractions derived from mice with three different expression levels of PrP. We analyzed wild-type mice, Prn-p0/0 mice in which the endogenous PrP gene has been ablated and which do not produce any PrP protein (19), and Tga20 mice which carry a wild-type PrP transgene and express ~10 times the normal level of PrP (14). Brain tissue was fractionated by differential centrifugation to yield a series of membrane fractions (P1, P2, P3) and a soluble fraction (S3). Synaptic membranes in which PrP is enriched (17) are present in the P2 fraction. To investigate the possibility that copper might be loosely bound to synaptic membranes, we subjected a portion of the P2 fraction to a single wash and re-centrifugation (P2+). Metal ion content was determined by ICP-MS, a technique that has high sensitivity (100 pg/g) and selectivity (ability to distinguish ions with similar charge-to-mass ratios).

No significant differences among mice of the three genotypes were found in the content of copper (Table I), zinc, or iron (not shown) in any of the brain fractions. The absolute values for total brain copper given in Table I are close to those reported by other investigators (23). Similar results were also obtained by atomic absorption spectroscopy (not shown). We noted that washing the P2 membrane fraction caused a consistent but variable reduction in the copper content (see P2+ entries in Table I), suggesting that a significant amount of the metal is weakly bound.

Enzymatic Activity and Copper Loading of SOD1—Measurements of SOD1 activity in brain extracts from Prn-p0/0, wild-type, and Tga20 mice using two different spectrophotometric assays did not reveal any significant differences among the three groups of animals (Fig. 1).

We also prepared primary cultures of cerebellar neurons from Prn-p0/0, wild-type, and Tga20 mice. Western blot analysis revealed no differences in the amount of SOD1 protein in cultures from the three groups of animals (Fig. 2A), indicating that PrP does not affect the steady state level of this enzyme. When SOD1 activity was examined in the same samples by a non-denaturing gel assay using nitro blue tetrazolium, no differences were observed (Fig. 2B). The identity of the single band of activity in Fig. 2B was confirmed by its absence in extracts from SOD1-deficient mice (data not shown). We also failed to detect differences in SOD1 activity among cultures from the three lines of mice using the xanthine-based spectrophotometric assay (data not shown).

To directly measure copper incorporation into SOD1, we metabolically labeled primary cerebellar cultures with 64Cu. When cell lysates were analyzed by non-denaturing gel electrophoresis, a single 64Cu-labeled band, previously identified as dimeric SOD1 (11), was observed (Fig. 2C). There was no difference in the intensity of this band from mice of the three genotypes. To confirm our results on copper incorporation in vivo, we analyzed incorporation of radioactivity into SOD1 from the brains of animals given intrathecal injections of 500 μCi of 64Cu. As with the cell culture experiments, no differences in copper incorporation were seen among wild-type, Prn-p0/0, and Tga20 mice (data not shown).

COX Activity—To examine a possible effect of PrP expression on a second cuproenzyme, we assayed the activity of COX, a ubiquitous mitochondrial enzyme. Prn-p0/0, wild-type, and Tga20 mice had brain COX activities of 0.650 ± 0.43, 0.612 ± 0.059, and 0.646 ± 0.022 μmol/min/mg protein, respectively (mean ± S.D., n = 4). Thus, as for SOD1, there was no difference in COX activity between mice with differing expression levels of PrP.

DISCUSSION

We report here that the amount of copper in subcellular fractions of brain is not significantly different among mice that express 0, 1, and 10 times the normal level of PrP. We also failed to observe any effect of PrP expression level on the...
Fig. 1. SOD1 activities in brain lysates from Prn-p0/w, wild-type, and Tga20 mice are similar, based on two different spectrophotometric assays. Assays are described under "Experimental Procedures." Bars show the mean ± S.D. of values from three (xanthine assay) or four (pyrogallol assay) brains.

Fig. 2. SOD1 protein, activity, and copper incorporation are similar in cultures of cerebellar neurons from Prn-p0/w, wild-type, and Tga20 mice. Lysates of cerebellar cultures were subjected to electrophoresis on a 10% polyacrylamide gel under denaturing (A) or non-denaturing (B and C) conditions. A, Western blot analysis of lysates using an anti-SOD1 antisera; B, gel-based assay for SOD activity was performed using nitro blue tetrazolium; C, autoradiography of lysates from 64Cu-labeled cultures.

Enzymatic activity and copper loading of SOD1 from brain or on the activity of a second cuproenzyme from brain, COX.

Several of these findings differ markedly from those reported by Kretzschmar and colleagues. These investigators reported a 10–15-fold reduction of copper in crude membranes, synaptosomes, and endosomes from the same strain of Prn-p0/0 mice as we utilized here (6), although in a subsequent publication a more modest 2-fold decrease was observed in synaptosomes (17). This group also reported that SOD1 activity in brain extracts and incorporation of 64Cu into SOD1 from cultured cerebellar neurons was ~50% of normal in Prn-p0/0 mice (9, 10). Moreover, SOD1 activity was found to be modestly elevated (~20%) in Tga35 mice, which have a PrP expression level (10). 64Cu incorporation into SOD1 was increased by 250% (10). 64Cu incorporation in these experiments was assessed by counting the amount of radioactivity immunoprecipitated by an anti-SOD1 antibody, so that the M, of the protein(s) to which the radioactivity was bound could not be confirmed, as in our experiments. It is not clear what other explanations might account for the discrepancies between our results and those of Kretzschmar and colleagues.

The results reported here require a re-evaluation of the role of PrPc in brain copper metabolism. First, our ICP-MS copper measurements indicate that PrPc is unlikely to be a major copper-binding protein in brain membranes. This conclusion is also supported by calculations which demonstrate that the total amount of PrPc in brain (~3 nmol/g (24)) is insufficient to account for binding of more than a fraction of the total amount of copper found in membrane fractions (~3 μg/g or 50 nmol/g, Table I). Given the relatively weak affinity of PrPc for copper (5–15 μM), it is possible that some copper bound to PrPc was released during the process of subcellular fractionation. In this case, however, the released metal would have been detected in other fractions which we analyzed. Our results also suggest that PrPc is not the primary carrier responsible for entry of copper into the brain via the blood-brain or blood-CSF barriers or for uptake of the metal into neurons from the extracellular space. If PrPc were an essential component of these global uptake mechanisms, then variations in PrP expression should be directly reflected in the copper content of brain fractions.

Our studies also indicate that PrPc does not play a role in the specialized trafficking pathways involved in delivery of copper to SOD1 and COX in brain. In mammalian cells, copper transported across the plasma membrane is delivered to cuproproteins via specific chaperone molecules, which bind copper and transfer it to the target protein by physical association (25). This mechanism is necessary because, due to its extreme toxicity, free copper is kept at negligible concentrations in the cytoplasm (~1 ion per cell) by binding to copper-scavenging proteins such as metallothionein (26). Lys7p in yeast and CCS in mammals have been identified as the chaperones that transfer copper to SOD1 and COX (27). However, PrPc could function in copper trafficking pathways upstream of such chaperones. If this is the case, however, the pathways involved are likely to be those that target cuproproteins other than SOD1 and COX.

The strongest piece of evidence supporting a role for PrPc in copper biology is the fact (now established by a number of laboratories) that copper binds to PrPc and induces a conformational change in the octapeptide repeat region (3–8). The results reported here therefore focus attention on other possible hypotheses about the connection between PrPc and copper. One possibility is that copper is an essential cofactor for a previously unidentified enzymatic activity of PrPc. Indeed, a recent report suggests that both bacterially produced and
brain-derived PrP<sup>C</sup> has a copper-dependent superoxide dismutase activity (28). However, cuproenzymes such as SOD1 have much higher affinities for copper than PrP<sup>C</sup> (femtomolar versus micromolar), and these enzymes, unlike PrP<sup>C</sup>, usually require partial denaturation for removal of bound copper (26, 29). In addition, they all contain copper centers characterized by metal bonding to sulfur atoms in methionine or cysteine residues, in addition to nitrogen atoms in histidine residues as for PrP<sup>C</sup> (30).

A much more likely function for PrP<sup>C</sup> is to serve as a reversible sink or carrier for copper ions. Consistent with this suggestion, the normal concentration of Cu<sup>2+</sup> in plasma and CSF (1–10 μM) is similar to the estimated κ<sub>d</sub> for copper binding to PrP<sup>C</sup>, and the concentration of the metal in brain tissue is estimated to be even higher (100 μM) (31). Most extracellular copper is bound to ceruloplasmin in plasma and CSF, and the remainder is complexed with albumin, peptides, and amino acids (30). Because these non-ceruloplasmin species have copper dissociation constants in the low micromolar range (expressed in terms of total copper), they should be able to readily transfer copper to and from PrP<sup>C</sup>. Further work will be necessary to explore whether PrP<sup>C</sup> functions as an endocytic receptor for cellular uptake of copper ions, as we have previously speculated (32), or whether it facilitates some other aspect of copper trafficking such as efflux from the cell or intracellular sequestration of the metal. Given the recent recognition that transition metals play a key role in a number of neurodegenerative disorders (33, 34), the connection between PrP and copper metabolism will continue to be an important subject for future investigations.

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