Prion infection impairs copper binding of cultured cells

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Abstract:

The molecular mechanism of neurodegeneration in transmissible spongiform encephalopathies (TSEs) remains unclear. Using radioactive copper ($^{64}$ Cu) at physiological concentration, we showed that prion infected cells display a marked reduction in Cu binding. The level of full length prion protein known to bind the metal ion was not modified in infected cells, but a fraction of this protein was not releasable from the membrane by phosphatidylinositol-specific phospholipase C (PIPLC). Our results suggest that prion infection modulates Cu content at a cellular level, and that modification of Cu homeostasis play a determinant role in TSE’s neuropathology.
**Introduction**

Prion diseases form a group of fatal neurodegenerative disorders including Creutzfeldt Jakob disease in humans, Scrapie and Bovine Spongiform Encephalopathy in animals (1). Most prion diseases are characterized by the accumulation of an abnormally folded isoform of the cellular prion protein (PrP\(^C\)), denoted PrP\(^Sc\), which is the major component of infectious prions (2). The formation of PrP\(^Sc\) from PrP\(^C\) is accompanied by profound structural and biochemical changes. PrP\(^C\), rich in \(\alpha\)-helical regions, is converted into a highly \(\beta\)-sheeted protein partially resistant to proteolytic digestion, PrP\(^Sc\) (3). Chemical analysis of the purified protein demonstrates that PrP\(^Sc\), like PrP\(^C\), possesses a C-terminal GPI anchor (4). Unlike PrP\(^C\), PrP\(^Sc\) is not releasable by PIPLC from brain membranes or from the surface of scrapie infected N2a cells (5).

PrP\(^C\) is a 253 amino acid protein highly expressed by neurons (6). Its amino-terminal region contains a repeated five octapeptides domain that binds Copper (Cu) (for reviews see (7)). In addition, Cu binding (8,9), as well as the activity of several antioxidant enzymes in different models (10,11), was directly related to the level of PrP\(^C\) expression (for review see (12,13)). Interestingly, PrP mutations that have additional copies of the octapeptide repeats induced neurodegeneration in transgenic mice (14). Based on these observations, it could be hypothesized that prion diseases are linked to an alteration of Cu metabolism that impacts the activity of Cu enzymes and/or the response of cells to oxidative stress.

Previously, we showed that neuronal cells infected with prions were more susceptible to oxidative stress through an alteration of physiological anti-oxidative cellular mechanisms (15). In the present study, we demonstrate that prion infection diminished Cu binding by the cells. In addition, we show that a fraction of PrP in infected cells was not readily released from the plasma membrane by PIPLC, a feature that may revealed the formation of misfolded PrP.

**Results and discussion**

In a recent work, we demonstrated, using \(^{64}\text{Cu}\), that binding of copper to the outer of the plasma cell membrane is related to the level of PrP\(^C\) expression in an inducible cell line (9). In addition, we showed that PrP was not directly involved in the delivery of Cu inside of the cell but served as a sink for Cu and bound metal ions as part of the acquisition of its active conformation and/or its physiological function. Here, we investigated the influence of prion generation on Cu binding using a similar paradigm, i.e. the study of the uptake of
physiological concentration of $^{64}$Cu by cultured cells. This was performed using the hypothalamic cell line GT1, which was eventually infected with the Chandler strain (GT1$^{Chl}$). As a control cell line, the GT1$^{Chl}$ treated with Congo red (GT1$^{Chl-CR}$) was used since this treatment allows for a cessation of PrP$^C$ conversion and removal of PrP$^{Sc}$ (15). It was noteworthy that all these lines expressed a similar level of PrP$^C$, while, as expected, only the GT1$^{Chl}$ accumulated the protease resistant PrP isoform, PrP$^{Sc}$ (Fig. 1A). The latter molecule could easily be detected in the cultures after deglycosylation even in absence of proteinase K digestion (Fig. 1B, lane 2). To demonstrate that most PrP$^{Sc}$ was cleaved in GT1$^{Chl}$ cells as in ScN2a (16,17), soluble (S) and insoluble (I) PrP molecules were separated by ultracentrifugation and revealed by western blot after deglycosylation (Fig. 1C, PK-). More than 90% of the insoluble PrP was actually cleaved and corresponded to PrP$^{Sc}$ molecules as confirmed by proteinase K digestion (Fig. 1C, PK+).

Binding of small concentration of $^{64}$Cu (1.6 µM) to the different cell lines was monitored by measuring, after different time points, the amount of radioactivity remaining associated with the cells (Fig. 2A, see Material and Methods). A significant difference between infected and control cell lines was apparent 10 hours after the beginning of the experiment. Following the incubation with $^{64}$Cu, the initial uptake of the metal ion was likely to be related to classical transport system such as CTR1 (18). In a previous work, we showed that the presence of PrP did not influence Cu uptake in this early phase. Subsequently, incorporation of $^{64}$Cu was found to be proportional to the level of PrP expression by the cells (9). This relates to the synthesis of new PrP molecules that incorporate metal ions and/or to the exchange of metal ions between PrP and other Cu binding molecules. Recently, it has been shown that octapeptide domain of PrP$^C$ have a copper reducing ability (19). The interaction of PrP$^C$ with copper could be necessary to reduce Cu (II) to Cu(I) on the plasma cell membrane and then presenting Cu(I) to the classical copper transport CTR1. We observed here that after 24h, Cu binding was significantly diminished in infected cells which accumulated high levels of cleaved PrP$^{Sc}$ (Fig. 2A). It is likely that PrP$^{Sc}$, which had lost its octapeptide region known to bind metal ions, would not by itself modify the amount of Cu associated with the cells. This is also in agreement with several studies in animals and in vitro showing that this isoform does not bind Cu and might be associated with other metal ions such as manganese or zinc (20-22). Therefore, it is puzzling that infected cells, while they have a normal amount of full length PrP, did not bind Cu in expected amounts.

In a previous work, we were able to demonstrate that the PIPLC release of GPI-anchor proteins, including PrP, reduced the amount of $^{64}$Cu associated with cell cultures, suggesting...
with other data that PrP was a major Cu binding GPI-anchored protein (9). To confirm these results in GT1 cells, cell cultures were incubated 30 hours with $^{64}\text{Cu}$, treated with PIPLC and the amount of $^{64}\text{Cu}$ still bound to the cells was measured (Fig. 2B). As expected, PIPLC treatment significantly decreased $^{64}\text{Cu}$ binding in GT1 and GT1$^{\text{Chl-CR}}$ and released radioactive Cu in the media (data not shown). After PIPLC treatment of infected GT1$^{\text{Chl}}$ cells, Cu binding was not modified (Fig. 2B). In fact, the level remained low but was still largely within the limits of detection of the method used. This indicated that Cu content in infected cells was not affected by the release of GPI anchored proteins, including PrP. However, we then checked by western blot if PIPLC effectively released PrP from the cell membranes and unexpectedly, it appeared that significantly less PrP was released from GT1$^{\text{Chl}}$ than from control GT1 cells (Fig. 3A and B). As reported on the bar graph, we also confirmed this result in control and scrapie infected N2a cells available in the laboratory (23). Importantly, the PrP molecules detected in these experiments could not correspond to PrP$^{\text{Sc}}$ which was N-terminally cleaved in our cultures and not recognized by P45-66 (Fig. 1B and C). The decrease of the PIPLC release of PrP$^{\text{C}}$ in infected cells may be the consequence of a modification of the cellular environment of the molecule as suggested before (24). It is possible that PrP$^{\text{Sc}}$ could be responsible for this modification of the cellular environment of PrP$^{\text{C}}$ and could interact/co-aggregate with PrP$^{\text{C}}$ and renders PIPLC cleavage inefficient. This result is reminiscent of that obtained with mutated PrP molecules, which just after synthesis are resistant to PIPLC cleavage (25). For mutated PrPs, this property has been explained by the fact that their GPI anchors become physically inaccessible to the phospholipase, as part of their conversion to PrP$^{\text{Sc}}$-like molecules (26). Importantly, this PIPLC resistance acquired in the endoplasmic reticulum was the earliest biochemical change detected in mutated PrPs until the acquisition of their PrP$^{\text{Sc}}$-like properties (25). Similarly, it is possible that the PrP “resistant” to PIPLC in infected cells represents an intermediate in the formation of PrP$^{\text{Sc}}$ and corresponds to a misfolded PrP generated in the endoplasmic reticulum, as a recent report suggests that this organelle plays an important role in the generation of PrP$^{\text{Sc}}$ (27). A speculative scenario would be that prion generation leads to the formation of a misfolded PrP that is unable to bind Cu and could not fulfil the physiological function of PrP.

The fact that prion infection has a dramatic effect on $^{64}\text{Cu}$ binding by the cells is important since Cu, as other transition metals, is believed to play an important role in the neuropathology of neurodegenerative disorders such as Parkinson’s disease, Alzheimer’s disease and Amyotrophic Lateral Sclerosis (28). By its ability to readily adopt two ionic states Cu(I) and Cu(II), Cu is required for the catalytic activity of a number of essential
enzymes such as Cu/Zn superoxide dismutase (Cu/Zn SOD) or cytochrome C oxidase, the majority of which catalyze oxidation-reduction reactions. Free Cu is also a toxic ion that generates hydroxyl radical, a highly reactive oxygen species involved in causing direct damage to nucleic acids, proteins, lipids as well as apoptosis (29). Both deficiency and excess in Cu leads to a number of pathological disorders such as Menkes syndrome or Wilson’s disease (30) which illustrates its physiological importance and duality in the central nervous system. The modification of Cu metabolism following prion infection is also reminiscent of previous works showing that prion infection strongly affected the Cu content in synaptosomes (31). In conclusion, metal ions could play an essential role in the pathogenesis of prion diseases and represent important targets for future therapeutic targets.
Materiel and Methods

Reagents

Pefabloc and proteinase K were purchased from Roche Diagnostics. Dulbecco’s Modified Eagle’s medium (DMEM) was from Invitrogen, and fetal calf serum (FCS) from Bio-Whitaker. All other reagents are from Sigma. Rabbit polyclonal antibody P45-66 raised against synthetic peptide encompassing mouse PrP residues 45-66 has been described earlier (32). SAF 60, 69 and 70, raised against the peptide sequence 142-160 of hamster PrP, were produced in the laboratory of J Grassi (CEA, France). A mixture of the three antibodies was used to enhance the detection of PrPSc. Secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Cell culture

Generation of GTI cells infected with the Chandler strain (GT1Chl) and cured with CR have been reported earlier (23,33). Cells were maintained at 37°C / 5% CO2 in DMEM supplemented with 5% FCS, 5% horse serum and antibiotics (penicillin-streptomycin).

Cellular 64Cu binding and release.

Cells were cultured in 35-mm Petri dishes. Culture medium was replaced by 2 ml fresh complete medium containing 0.1 µg 64Cu/ml (2 µCi/ml) (CIS bio international, Gif-sur-Yvette, France; specific activity 20 mCi/mg) to evaluate Cu binding. Cells were incubated at 37°C under 5% CO2. The radioactive medium was removed after 0, 8, 11, 20, 27 and 30 h. Cells were rinsed twice with 2 ml diluted Puck’s saline A solution (Invitrogen), and harvested. Each dish was then rinsed with 1 ml Puck’s saline A solution. The final 2 ml obtained for each dish were counted for 2 min using a Packard Cobra III mono well gamma counter (Packard Instrument Company, Meriden, CT). Data were analyzed using a “self made” computer half-life calculation program to obtain results as µCi 64Cu incorporated or retained per mg protein.

Insolubility and proteinase K resistance

Cells were lysed for 30 min at 4°C in lysis buffer (LB, 150 mM NaCl, 0.5 % Triton X-100, 0.5 % sodium deoxycholate, 50 mM Tris pH 7.4) containing different protease inhibitors (1µg/ml pepstatin, 1 µg/ml leupeptin and 2 mM EDTA). After a low speed centrifugation
(8,000 g for 4 min) to remove the debris, the lysates were centrifuged at 70,000 rpm for 30 min in the TLA 100.4 rotor of a Beckman Optima TL ultracentrifuge to separate detergent-soluble and detergent-insoluble protein. Fractions were then treated with N-glycosidase F (0.01 units/ml) for 16 h at 37 °C prior to western-blot analysis. For protease resistance, cell lysates were spun as described above and then each fraction was treated with 16 µg of proteinase K per mg of total protein for 30 min at 37 °C and digestion was stopped by the addition of Pefabloc (1mM) for 5 min on ice. The different fractions were western blotted as described below.

**Western blotting**

Cells were lysed for 30 min at 4°C in LB plus protease inhibitors. Lysates were clarified by centrifugation (8,000 g for 4 min) and when indicated were eventually treated with N-glycosidase F. Samples were loaded onto 12% SDS-PAGE and the proteins were transferred onto Immobilon-P membranes. PrP was detected by using the antibodies indicated above. For quantitation, films were analyzed using Sigma Scan Image Analysis Software.

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References

31. Herms, J., Tings, T., Gall, S., Madlung, A., Giese, A., Siebert, H., Schurmann, P.,
222-230
34. Chen, S. G., Teplow, D. B., Parchi, P., Teller, J. K., Gambetti, P., and Autilio-
Figure legends

Figure 1: PrP\textsuperscript{C} and PrP\textsuperscript{Sc} detection in cell cultures.

**Panel A:** Lysates of GT1, GT1\textsuperscript{Chl} and GT1\textsuperscript{Chl-CR} cells were analyzed by western blotting before (PK-) and after (PK+) proteinase K digestion to detect PrP\textsuperscript{C} and PrP\textsuperscript{Sc}, respectively. Full length PrP\textsuperscript{C} revealed using P45-66 was present in similar amount in the three cell lines, while PrP\textsuperscript{Sc} detected with SAF mix antibodies was detected only in GT1\textsuperscript{Chl}. Equivalent amounts of protein of the lysate were used for each lane of the PK- and the PK+ panels: 15\mu g and 150\mu g, respectively.

**Panel B:** Lysates of GT1 and GT1\textsuperscript{Chl} cells were analyzed by immunoblot using SAF mix after deglycosylation with PNGase F to reduce the heterogeneity of the bands. Two main bands were detected in the GT1 (lane 1). They represented full-length deglycosylated PrP migrating around 27 kDa, and the C-terminal PrP fragment produced by cleavage at codon 111/112 and migrating at 18 kDa. In GT1\textsuperscript{Chl}, an additional band of 20 kDa (star) corresponded to PrP\textsuperscript{Sc} cleaved around codon 88 ((16,17,34).

**Panel C:** Lysates of GT1 and GT1\textsuperscript{Chl} cells were subjected to high speed centrifugation to separate soluble (S) from insoluble (I) fractions. The fractions were western blotted with SAF mix before (PK-) or after (PK+) proteinase K digestion. Most of insoluble PrP corresponded to cleaved PrP molecules (star) and to PrP\textsuperscript{Sc} as this band was also proteinase K resistant. Molecular weights on the left are in kDa.

Figure 2: Binding of $^{64}$Cu to the culture and effect of PIPLC digestion.

**Panel A:** 0.1 \mu g of $^{64}$Cu/ml was added to cell culture medium. Cells were incubated for 0.8, 11, 20, 27 and 30 hours in radioactive media, rinsed, harvested and $^{64}$Cu binding was measured. The data obtained from three independent experiments were plotted. Bars represents mean +/- SD; *: GT1 or GT1\textsuperscript{Chl-CR} vs. GT1\textsuperscript{Chl}, p<0.001 (Student’s t test).

**Panel B:** Cells incubated for 30h with $^{64}$Cu/ml were treated with 0.2 U/ml PIPLC at 37\degree C for 2h just before measuring the radioactivity associated with the lysates. The results from three independent experiments were used to obtain the bar graph. Bars represent mean +/- SD; *: Control vs. PIPLC treated cells, p<0.01 (Student’s t test). PIPLC treatment significantly decreased Cu binding in GT1 or GT1\textsuperscript{Chl-CR} cells but not in GT1\textsuperscript{Chl}.
Figure 3: Released of PrP following PIPLC digestion

Panel A: GT1 and GTChl cells were treated with (+) or without (-) 0.2 U/ml PIPLC for 2 hours at 37°C. Medium (M) and cells (C) were collected and analysed for their PrP content using P45-66. The three PrP glycoform associated with the cell lysates are clearly visible in the (C) lanes. As described before (32), PIPLC released preferably higher glycosylated isoforms that migrated slightly slower in SDS/PAGE following the loss of the lipid anchor.

Panel B: PrP bands from three separate experiments were quantitated by densitometry, and the amount of PrP released by PIPLC was plotted as a percentage of the total amount of PrP. Similar experiments were performed on N2a and ScN2a cells (see text) and plotted similarly. Bars represent mean +/- SD; *: Control vs. infected cells, p<0.01 (Student’s t test).
Rachidi et al., Figure 1
Rachidi et al., Figure 2
A

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<tr>
<th>PIPLC (-)</th>
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<tr>
<td>GT1</td>
<td>GT1^{Chl}</td>
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<tr>
<td>C</td>
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B

% of PrP released by PIPLC

- GT1
- GT1^{Chl}
- N2a
- ScN2a

Rachidi et al., Figure 3
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