Summary of the Ph.D. Thesis

Investigation of the role of prion protein in protection against transition metal induced toxicity

Pradeep Kumar Reddy Cingaram

Supervisor: Dr. Ervin Welker
Co-supervisor: Dr. Elfrieda Fodor

Doctoral School of Biology
University of Szeged

Institute of Biochemistry
Biological Research Center of the Hungarian Academy of Sciences

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Introduction

Spongiform degeneration and neuronal loss are characteristic histopathological hallmarks found in the brains of individuals suffering from transmissible spongiform encephalopathy's (TSE) or prion diseases. Prion diseases are a family of rare and fatal neurodegenerative disorders that affect humans and many other mammals. They include Creutzfeldt-Jacob disease (CJD), Gerstmann Straussler syndrome (GSS), fatal familial insomnia (FFI) and kuru in humans, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk and scrapie in sheep. Prion diseases are characterized by the deposition of an abnormal, protease resistant, infectious pathogenic "scrapie" isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}) in brain and in other tissues.

The normal cellular isoform of the prion protein, PrP^{C}, is a highly conserved, approximately 250 amino acid containing glycoprotein that is widely expressed in the body: predominantly in neurons and glia of the CNS and can also be found in several peripheral tissues, and leukocytes. PrP^{C} possesses two N-glycosylation sites and is GPI-anchored to the outer leaflet of the plasma membrane. The exact cellular functions of PrP^{C} are still unknown. However, in the last several years, various biological functions have been suggested for the protein, which include copper metabolism, signal transduction, antioxidant activity, neurotransmitter metabolism, neurogenesis, immune cell activation, cell adhesion, and homeostasis of trace elements.

The interactions of PrP with various transition metals have been implicated not only in the normal physiology of the protein but also in both TSE and Alzheimer's, as being a direct or indirect contributing factor for triggering a neurodegenerative condition. Both *in vivo* and *in vitro* studies have demonstrated that PrP^{C} binds divalent cations. The most encountered site is the OR of PrP^{C} which can bind copper, zinc, manganese, nickel and iron; among which copper shows the highest binding affinity to the OR region. Majority of the earlier hypotheses about the normal cellular function of PrP^{C}, addressed the protein as a Cu^{2+}-binding protein. In the presence of sub-stoichiometric metal concentrations or acidic pH, the imidazole nitrogen atoms are the only true effective donor atoms for both copper and zinc. At neutral or basic pH and in the presence of concentrations of copper (at least equimolar with respect to the peptide), all histidines can behave as independent coordination sites and PrP^{C} can bind up to six Cu^{2+} ions *in vivo.* Zn^{2+} is not able to displace amide protons and thus forms less stable complex in respect to Cu^{2+}.

Free transition metal ions are especially highly effective in generating reactive oxygen species (ROS) that can induce lipid peroxidation and protein oxidation, leading to cellular

damage. Many reports showed a protective role of PrP^C against cellular stresses, especially against oxidative damage, which is perhaps one of the most widely accepted functions of PrP^C. Remarkably, the loss of antioxidant defense was suggested to play a major role in scrapie-infected cells and prion diseases. Regarding the mechanisms of these protective effects of PrP^C, it was shown that cultured cells derived from *Prnp*$^{-/-}$ mice were more sensitive to oxidative damage and exhibited reduced superoxide dismutase (SOD) activity when compared to the PrP expressing wild type. Furthermore, recombinant PrP^C refolded in the presence of Cu^{2+} was reported to have SOD activity, although other authors found neither decreased SOD activity in *Prnp*$^{-/-}$ mice nor SOD activity with recombinant PrP^C. In addition, experiments using genetically modified mice, as well as cross between PrP^C overexpressing and SOD-deficient mice argue against such a role for PrP^C *in vivo*.

Alternatively, it is possible that the binding of Cu^{2+} or Zn^{2+} to PrP^C that induces its endocytosis, is a signal for triggering antioxidative defense even though non-oxidative mechanisms are also considered. Although these mechanisms are not yet clear, a protective effect for PrP^C or its fragments in metal-induced toxicity has been reported in few studies by using various model systems. However, the data are not absolutely univocal in the different systems used.

Main objectives of the thesis

The aim of the presented studies in the current thesis is to examine the role of PrP in condition of metal stress induced by various concentrations of Cu^{2+}, Zn^{2+}, Mn^{2+}, and Co^{2+}, using the hippocampus-derived mouse neuronal cells, Zpl (*Prnp*$^{-/-}$) and ZW (*Prnp*$^{+/+}$), and the SH-SY5Y human neuroblastoma cells as model systems.

The hippocampus-derived, *Prnp*$^{-/-}$ ablated, Zpl 2-1 cells have been shown to be more vulnerable to serum deprivation and oxidative damage induced by H_2O_2 than its PrP-expressing counterpart, ZW cells. Furthermore, reintroduction of PrP restored the viability of Zpl cells in this context. Thus, this model system seems to be relevant for assessing the effect of PrP^C on metal-induced toxicity as well. SH-SY5Y cells have low to undetectable endogenous PrP expression, as such could serve as a model where the effects of the presence of PrP could be studied in metal-stress conditions by re-introduction of its expression.

The main objective of the thesis was to test whether PrP has any general protective role against metal-induced toxicity in these systems, using the four different metals.

The specific aims of my Ph.D. thesis were to answer the following questions:

1) Are the PrP lacking Zpl 2-1 cells more susceptible to Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} induced toxicities than the control ZW cell line?
2) Does PrP expression generated in Zpl cells confer any elevated resistance to the cells against metal-induced toxicity?
3) Would the PrP expression generated SH-SY5Y cells, as a different model system, confer increased resistance to the cells against Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} metal-induced toxicities?

Methods

To address the above questions we chose methods that report on the general health of the cells, rather than looking at a specific pathway involved: such as alamarBlue viability assay, propidium iodide-based dye exclusion assay and morphological examination of the cells. Also, we extended the studies to four different metals, which were applied in various concentrations: Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+}.

Specific methods used were:

- Cell culturing and transfection of cells and generation of stable transgenic cells.
- Western blot analysis.
- Immunocytochemistry.
- Metal ion treatment of cells.
- Cell viability assays and cell morphology.
- Cell-death assays.

Results

1. Prior to testing the metal stress conditions on ZW 13-2 and Zpl 2-1 cells, I have verified the expression levels of the prion protein in the cell cultures maintained in our laboratory using immunoblotting and immunocytochemical analyses. ZW 13-2 cells proved to have a high level of PrP^{C} expression with proper N-glycosylation, as confirmed by western blot of both untreated and PNGase treated samples. Contrary, in case of Zpl 2-1 cells, as expected for the PrP-knockout cell line, I have not found any detectable PrP levels. Expression and localization of the prion protein in the two cell lines was further tested by immunocytochemical analysis, which proved the plasma membrane localization of the PrP in ZW cells.

2. To investigate the role of PrP^C under transition metals-induced toxicity, we examined Zpl 2-1 and ZW 13-2 cells under Cu^{2+}, Zn^{2+}, Mn^{2+} or Co^{2+} treatments using a range of concentrations of each metal ion and alamarBlue-based cell viability assay. After 24 h of metal treatments of various concentrations, cells proved to be sensitive at above 200 μM to copper, manganese and cobalt and above 50 μM in the case of zinc. Nevertheless, Zpl 2–1 cells were significantly more susceptible to Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} toxicities than ZW 13–2 cells at all concentrations of the metal ions that fall into the toxic ranges for the cells.
3. Additionally, the morphological features of ZW 13–2 and Zpl 2–1 cells differed when exposed to various concentrations of Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+}. Using light microscopy, ZW 13–2 and Zpl 2–1 cells exposed to these metals revealed irregular shrinkage and cell rounding as compared to the untreated control cells and started to detach from the surfaces of culture dishes gradually with increasing concentrations of transition metals. However, the morphological changes as a response to metal treatments started to appear earlier i.e. from lower concentrations of the respective metals in case of Zpl cells as compared to ZW cells, indicating that PrP-expressing ZW 13-2 control cells are more resistant to the transition metal stress.
4. To confirm whether the presence of the prion protein in itself is solely responsible for the protective effect against transition metals induced toxicity found in the case of ZW 13-2 and Zpl 2-1 cells, we used the *Prnp*$^{-/-}$ (Zpl 2–1) knock out cell line and re-introduced the *Prnp* gene into the cells, by stably transfecting it with a DNA construct encoding mouse PrP using the Sleeping Beauty (SB) transposase system. An only-EGFP-expressing vector was also used for the purpose to generate control cells (Zpl 2-1-vector) along with the Zpl 2-1-PrP cells. By using Western blot and immunocytochemical analyses, the generated stable Zpl 2-1-PrP transgenic cells proved to possess comparable levels of PrP expression to the wild type ZW 13-2 cell the expressed PrP had similar localization and glycosylation to the PrP in the wild type cell, making the generated Zpl 2-1-PrP cell suitable for a comparative study on the prion protein.
5. To verify whether the presence of cellular prion protein was the only accountable factor for the difference found in the susceptibilities of PrP-expressing ZW 13–2 cells and *Prnp*$^{-/-}$Zpl 2–1 cells against metal toxicity, I treated Zpl 2-1-PrP and Zpl 2-1-vector cells with the same range of concentrations of Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} as the ZW and Zpl cells and assessed their survival using alamarBlue-based assay. Treatment

with these metals significantly decreased cell viability in both Zpl 2-1-PrP and Zpl 2-1-vector cells compared to the untreated controls at the same metal ion concentrations. These results showed that the PrP expressing Zpl 2–1-PrP cells are not significantly more resistant to Cu^{2+}, Zn^{2+}, $Mn^{2+}$and Co^{2+} induced toxicities than its control Zpl 2-1-vector cells, even though the PrP expression in Zpl 2-1-PrP cells is present (and at a level comparable to that in the ZW cells), whereas in the Zpl 2-1-vector cells it is not present. Given that the PrP expression levels of the two types of cells, ZW 13-2 and Zpl 2-1-PrP, are comparable these findings argue that PrP was not the sole reason for the increased resistance of ZW 13–2 cells compared to Zpl 2–1 cells when exposed to metal stresses.

6. Subsequently, I tested whether the generated stable cells differed in their morphological features when exposed to different doses of Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} using light microscopy. The morphological features of Zpl 2-1-PrP and Zpl 2-1-vector cells exposed to each of the four metals revealed irregular shrinkage and cell rounding compared to the untreated control cells and the cells gradually detached from the surface of the culture dishes in a dose-dependent manner for Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} treatments, starting at the same levels of metal concentrations for both types of cells.
7. As a complementary approach to cell viability and cell morphological assays, we attempted to check the effect of the presence of PrP^{C} on transition metal induced toxicity by assessing the extent of cell death caused by the metal treatment. ZW 13–2, Zpl 2–1, Zpl 2-1-PrP and Zpl 2-1-vector cells were treated with the four transition metal ions Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} for a period of 24 h followed by measurement of cell death using propidium iodide (PI) exclusion assay. Exposure to Cu^{2+}, Zn^{2+}, and Mn^{2+} for 24 h resulted in increase of PI positive cells in most types of cells and conditions based on the average number of PI stained dead cells. However, the number of PI positive cells was significantly less in ZW 13-2 as compared to Zpl 2-1 cell lines in case of Cu^{2+}, Zn^{2+} and Mn^{2+} but not in case of Co^{2+} treatments. Co^{2+} was not significantly toxic to either Zpl 2-1 or ZW 13-2 cells compared to untreated control cells. In case of Zpl 2-1-vector and Zpl 2-1-PrP cells, however, such results could not be observed; the Zpl 2-1-PrP cell population had significantly more PI positive cells compared to Zpl 2-1-vector cells when treated by Cu^{2+}, Zn^{2+} and Co^{2+}, with both types of cells being unresponsive to manganese treatment. These data indicate that

PrP^C-expressing Zpl 2-1-PrP cells did not gain more resistance to any of the metals tested and at the concentrations applied, compared to PrP-lacking Zpl 2-1-vector cells.

8. In order to investigate the role of PrP in metal-induced toxicity and to test the observations made on Zpl cells, namely that expression of PrP in itself did not restore or increase the resistance of the cells to transition metals' toxicities compared to the wild type ZW cells, we extended the studies to another model system, the SH-SY5Y human neuroblastoma cells. . When we generated PrP-expressing and control vector-only-expressing cells from these cells and applied the same metal ion concentration treatments as in the case of ZW and Zpl cells, we found that PrP expressing SH-SY5Y-PrP cells were in general significantly more resistant to Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} induced toxicities, than SH-SY5Y-vector cells or SH-SY5Y cells. These results seem to argue for a protective role of PrP against metal induced toxicities in this model system.

Summary of findings and conclusions

- Based on the obtained results we can conclude the followings PrP expressing wild type ZW 13–2 cells are significantly more resistant to Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} induced toxicities than PrP- ablated Zpl 2–1 cells, and also they are morphologically less affected by Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} induced stresses than PrP- ablated Zpl 2–1 cells.

When introducing PrP-expression into PrP-ablated Zpl cells in an attempt to draw a more definite link between PrP expression and cellular resistance against metal toxicities we found that:

- PrP expressing Zpl 2–1-PrP cells are not significantly more resistant to Cu^{2+}, Zn^{2+}, $Mn^{2+}$and Co^{2+} induced toxicities than its control Zpl 2-1-vector cells, as assessed by either viability assay or morphological examination.
- PrP expressing Zpl 2-1-PrP and its control Zpl 2-1-vector cells are more susceptible to Cu^{+2}, Zn^{+2} and Co^{+2} toxicity than ZW 13-2 cells.
- Zpl 2-1-PrP cells are not more resistant to Cu^{+2}, Zn^{+2} and Co^{+2} induced cell death than Zpl 2-1-vector cells.

When testing the effects of Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} on another cell model system, the SH-SY5Y human neuroblastoma cells that have low to undetectable PrP expression levels by generating stable transgenic cells with induced PrP-expression or induced vector-only-expression (as control) from the mother SH-SY5Y cells, we found that:

- PrP expressing SH-SY5Y-PrP cells are significantly more resistant to Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} induced toxicities than SH-SY5Y and SH-SY5Y-vector cells.

Summarizing, although ZW cells established from WT mice are more resistant to all four metal ions tested, we could not establish a clear link between prion protein expression and an increased resistance to metal toxicity, since PrP expression does not confer increased resistance to Zpl cells as compared to GFP expression alone. Thus, the increased resistance of ZW cells are either not related to PrP^C expression, or at least PrP^C expression is not a sole factor necessary for the increased resistance. In SH-SY5Y cells elevated levels of PrP-expression confer higher resistance to metal toxicities compared to the wild type or the vector-only-expressing cells. Altogether, our results do not seem to support a ***general*** protective role for PrP against transition metal toxicity and also, we emphasize the necessity of extreme care when comparing cells derived from PrP knock-out and wild type mice.

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Other article:

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