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Prion protein prevents heavy metals overloading of cells and thus protects them against their toxicity

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Summary. – Physiological function of a prion protein (PrP) is not known yet. Regarding the relation of PrP to heavy metals it is known that PrP is able to bind divalent ions of copper, zinc, manganese and nickel through its octarepeat region. It has been hypothesized but not yet confirmed that PrP could play a role in copper metabolism. In this study, cells expressing human full-length PrP (HuPrP1) and PrP-knockout ($PrP^{0/0}$/1) cells were incubated with various concentrations of copper, zinc, manganese and nickel for 4 days and then were assayed for intracellular content of these metals and cell viability. The results showed that HuPrP1 cells accumulated less heavy metals than $PrP^{0/0}$/1 cells when concentrations of heavy metals exceeded physiological level. In conclusion, HuPrP1 cells are more resistant to chronic overload with copper, manganese, zinc or nickel than $PrP^{0/0}$/1 cells. The resistance to metals overload is caused solely by the presence of PrP, since HuPrP1 and $PrP^{0/0}$/1 cells differ only in the expression of PrP. These results indicate that one of the functions of PrP can be the modulation of trace heavy metal concentrations in cells and protection of cells against heavy metals overload and subsequent oxidative stress.

Keywords: prion protein; copper; manganese; zinc; nickel; oxidative stress

Introduction

Prion protein (PrP) is normally expressed in neurons and glia in the brain and spinal cord (Moser *et al*., 1995). Minor expression was observed in the peripheral cells and tissues, i.e. in leukocytes, enteroglial cells of the myenteric plexus of intestine, in heart and skeletal muscles, in lungs, spleen, kidneys and genitals (Albanese *et al*., 2008; Ford *et al*., 2002; Horiuchi *et al*., 1995; Bendheim *et al*., 1992).

The physiological function of PrP is not known yet. Several strains of PrP-knockout mice were generated, but none of them revealed any developmental or behavioral abnormalities, suggesting an idea that the function of PrP is not essential nor is redundant (Linden *et al*., 2008; Bueler *et al*., 1992). Among possible PrP functions, a regulation of circadian rhythm, immunomodulatory functions, memory modulation, role in cell signaling and cell adhesion or regulation of apoptosis were suggested (Linden *et al*., 2008; Wu *et al*., 2008; Isaacs *et al*., 2006; Bounhar *et al*., 2001; Tobler *et al*., 1996; Bueler *et al*., 1992).

It is known that PrP is able to bind divalent ions of copper and other metals, like zinc, manganese and nickel through its octarepeat region. After binding copper, PrP is rapidly endocytosed, but the fate of copper is not known. Some authors argue that PrP delivers the copper for Cu-Zn superoxide dismutase (SOD) or that PrP has its own SOD activity (Brown *et al*., 1999, 1997a), but these results were questioned (Westergard *et al*., 2007; Jones *et al*., 2005).

Uptake of copper into the cell was considered as one of the potential physiological functions for PrP, but this was not confirmed (Brown, 1999). Another study showed that the majority of copper bound to PrP remains on the cell

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Abbreviations: PrP = prion protein; HuPrP1 cells = cells expressing full-length human prion protein; $PrP^{0/0}$/1 cells = cells without gene for prion protein; SOD = superoxid dismutase

surface and is not delivered into the cell cytoplasm (Rachidi *et al*., 2003). Possible connection of PrP to the metabolism of trace metals is illustrated by a fact that PrP expression is upregulated in response to elevated copper and manganese concentrations, and the cells expressing PrP are more resistant to oxidative stress caused by manganese (Choi *et al*., 2010; Varela-Nallar *et al*., 2006; Armendariz *et al*., 2004). According to these results, PrP expressing cells accumulate less manganese than PrP-knockout cells (Choi *et al*., 2007). Similarly, PrP expressing cells are more resistant to higher concentrations of zinc than PrP-free cells, but the amounts of intracellular zinc were the same in both cell lines, which could be caused by re-localization of zinc into vesicles (Rachidi *et al*., 2009). Other suggestion for PrP function linked to copper metabolism is the buffering of copper in the synaptic cleft and its transport back to the presynaptic cytosol. The support for this theory is that PrP is present in elevated amount at synapses (Sales *et al*., 1998) and synapses are the sites with the highest concentration of copper in mammalian body (Hartter and Barnea, 1988). Moreover, metal imbalance, especially copper and manganese seems to be one of the early hallmarks of prion diseases (Thackray *et al*., 2002).

In this work, we used a cell model to examine the role of PrP in the metabolism of selected divalent heavy metals. We compared the contents of Cu, Zn, Mn and Ni in PrP expressing and PrP-knockout cells, cultivated in medium with various concentrations of these metals and the effect of PrP expression on cell viability.

Materials and Methods

Cell cultures. In this study, we used PrP knockout cell line $PrP^{0/0}$/1 and human prion protein expressing cell line HuPrP1, both of fibroblast origin, prepared as described previously (Prčina *et al*., 2010). The cells were grown in DMEM (Gibco) supplemented with 10% FBS (Gibco), 2 mmol/l L-glutamine (PAA) and 50 μg/ml gentamycin (Gibco) and incubated at 37°C in a humidified incubator in atmosphere containing 5% CO_2.

Experimental design. For measurements of intracellular metals content, cells were grown in medium containing 2, 20 and 200 μmol/l $CuCl_2$, 200 μmol/l $NiCl_2$, 80 μmol/l $ZnSO_4$ and 100 μmol/l $MnCl_2$ for 24 hr. Fully confluent cultures on total area of 300 cm^2 were rinsed twice with pre-warmed PBS and harvested by scraping. Cells were then rinsed with PBS again, pelleted at 200 g and vacuum dried. The copper content in the samples was measured in tetraplicates by inductively coupled plasma-atomic emission spectrometer at the Institute of Laboratory Research on Geomaterials, Faculty of Natural Sciences of Comenius University, Bratislava. The measured values were related to samples weight.

Cell viability assay. For determination of cell viability after exposure to elevated copper concentrations, we measured the level of intracellular ATP. Cells were cultured for 4 days in medium supplemented either with 2–300 μmol/l $CuCl_2$, 120–200 μmol/l $NiCl_2$, 20–100 μmol/l $MnCl_2$ or 20–100 μmol/l $ZnSO_4$. Determination of cell survival was performed according to manufacturer's protocol using CellTiter-Glo® Luminescent cell viability assay (Promega), which is based on quantitation of ATP present in metabolically active cells. All measured values were expressed in percentage related to the control grown in medium not supplemented with metal salts.

Western blot. PBS rinsed cells were scraped and lysed in lysis buffer (50 mmol/l Tris-HCl, pH = 7.5, 150 mmol/l NaCl, 2 mmol/l EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate). Fifty μg of total cellular proteins were separated by PAGE in a 12% gel under reducing conditions and electroblotted to a PVDF membrane in 10 mmol/l N-cyclohexyl-3-aminopropanesulfonic acid (pH 11). The blot was blocked in 5% non-fat milk in PBS for 1 hr and incubated with the MAb SAF32 (SPI Bio; diluted 1:2,000 in PBS) overnight at 4°C, followed by polyclonal HRP-conjugated goat anti-mouse IgG (1:5,000; DAKO). The blot was developed with Super signal west pico chemiluminescent substrate (Pierce) and scanned with a LAS3000 imaging system (FUJI Photo Film).

Statistical analysis. The statistical significance of differences was evaluated by Student´s t-test. Differences with $P \leq 0.05$ were considered significant.

Results

PrP expressing cells accumulate less copper, manganese and nickel than PrP-knockout cells

To determine the impact of prion protein expression on accumulation of divalent metal ions in the cells, we measured the intracellular content of Cu, Mn, Zn or Ni in $PrP^{0/0}$/1 and HuPrP1 cells treated with individual metals using ICP-MS. Results showed a significant difference in content of Cu, Mn and Ni between the examined cell lines cultivated in high concentrations of these metals, while there was almost no difference in Zn content. No differences in levels of accumulated copper were observed between HuPrP1 and $PrP^{0/0}$/1 cells, when they were cultivated in low concentrations of copper (2 μmol/l and 20 μmol/l). However, when cultivated with 200 μmol/l Cu, HuPrP1 cells accumulated about 3-fold less amount of copper compared to $PrP^{0/0}$/1 cells. The difference was shown to be statistically significant (Fig. 1a). We obtained similar results with another two metals, Mn and Ni. Similarly to copper, HuPrP1 cells which were treated with high concentration of Mn (100 μmol/l) accumulated 3-fold less amount of manganese than $PrP^{0/0}$/1 cells. This difference was statistically significant (Fig. 1b). Furthermore, HuPrP1 cells cultivated in 200 μmol/l nickel accumulated 1.6-fold less Ni when compared to their $PrP^{0/0}$/1 counterparts. This

difference was also statistically significant (Fig. 1d). However, the differences between HuPrP1 and PrP$^{0/0}$/1 cells in content of accumulated Zn were not significant in the cells cultivated in 80 μmol/l $ZnSO_4$ (Fig. 1c).

PrP expressing cells are more resistant to high concentrations of copper, manganese, zinc and nickel than PrP-knockout cells

To explore the possible protective effect of the prion protein against the heavy metals toxicity, we cultivated PrP$^{0/0}$/1 and HuPrP1 cells in medium containing Cu, Mn, Zn or Ni and after 4 days of cultivation we measured the level of ATP as a marker of cell viability. The measurements showed that HuPrP1 cells are more resistant to cytotoxicity caused by the Cu, Mn, Zn and Ni. In the PrP$^{0/0}$/1 cells some dead cells were observed in light microscope (data not shown) after incubation with 200 μmol/l copper, but cell death was mostly prominent in cell culture incubated in 300 μmol/l copper (Fig. 2a). In both these concentrations, HuPrP1 cells were more resistant to metal toxicity, with viability of 1.6-fold in 200 μmol/l copper and 3,8-fold in 300 μmol/l copper higher, than in PrP$^{0/0}$/1 cells. The difference in viability of examined cells was statistically significant. For manganese, the threshold was at the concentration of 80 μmol/l, when HuPrP1 cell viability was 9-fold higher than in PrP$^{0/0}$/1 cells and in medium with 100 μmol/l manganese the ratio between the number of viable HuPrP1 and PrP$^{0/0}$/1 cells was 13 (Fig. 2b). Statistically significant difference between the viability of the two cell lines cultivated with zinc appeared at the zinc concentration 100 μmol/l, when there was 1.9-fold more living HuPrP1 cells compared to PrP$^{0/0}$/1 cells (Fig. 2c). Further, after exposure to nickel, the amount of viable PrP$^{0/0}$/1 cells in comparison to HuPrP1 cells gradually decreased reaching lowest amount (16% of the control) at the concentration 200 μmol/l Ni (Fig. 2d). The differences were observed in all analysed concentrations and were shown to be statistically significant (Fig. 2d).

Discussion

Previously published results are quite contradictory in the opinion regarding the role of PrP in copper and other metals metabolism, especially its role in regulation or even participation in SOD activity. It is known that after copper binding, PrP is rapidly endocytosed from the cell surface (Brown *et al*., 1997b; Pauly and Harris, 1998). Together with the knowledge that PrP-knockout cells are more sensitive to oxidative stress than PrP expressing cells, these properties of PrP led to assumption that PrP could be a copper transporter and may influence the activity of Cu/Zn SOD (Brown *et al*., 1999; Brown *et al*., 1997a).

Experiments performed on neuronal cells showed that PrP expression increases the level of copper in the cells and the copper uptake depends on the level of PrP expression and on clathrin-mediated endocytosis (Urso *et al*., 2010; Brown, 1999). The upregulation of PrP expression and subsequent enhancement of Cu uptake was detected under reduced Cu availability, explained as the mechanism to sustain the copper delivery for Cu/Zn SOD (Urso *et al*., 2012). These results are in contrast with our data, since we have found out

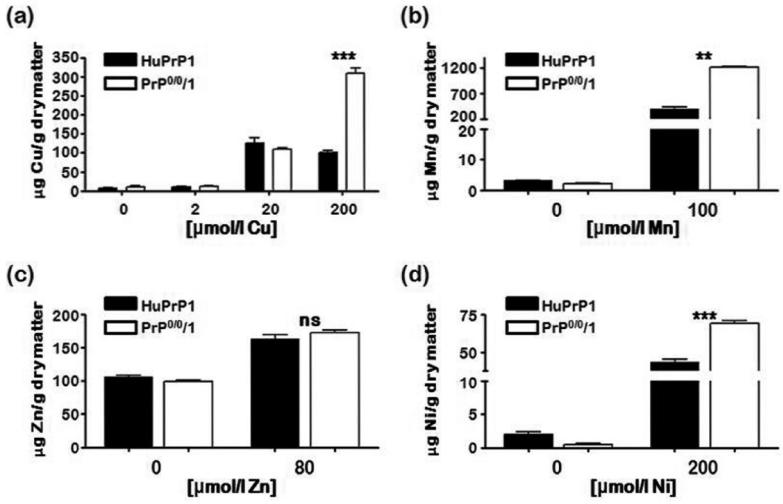

Fig. 1
Accumulation of heavy metals by HuPrP1 and PrP$^{0/0}$/1 cells
Cells were cultivated in media with various concentrations of Cu, Mn, Zn and Ni (a-d) or without them for 24 hr and the metal content was assayed in dried cells.

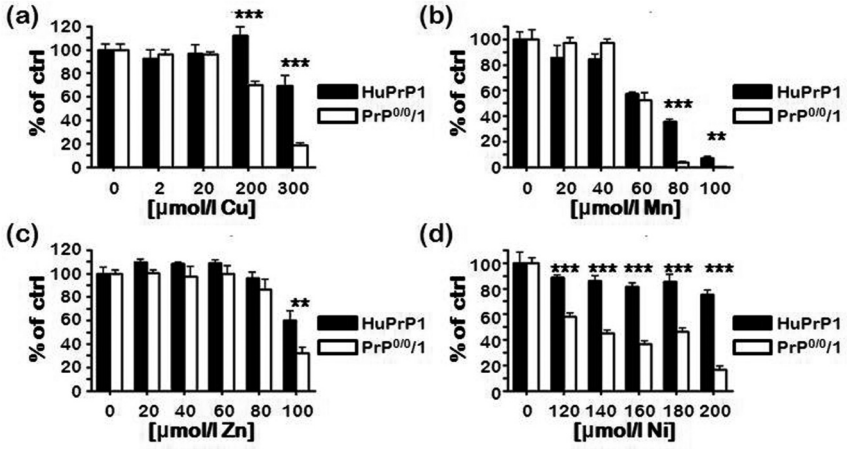

Fig. 2
Viability of HuPrP1 and PrP$^{0/0}$/1 cells after cultivation in various concentrations of heavy metals quantified through ATP production
Cells were cultivated in media with various concentrations of Cu, Mn, Zn and Ni (a–d) or without them for 4 days and the cell viability was then assayed and expressed in percentage relative to control.

that PrP$^{0/0}$/1 cells accumulate more copper, manganese and nickel compared to HuPrP1 cells and as a result, PrP$^{0/0}$/1 are more sensitive to copper, manganese and nickel toxicity. In wide range of Cu concentrations in culture medium, there were no conditions favouring higher uptake of copper into the cells expressing PrP, HuPrP1 cells were protected against accumulation of copper in the environment with excess of this metal. We tested another 3 divalent metals, two microelements (Mn, Zn) and nickel, whose role in mammalian body is not known, and obtained similar results. In high concentration of metal ions, PrP$^{0/0}$/1 cells accumulated significantly more manganese and nickel, when compared to HuPrP1 cells. However, there was no difference in zinc content between the two cell lines. It can be argued that our fibroblast cell models are not suitable for experiments with PrP, which is predominantly expressed in neuronal cells and that the published results acquired from neuronal cell models are more reliable. However, other results obtained with our cell models showed that PrP$^{0/0}$/1 cells accumulate more manganese than HuPrP1 cells and this is in agreement with experiments performed on neuronal cells (Choi *et al.*, 2007). Moreover, HuPrP1 cells were more resistant to metals toxicity than PrP$^{0/0}$/1 cells, what is in correspondence with previously published results suggesting that PrP participates on metals efflux rather than on transport of metal ions into the cell. The arguments supporting this theory include an upregulation of PrP as response to copper or manganese overload, lowered accumulation of manganese and higher resistance of PrP expressing cells to manganese and zinc toxicity (Choi *et al.*, 2007, 2010; Rachidi *et al.*, 2009). Our results thus showed that the PrP-mediated prevention of metal accumulation does not require neuronal-specific factors, and the pathway or pathways responsible for this effect are common for several cell types. This phenotype is caused solely by the presence of PrP, since HuPrP1 and PrP$^{0/0}$/1 cells differ only by the expression of PrP. We know that at least in our case, the lowered accumulation of copper, manganese and nickel by PrP expressing cells is not caused by releasing PrP with bound metal into medium, as predicted Choi and coworkers (Choi *et al.*, 2007). We precipitated proteins from the medium used for cultivation of HuPrP1 cells with all tested metals after 48 hr of cultivation, and tested the presence of PrP using MAb SAF32 (epitope in octarepeats), but we could not detect any PrP signal (data not shown).

It seems that the PrP-mediated resistance against zinc toxicity is based on different mechanism than the resistance against other metal ions. Although PrP-expressing cells are more resistant against zinc toxicity, they do not show altered levels of intracellular zinc compared to control cells without expression of PrP. Instead of possible efflux of zinc or preventing the zinc uptake authors observed relocalization of intracellular zinc into vesicles (Rachidi *et al.*, 2009). We observed the same effect after exposure of our cell lines to increased concentrations of zinc. HuPrP1 cells survived higher Zn concentrations, but did not accumulate less Zn than PrP$^{0/0}$/1 cells. We do not know the exact principle behind the resistance of PrP expressing cells to Zn toxicity. There is a question whether it is the relocalization of Zn inside the cell as reported previously (Rachidi *et al.*, 2009), or completely different mechanism. Other question is whether the effect of undecreased accumulation of Zn is common for all PrP expressing cells, like in case of Mn, or is restricted only to the non-neuronal cells expressing recombinant PrP.

Our results indicate that PrP could act as a buffering protein, preventing a cell damage caused by heavy metal toxicity.

One of the highest concentrations of copper, manganese and zinc is present at synapses, structures with elevated amount of PrP. Nerve endings release copper into the synaptic cleft upon depolarization, and the concentration can locally reach 250 μmol/l (Kardos *et al.*, 1989). Manganese is released into the synaptic cleft, probably from glutamatergic neuron terminals, and may influence synaptic neurotransmission (Takeda *et al.*, 2002). Furthermore, zinc can rise to micromolar levels in the proximity of axon terminals after releasing from synaptic vesicles (Sensi *et al.*, 2009). In such extremely elevated concentrations of these metals, cells are exposed to their toxic effects.

Differences between HuPrP1 and $PrP^{0/0}$/1 cells in accumulation of Ni can be considered as the mimicking effect of Ni due to its divalency and other similarities to other tested metals, because Ni has no known function in mammalian body, and it is surely not present in any tissue or cell compartment in such high concentrations as we used in our experiments.

Conclusion

In this study, we examined and confirmed that the function or at least one of the functions of the PrP could be connected to metals metabolism, mainly the metabolism of copper and manganese and possibly zinc. The effect of PrP expression was prominent after cultivation of cells in high concentrations of tested metals for longer time. Our results indicate that the role of PrP could be in detoxificating cells from heavy metals, mainly at synapses. We hypothesize that prion protein could thus act as the buffering agent, preventing oxidative damage of the cell after local elevation of the concentration of these metals.

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