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Copper reduction by copper binding proteins and its relation to neurodegenerative diseases

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Abstract

Increasing evidence supports an important role for metals in neurobiology. In fact, copper binding proteins that form bioinorganic complexes are able to display oxidant or anti-oxidant properties, which would impact on neuronal function or in the triggering of neurodegenerative process. Two proteins related to neurodegenerative diseases have been described as copper binding proteins: the amyloid precursor protein (APP), a protein related to Alzheimer's disease, and the Prion protein (PrP), related to Creutzfeldt-Jakob disease. We used different synthetic peptides from APP and PrP sequences in order to evaluate the ability to reduce copper. We observed that $APP_{135-156}$, amyloid-β-peptide ($A\beta_{1-40}$), and PrP_{59-91} all have copper reducing ability, with the $APP_{135-156}$ peptide being more potent than the other fragments. Moreover, we identify His, Cys and Trp residues as key amino acids involved in the copper reduction of Aβ, APP and PrP, respectively. We postulated, that in a cellular context, the interaction of these proteins with copper could be necessary to reduce copper on plasma membrane, possibly presenting Cu(I) to the copper transporter, driving the delivery of this metal to antioxidant enzymes. Moreover, protein-metal complexes could be the catalytic centers for the formation of reactive oxygen species involved in the oxidative damage present both in Alzheimer's and Prion disease.

Abbreviations: Aβ – amyloid-β peptide; AD – Alzheimer's Disease; APP – amyloid precursor protein; PrP – prion protein.

Introduction

Copper cellular homeostasis is a finely regulated phenomenon, as indicated by the free cytoplasmic copper concentration, estimated to be less than 10^{-18} M (Rae *et al.* 1999). This fine-tuning involves many proteins localized at different biological levels: plasma membrane, organelles and cytoplasm. Recently, in the cytoplasm, a new family of soluble copper chaperones, is emerging that act in the trafficking of the copper ions (O'Halloran & Culotta 2000; Rosenzweig & O'Halloran 2000). These copper chaperones deliver copper to different proteins that need this metal to obtain an optimal specific activity, such as Cytochrome oxidase, in the mitochondria membrane, and Copper-Zinc Superoxide Dismutase (SOD), in the cytosol (O'Halloran & Culotta 2000; Rosenzweig & O'Halloran 2000). In particular, SOD in its copper-zinc bound form, acts to limit reactive oxidative species (ROS) by catalyzing the dismutation of superoxides to a less reactive intermediate (i.e., H_2O_2), and thus regulates the redox status of the cell (Miranda *et al.* 2000), which would have an impact on processes such as development, life span, aging and neurodegenerative diseases (Castagne *et al.* 1999). Therefore, the study of the mechanisms involved in copper homeostasis is required to understand the regulation of normal cellular processes.

Table 1.

Protein fragment	localization	Proposed function	Copper affinity
APP $_{135-156}$	Extracellular	Copper Reductase	Nanomolar
Aβ $_{1-40}$	Juxta and intramembrane	Neurotoxic	Picomolar
PrP $_{59-96}$	Extracellular	Neuroprotective; Copper Reductase; SOD activity	Fentomolar

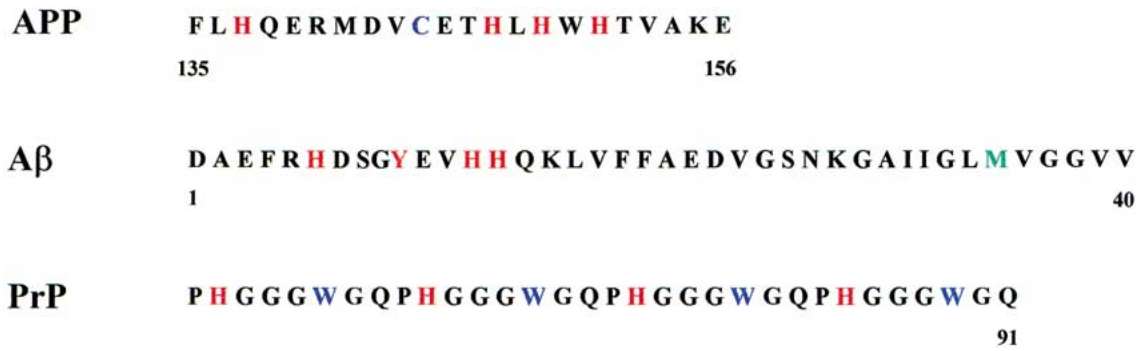

Fig. 1. Copper binding domains. The amino acid sequence of copper binding domains of APP, Aβ peptide and Prion protein are illustrated. The red letters represent amino acids involved in the copper coordination; blue letters represent amino acids involved in electron transfer and green letter represent possible amino acids involved in electron transfer.

Two proteins related to neurodegenerative diseases have been described as copper binding proteins: The amyloid precursor protein (APP), a protein related to Alzheimer's disease (AD) (Selkoe 1997), and the Prion protein (PrP), related to the Creutzfeldt-Jakob disease (Prusiner 1991) (Table 1). We and others have suggested that these proteins may be involved in the copper transport from the extracellular to the intracellular space (Multhaup *et al.* 1996; Ruiz *et al.* 1999, 2000), similarly to the copper incorporation system that operates in yeast (Hassett & Kosman 1995). However, under certain adverse circumstances, these proteins may gain pro-oxidant activity through incorrect metal acquisition (Estevez *et al.* 1999; Bush 2000), explaining some aspects related to oxidative damage that appear in neurodegenerative diseases (Miranda *et al.* 2000; Bush 2000). With this in mind, we decided to compare the capacity to reduce copper of the copper binding domains from APP, Aβ and PrP. Our results established that all of these fragments were able to reduce copper, with APP and Prion being more potent than the Aβ peptide, having Cys, Trp and His, the corresponding key amino acids for their reducing capacity. Moreover, we found that copper reducing capacity is not strictly related to amyloid structure formation.

Experimental

Materials

Synthetic peptides corresponding to the copper binding domain of the wild-type human PrP sequence (PrP_{59-91}) and its mutant $PrP_{Trp\rightarrow Ala}$ were obtained from Genemed Biotechnologies, Inc. (San Francisco, CA). Peptides corresponding to human wild-type $APP_{135-156}$ sequence and $A\beta_{1-40}$ (human and rat sequence) were obtained from Chiron Corp. (Emeryville, CA). Variant APP peptides containing a cysteine to serine substitution ($APP_{Cys144\rightarrow Ser}$) were obtained from BioSynthesis Inc. (Lewisville, TX). All peptides were purified by reverse phase high performance liquid chromatography. Peptides were freeze-dried and stock solutions were dissolved in H_2O stored at -20 °C and used within 10 days.

Copper reduction assay

Copper reduction was analysed using bathocuproine disulfonate (BC) (Merck) as a Cu^{1+} indicator molecule (Multhaup *et al.* 1996; Ruiz *et al.* 1999). The Cu^{1+}-BC divalent complex has a maximum absorbance at 480 nm when scanned from 230 to 680 nm. For the assay, samples were incubated in PBS (pH 7.4) at 37 °C for 60 min with Cu^{2+} (copper chloride), 360 μM BC and various reducing agents including vitamin C as control. Cu^{1+} formation was monitored at 480 nm (Multhaup *et al.* 1996; Ruiz *et al.* 1999). H_2O used in buffer preparations was submitted to E-pure resin system (Barnstead, Dubuque, IA) to eliminate organic compounds and Chelex-100 resin (Bio-Rad, Hercules, CA) to eliminate anions and cations. After this treatment, the resultant H_2O had a conductance value of 18 MΩ/cm. All the solvents used were Cu^{1+} free by copper reduction assay.

Aggregation assay: turbidity

The aggregation assay was carried out as described by Inestrosa, *et al*. (1996). Stock solutions were prepared by dissolving lyophilized aliquots of the $APP_{135-156}$, $A\beta_{1-40}$ and PrP_{59-91} in water and added to aqueous buffer (phosphate saline buffer, PBS, pH 7.4) in the absence or presence of $CuCl_2$ and incubated at 37 °C for 4 days. Aggregation was measured by turbidity at 405 nm vs. buffer blank.

Congo Red staining and polarized light microscopy

The samples were submitted to staining with Congo Red as was previously described (Alvarez *et al.* 1997). Briefly, the samples were stained during 20 min with a Congo Red Basic solution (80% ethanol, 0.005% NaOH, saturated NaCl). Then, they were dehydrated with 100% ethanol and 100% xylol. The samples were analyzed by polarized light microscopy.

Results

APP, Aβ and Prion protein reduces copper

We analysed the copper-reducing capacity of the wild-type Aβ, APP and PrP fragments corresponding to copper binding domains (Figure 1). The different peptides and other compounds were incubated with copper in the presence of bathocuproine for 1 h at 37 °C. Results indicate that APP, Prion and Aβ peptide present copper reducing activity in comparison to Vitamin C, a strong reducing agent (Figure 2). The APP and PrP fragments presented higher activity than Aβ. In the same experiments BSA, AChE or angiotensin did not present any activity, displaying values similar to the background (data not shown). The reducing capacity of Aβ, APP and Prion fragments was found to be concentration-dependent as shown in Figure 3.

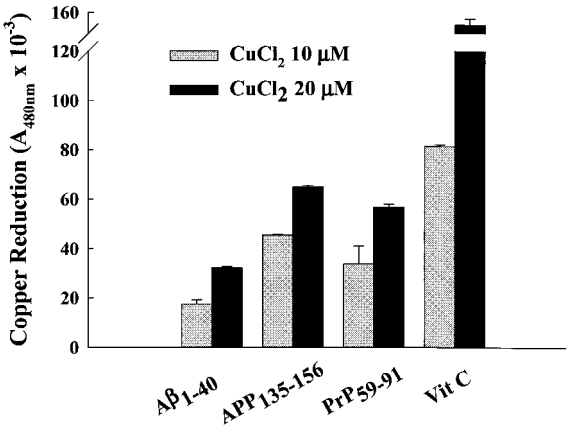

Fig. 2. Copper reduction by APP, Aβ and Prion fragments. Samples containing the Cu(I) indicator BC (360 μM), Cu(II), and different peptides fragments (5 μM) in PBS (pH 7.4) were incubated at 37 °C for 60 min. Vitamin C (5 μM) was included as positive control. Values represent the means (± SEM) of three different experiments.

Mutant APP, Aβ and Prion protein show decreased capacity to reduce copper

We then studied the putative amino acids present within the APP, Aβ and PrP peptides that should be involved in the copper reduction. To evaluate them, we used the rat Aβ peptide sequence which has three amino acid changes in the Aβ structure (Arg5→Gly, Tyr10→Phe and His13→Arg) in comparison with the human Aβ sequence and two mutant peptides carrying specific amino acid substitution: One at the only cysteine present in the $APP_{135-156}$ fragment (APPCys144→Ser) and another at the four tryptophans present in the PrP fragment (PrPTrp→Ala). All mutant peptides had less copper reducing ability compared with their wild-type counterparts (APP and PrP) or the human counterpart (Aβ) (Figure 4). The loss of copper reduction capacity was dramatic for APPCys144→Ser and PrPTrp→Ala, suggesting that these residues are key elements in the electron donor chemistry. The copper reducing ability displayed by rat Aβ was 50% of human Aβ suggesting that additional residues contribute to the effect.

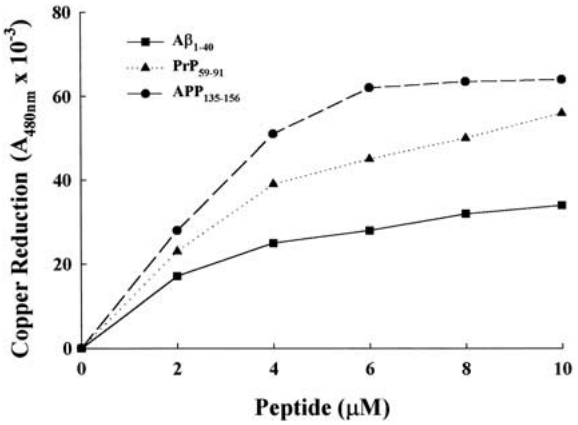

Fig. 3. Dependence of Cu (I) formation by peptide concentration. Cu (I) formation is dependent on the concentration of APP (●), Aβ (■) and PrP (▲) used. APP135-156, Aβ1-40 or PrP (0–10 *mu*M) were incubated with 10 μM Cu (II) and BC (360 μM) in PBS at 37 °C for 60 min. Cu(I) formation was monitored as the increase in absorbance at 480 nm. Values represent a representative experiment.

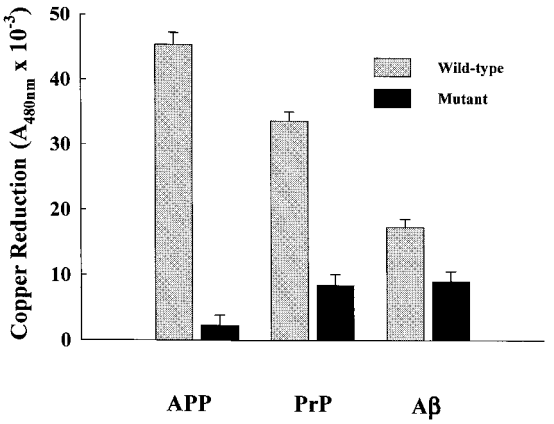

Fig. 4. Key amino acids of APP, Aβ and PrP involved in copper reduction. Mutant and wild-type peptides of APP, Aβ or PrP sequences were incubated in PBS at 37 °C for 60 min with 10 μM Cu (II) and 360 μM BC. Cu (I) formation was monitored as the increase in absorbance at 480 nm. Values represent means (± SEM) of three different experiments.

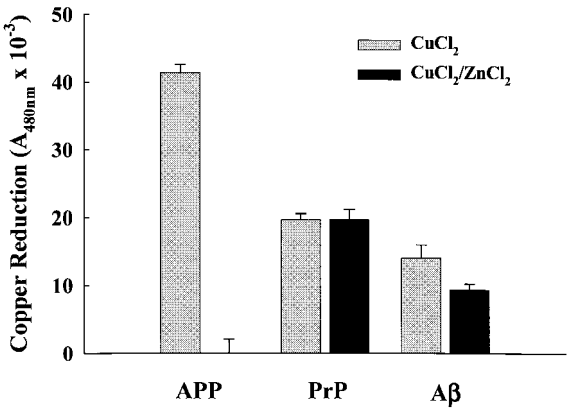

Fig. 5. Differential effect of zinc on copper reduction by APP, Prion and Aβ peptides. APP, Aβ, and Prion protein were preincubated with or without $ZnCl_2$ (10 μM) at 18 °C for 15 min. Then the peptides were incubated with 10 μM Cu (II) and BC (360 μM) in PBS at 37 °C for 60 min. Cu(I) formation was monitored as the increase in absorbance at 480 nm. Values represent the means (± SEM) of three different experiments.

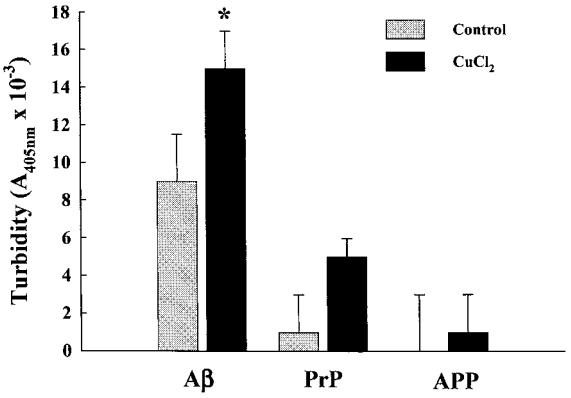

Fig. 6. Effect of copper on peptide aggregation. APP, Aβ, and Prion protein were incubated in the absence (◯) or presence of $CuCl_2$ (10 μM) at 37 °C for 4 days. Aggregation was measured by turbidity at 405 nm vs. buffer blank. Values represent the means (± SEM) of three different experiments. * indicates a statistical significance or $P < 0.05$ (Student's *t*-test).

Differential effect of zinc on copper reduction by APP, PrP an Aβ

Since zinc is more available than copper in synaptic clefts (Frederickson 1989; Frederickson & Bush 2001; Hartter & Barnea 1988), we evaluated the copper reduction ability of these peptides in the presence of zinc, as a possible competitor of the copper-peptide interaction. Different peptides were incubated with copper in the absence or presence of equimolar concentration of zinc (Figure 5). The copper reducing ability of APP was dramatically inhibited by the presence of zinc (100% inhibition). No effect was observed for the PrP, and only 30% of inhibition was observed for the Aβ peptide.

Copper induces the formation of amyloid aggregates of Aβ peptide

We evaluated amyloid formation by the $A\beta_{1-40}$, $APP_{135-156}$ and PrP_{59-91} fragments in the presence of $CuCl_2$. The samples were incubated without stirred at 37 °C for 4 days and the formation of aggregates was monitored by the increase of turbidity at 405 nm. As expected, $A\beta_{1-40}$ in the absence or presence of

copper presented a higher turbidity signal (Figure 6). Instead, the APP and PrP fragments displayed only a minor signal. Finally, we evaluated the amyloid characteristics of these aggregates using Congo red and polarized light microscopy. As shown the Figure 7, the $A\beta_{1-40}$ aggregates formed in the absence or presence of copper presented apple green birefringence indicating the amyloid character of these aggregates. However, the APP and PrP samples did not present these characteristics (data not shown), suggesting that the small turbidity signal observed with these samples must correspond to non-amyloid aggregates.

Discussion

The physiological function of APP, Aβ and PrP has not yet being established; however the accumulated evidence so far supports the idea these proteins may be playing a role as copper binding proteins. First, these proteins coordinate copper forming bioinorganic complexes with high Kd values (Hesse *et al.*, 1994; Atwood *et al.*, 2000; Jackson *et al.*, 2001), suggesting a possible *in vivo* relevance. In fact, PrP retains copper bound to its structure when it is isolated from rat and human brain tissue (Brown *et al.* 2001). Second, they contain in their structure clusters of amino acids classically involved in copper binding coordination such as histidines and cysteines. Third, we and others have established that copper binding fragments of APP, Aβ and PrP can reduce copper (Multhaup *et al.* 1996; Huang *et al.* 1999a; Ruiz *et al.* 1999, 2000; Opazo *et al.* 2000), supporting the argument that these bioinorganic complexes are redox-active. In the present work we compared their copper reducing abilities and found that APP was the more potent of the three copper binding peptides. Moreover, we found a differential inhibitory effect of zinc on the copper reduction, which was more potent on APP, suggesting that this fragment could not reduce copper *in vivo* at the synaptic cleft, where zinc is ordinarily more available than copper (Frederickson 1989; Frederickson & Bush 2001; Hartter & Barnea 1988). Interestingly, Aβ was partially affected by zinc and PrP was practically unaffected, in agreement with the strong and selective copper affinities recently described (Stockel *et al.* 1998; Atwood *et al.* 2000; Jackson *et al.* 2001). PrP is normally anchored to the neuronal plasma membrane by a GPI anchor domain (Stahl *et al.* 1987). The N-terminal copper binding domain seems to be involved in a novel role because it is among the best conserved regions of PrP among mammals (Wadsworth *et al.* 1999). Our observations demonstrate that synthetic peptides containing the copper binding domain of PrP, corresponding to four repeats of Pro-His-Gly-Gly-Gly-Trp-Gly-Gln (PrP_{59-91}), have the ability to reduce to Cu(II) to Cu(I) through the tryptophan residues. The Aβ peptide, a 40 amino acid peptide involved in the pathology of Alzheimer's disease (Selkoe 1997) showed copper-reductase activity. One of the elements probably involved in its reductive activity is the presence of two His residues in this sequence, at positions 13 and 14, which seem to play a key role in copper binding (Curtain et al. 2001). In fact, Aβ derived from the rat sequence (His13$\rightarrow$Arg) possessed a 50% lower copper reducing activity than the Aβ derived from the human sequence. In agreement with this result, Aβ derived from human sequence presents a minor copper reducing activity in presence of exogenous histidine (Opazo *et al.* 2000). Previous reports showed that Aβ-peptide reduces copper (II) to copper (I) with hydrogen peroxide production and probable hydroxyl radical formation suggested by TBARS positive signal (Huang *et al.* 1999a). The hydroxyl radical formed in this process may be generated via the Haber-Weiss reaction (Halliwell & Gutteridge 1990). Free radical-generating systems are able to catalyze the oxidative modification of proteins when Fe(III) or Cu(II) are incubated in the presence of O_2 and an appropriate electron donor (Halliwell & Gutteridge 1990). In fact, the conversion of superoxide radicals ($O_2^{\cdot -}$) and H_2O_2 to the highly cytotoxic hydroxyl radical ($HO^{\cdot}$) can only take place when catalytic concentrations of transition metals are present. The transition metals accumulated in senile plaques may be a direct source of reactive oxygen species (Smith *et al.* 1996) associated to the oxidative stress observed in Alzheimer's disease (Miranda *et al.* 2000). In fact, transgenic mice that overexpress APP exhibit amyloid deposition associated to redox-active iron and oxidative damage markers (Smith *et al.* 1998). In agreement with this data, Lovell *et al.* (1998) found that copper, iron and zinc are concentrated within the core and periphery of senile plaque deposits. On the other hand, transition metals directly stimulate aggregation of Aβ (Dyrks *et al.* 1992; Atwood *et al*. 1998) probably by increasing Aβ species that enhance neurotoxicity (Iversen *et al*. 1995). These results indicate that the unbalance of metal ion homeostasis (Nuñez *et al.* 2000) may play a role in the pathogenesis of Alzheimer's disease (Atwood *et al*. 1998; Sayre *et al.* 1999).

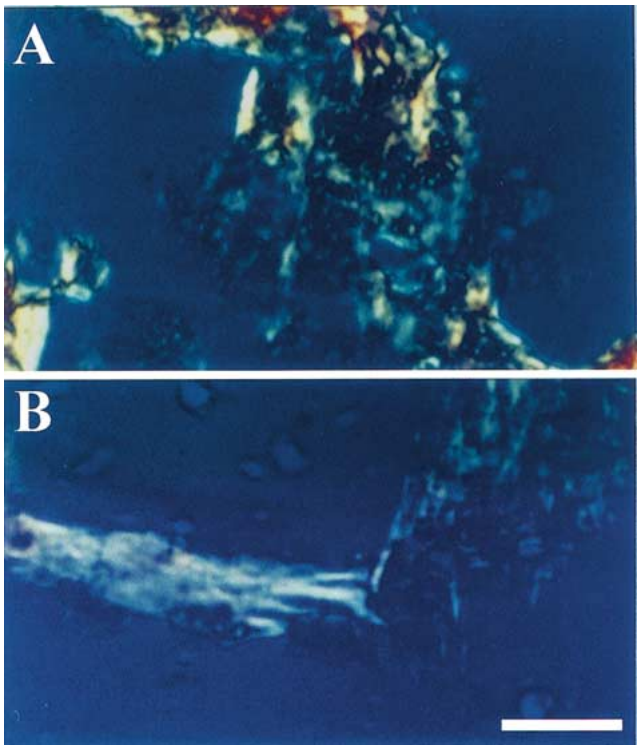

Fig. 7. Congo red staining of amyloid aggregates. Aβ_{1-40} (10 μM) was incubated in the absence or presence of $CuCl_2$ (10 μM) and the aggregates formed were stained with Congo red staining and the tinctorial pattern analyzed under polarized light. (A) Aβ fibrils, (B) Aβ fibrils formed in the presence of copper. The bar represents 50 μm.

An initial report, by Hensley and coworkers, suggested that the Aβ peptide itself spontaneously generates free radicals that can damage cells (Hensley *et al.* 1994).

Recently, these results were described as artifactual due to contaminants in some of the preparations (Dikalov *et al.* 1999). Moreover, Dikalov and coworkers showed that the Aβ peptide potentiates metal-catalyzed oxidation of hydroxylamine derivatives. Interestingly, Aβ peptide produces hydrogen peroxide through copper or iron reduction with a concomitant TBARS signal probably by hydroxyl radical formation (Huang *et al.* 1999a) via Fenton-like or Haber-Weiss reactions (Halliwell & Gutteridge 1990). Moreover, we found that amyloid fibrils reduces copper, suggesting that the oxygen radical species can be generated on an initial and a late step of amyloid formation (Opazo *et al.* 2000). Also, APP reduces copper (II) to copper (I) (Figure 2) (Multhaup *et al.* 1996; Ruiz *et al.* 1999) and subsequent exposure to hydrogen peroxide results in reoxidation of Cu(I) and site-specific cleavage of APP (Multhaup *et al.* 1998). In a cellular context, the favorable or deleterious effects of the copper-reducing activities of PrP, Aβ and APP will depend on the extent of PrP and APP expression or Aβ amyloid accumulation. In this sense the copper-reducing activity of PrP, APP and Aβ peptide should serve a favorable physiological function, possibly presenting Cu(I) to the copper plasma membrane transporter (Zhou & Gitschier 1997). In unfavorable conditions, an abnormal increase of APP or an accumulation of Aβ peptide into amyloid fibrils, may increase the reduction of copper, generating a concomitant increase in Cu(I) levels and free radicals and consequently causing oxidative damage (Multhaup *et al.* 1998; Huang *et al.* 1999b; Ruiz *et al.* 1999; Opazo *et al.* 2000), such as lipid peroxidation (Nunomura *et al.* 2001; Practicò *et al.* 2001) or protein oxidation, which will change protein function and increasing degradation (Davies et al. 1987).

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