BioMetals **16:** 215–223, 2003.

Iron-induced oxidative stress modify tau phosphorylation patterns in hippocampal cell cultures

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Published on line: September 2002

Key words: Alzheimer's disease; brain hippocampal cells, Cdk5/p35 system, iron, neurodegeneration, oxidative stress, tau phosphorylation

Abstract

Oxidative stress phenomena have been related with the onset of neurodegenerative diseases. Particularly in Alzheimer Disease (AD), oxygen reactive species (ROS) and its derivatives can be found in brain samples of postmortem AD patients. However, the mechanisms by which oxygen reactive species can alter neuronal function are still not elucidated. There is a growing amount of evidence pointing to a role for mitochondrial damage as the source of free radicals involved in oxidative stress. Among the species that participate in the production of oxygen reactive radicals, transition metals are one of the most important. Several reports have implicated the involvement of redox-active metals with the onset of different neurodegenerative diseases such as Alzheimer's Disease (AD), Progressive Supranuclear Palsy (PSP), Amyotrophic Lateral Sclerosis (ALS) and Parkinson's Disease (PD). On the other hand, our previous studies have indicated that Aβ-induced deregulation of the protein kinase Cdk5 associated with tau protein hyperphosphorylation constitute a critical pathway toward neurodegeneration. In the current paper we have shown that iron induces an imbalance in the function of Cdk5/p25 system of hippocampal neurons, resulting in a marked decrease in tau phosphorylation at the typical Alzheimer's epitopes. The loss of phosphorylated tau epitopes correlated with an increase in 4-hydroxy-nonenal (HNE) adducts revealing damage by oxidative stress. This effects on tau phosphorylation patterns seems to be a consequence of a decrease in the Cdk5/p25 complex activity that appears to result from a depletion of the activator p25, a mechanism in which calcium transients could be implicated.

Introduction

Oxidative stress can be defined as the loss of the balance between the systems that produce reactive oxygen species (ROS) and the antioxidant machinery. The production of ROS is the result of oxidative processes affecting cellular and biochemical integrity in neuronal cells. This imbalance occurs in several human neurodegenerative diseases and in animal models that mimics brain disorders (Markesberry 1997; Butterfield *et al.* 2001; Varadarajan *et al.* 2000). Neurodegenerative diseases involve protein aggregation, phenomena such as those occurring in Alzheimer's disease (AD) (Smith *et al.* 2000; Joseph *et al.* 2001; Maccioni *et al.* 2001a, b), Huntington's disease (HD) (Deckel 2001; Sayre *et al.* 2001), amyotrophic lateral sclerosis (ALS) (Estevez *et al.* 1999; Julien 2001), prion disorders such as Creutzfeld-Jacob disease (CJD) (Brown 2001), and disorders with aggregated α-synuclein such as Parkinson's disease (PD) and frontotemporal dementia (FTD) (Goedert *et al.*

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2001). In addition to aggregated α-synuclein in PD and FTD, other protein aggregates have been detected. These included β-amyloid peptide and tau protein in AD, hungtintin in HD and prions in CJD. The relationship between oxidative stress and protein aggregation still remains to be elucidated. In human aging there is an increase in oxidative stress markers and damage to cellular proteins, DNA and lipids (Christen 2000). The central nervous system (CNS) appears to be particularly vulnerable to ROS damage. A number of factors that contribute to the high vulnerability of the CNS to oxidative damage include a decreased level of the natural antioxidant glutathione in neurons, membranes containing a high proportion of polyunsaturated fatty acids (Hazel & Williams 1990), and a relatively increased oxygen requirement due to the high metabolic activities of the brain (Benzi & Moretti 1995).

During the last 10 years an increasing number of papers have dealt with the relationships between the presence of transition metals and neurodegenerative disorders (reviewed in Lynch *et al.* 2000; Qian & Shen 2001). Multiple lines of evidence implicate redox-active transitions metals as mediator of oxidative stress and ROS production in neurodegenerative disorders (Perez *et al.* 1998; Sayre *et al.* 1999; Quintana *et al.* 2000). Several studies have been conducted to analyze the roles of Fe, Cu, Mn and Zn in the production of oxygen and nitrogen reactive species, and oxidative stress damage to cells as related to neurodegeneration. Particularly iron has been implicated in the ethiopathology of several degenerative diseases of neuronal systems such as Alzheimer disease (Lovell *et al.* 1998), Parkinson (Jellinger 1999), progressive supranuclear palsy (PSP) (Perez *et al.* 1998) and cataracts (Goldstein *et al.* 2000). Iron is primarily stored in ferritin, although elevated levels of iron seem not to correlate with an increase in ferritin levels or the transport protein transferrin in Alzheimer's patients (Fischer *et al.* 1997). In this paper we analyze the changes in the levels of Cdk5 and p25 in hippocampal neuronal cells and tau hyperphosphorylation patterns in response to acute iron treatments. Our results indicate that iron-mediated oxidative stress induces oxidative stress markers such as HNE adducts and hemoxygenase-1, as compared with untreated neuronal cells and control cells treated with the iron chelating agent deferoxamine mesylate (Deferal). Furthermore, iron does not modify the intraneuronal level of Cdk5, even though a decreased pool of p25 was observed. The phosphorylation at Tyr-15 in Cdk5 was also assessed, revealing that iron did not produce any effect on the phophorylation dynamics. Interestingly, iron treatment of hippocampal cells with 20 μM Fe produced a significant decrease in the exposure of Alzheimer's type epitopes as analyzed with PHF-1 and AT-8 antibodies. The data suggests that the pathway involved in iron-mediated oxidative damage could involve different mechanisms than those implicated for β-amyloid induced oxidative stress.

Materials and methods

Primary cell cultures

Hippocampal neuron cell cultures were prepared from E18.5 rat embryos (Banker & Cowan 1977). Briefly, the hippocampus was dissected and then incubated in 0.25% trypsin-EDTA during 10 min at 37 °C. After trypsin digestion the tissue was washed with HBSS (GIBCO-BRL) solution and then disaggregated using a fire polished Pasteur pipette. Neurons were plated over poly-L-lysine coated coverslips at a 5,000 cells cm^2 for immunofluorescence experiments and 15,000 cells cm^2 for Western blots analyses. Cultures were maintained in 10% bovine serum until 3 h after plating, when the culture medium was replaced with medium containing the N2 supplement (GIBCO-BRL) (Bottenstein & Sato 1979). Cells were maintained in culture for 5 days, and the N2 medium was replaced every 48 h.

Iron treatment

Iron was supplied as iron citrate at 20 μM in a medium containing N2 supplement. For iron chelating experiments the drug Desferal (deferoxamine mesylate) (Sigma) was supplemented at 100 μM in a medium containing N2 supplement. Iron and iron-chelating treatments were performed during 24 h.

Immunoblots

After iron treatments, neurons were homogenized in RIPA buffer and the protein concentration determined by using the Bradford analyses (Bradford 1976). Equal quantity of each sample was resolved into 10% PAGE-SDS gels (Laemmli 1974). After transfer onto nitrocellulose membranes, samples were blocked in 5% non-fat dry milk and then incubated with the primary antibodies for 2 h at room temperature, or overnight at 4 °C. After three washing steps with PBS-Tween

(0.05%), membranes were incubated with peroxidase-conjugated secondary antibodies (Sigma). Finally, detection was performed using the chemiluminiscence system (ECL, Amersham Pharmacia) and samples were analyzed in a molecular imager FX (Biorad) of the Millennium Institute CBB core facility. We used the following primary antibodies: AT8 that recognize a phosphorylated epitope on tau protein; Tau-1 that recognize an unphosphorylated epitope of tau; PHF1 that recognize an Alzheimer's type phosphorylated epitope on tau protein, and Tau5 that recognize a normal phosphorylation epitope on tau protein conformation. A β-actin antibody (Sigma) was used to normalize the amount of protein loaded on each well.

Immunoflourescences

Cell cultures were fixed in 4% paraformaldehyde/4% sucrose during 15 min at 37 °C. After fixation, samples were permeabilized with 0.2% Triton X-100 at room temperature during 5 min. Samples were then washed three times in PBS and blocked with 5% BSA during 1 h at room temperature. Primary antibodies were diluted in 1% BSA and incubated in a wet chamber for 2 h at room temperature or overnight at 4 °C. After three washings with PBS, preparations were incubated with fluorescein or rhodamine-conjugated secondary antibodies (Sigma) during 1 h at room temperature (Capote & Maccioni 1998). Finally, samples were washed with PBS and mounted with Prolong mounting media (Molecular Probes). Additionally, F-actin was detected with rhodamine-conjugated Phalloidin at 5 μg ml (Sigma). Images were obtained from a Zeiss confocal microscope.

Results and discussion

Overall morphology of iron-treated hippocampal neurons

We first analyzed the effect of iron treatment on the general morphological features and cytoskeleton organization in cultured hippocampal cells. For such purpose, neuronal cultures maintained for 4 days *in vitro* (DIV) were incubated with increasing concentrations of iron ranging from 0 to 80 μM (data not shown). This study indicated that the optimal working iron concentration to be used for subsequent experiments was 20 μM, which is in the physiological range of iron concentration. Hippocampal neurons were at stage three of brain development by the time of iron administration (Dotti *et al.* 1988). There were not significant morphological differences between iron-treated and control cells, as analyzed through immunofluorescence using a monoclonal antitubulin FITC conjugated antibody and staining with rodhamine-Phalloidin for actin filaments (data not shown). Thus, cytoskeleton staining of microtubules and actin microfilaments showed no variations between treated and control groups. Iron does not appear to produce major changes in the cytostructure, therefore funcional aspects of iron-treated neurons (Maccioni *et al.* 2001b) was investigated in the context of the experiments described below.

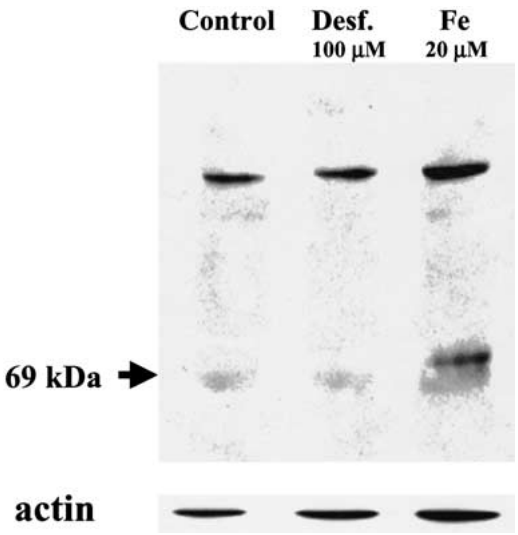

Fig. 1. Western blots of cells extracts from hippocampal neurons incubated in the presence or absence of iron. Neuronal cells were incubated in the absence of iron (control), or in the presence of 100 μM Deferal or 20 μM iron. Cells were homogenized and subjected to Western blot assays by using an anti-HNE adduct rabbit polyclonal antibody. The migration of actin as internal reference is indicated. A protein band of around 69 kDa was apparent after exposure of neuronal cells to iron.

A marker for lipid-peroxidation is increased in iron-treated neurons

It was of importance to analyze if iron-treatment produced an increase in the oxidative stress response within the hippocampal neurons. For such purpose we measured the levels of 4-HNE adducts. 4-HNE is an aldehyde product of lipid peroxidation that can damage primary neuron cell cultures (Mark *et al.* 1997), and can induce cross-linking of cytoskeletal proteins. As it is shown in the Figure 1, there is a marked increase in the amount of HNE-adducts in samples

derived from iron-treated cells. Controls using the iron chelating agent Desferal showed similar 4-HNE adducts levels as those observed in untreated controls, thus supporting the observation that the increase in HNE levels is due to iron overload. This increase in the amount of HNE-adducts found in treated cells also suggests that iron is triggering the oxidative stress response inside the neurons, and affecting lipids of the cell membrane. There is a vast amount of reports dealing with the importance of oxidative stress in the pathology of Alzheimer's disease (reviewed in Markesberry 1997; Smith *et al.* 2000). The results that imply free radical oxidative stress in AD includes: (i) increased levels of redox-active metals ion in AD brain; (ii) increased lipid peroxidation as detected by an increase in HNE adducts; (iii) increased protein, DNA and RNA oxidation, and upregulation of antioxidant enzymes; and (iv) extensive amounts of peroxynitrite and advanced glycation end products (AGE)-modifications. These studies point to the effect of iron treatment over hippocampal neurons in culture, due to an increase in oxidative stress of the cell. An increase in the amount of HNE-adducts in the iron-treated neurons support this conclusion. HNE-adducts are generated in response to lipid peroxidation induced by an increase in oxidative stress.

The levels of Cdk5 remained unchanged after iron treatment

Previous findings of our laboratory have implicated a deregulation of the Cdk5 enzyme in the pathway of Aβ-mediated neurotoxicity (Alvarez *et al.*, 1999, 2001). Studies have also revealed that an activation of the Cdk/p35 protein complex mediated by a single site Cdk5 phosphorylation is responsible for the anomalous overactivation of this protein kinase (Alvarez *et al.* 2001; Patrick *et al.* 2000). Additionally it has been shown that the generation of free radicals in neurons treated with Aβ may be important in the role of neurotoxicity (Behl *et al.* 1994; Harris *et al.* 1995; Mattson *et al.* 1995 a, b; Sagara *et al.* 1996). In this context, we decided to analyze the Cdk5 and the phospho-Cdk5 (at Tyr^{15}) levels (Zuckerberg *et al.* 2000) in the iron-treated cells. Looking at the total levels of this kinase we found no significant variations in the amount of Cdk5 (Figure 2A). Moreover, the levels of phosphorylated Cdk5 also remained unchanged as analyzed with antibody that recognize phosphorylated Tyr^{15} epitope on this protein (Figure 2B). However, when we analyzed the levels of p35, one of the neurospecific activators for Cdk5 we found that although p35 did not change its levels within the range of iron concentrations used, the expression of the soluble fragment p25 lacking the N-terminal p35 protein moiety decreased (Figure 2C). It has been reported that soluble p25 could act as a regulatory protein controlling Cdk5 activity (Patrick *et al.* 2000). Taken collectively these results suggest that iron-treatment of hippocampal cells produce a decrease in the activity of the Cdk5/p25 complex.

Iron alter the phosphorylation patterns of brain tau protein

Tau total levels were not altered in the cells treated with iron as shown with a phosphorylation independent antibody Tau-5 (Figure 3A). However, when we looked for the phosphorylated forms of the tau protein, we unexpectedly found a significant decrease in phosphorylated tau as shown with the AT8 antibody that recognize tau epitopes of Alzheimer's type (Figure 3B). Data of Figure 3 indicates that tau protein loses its hyperphosphorylation upon iron treatment. Studies have indicated that Cdk5 driven hyperphosphorylation occurs at residues Ser^{202}-Pro, Thr^{205}-Pro and Ser^{235}-Pro (Alvarez *et al.* 1999). Consistent with the latter result, the amount of tau protein in its hypophosphorylated form was increased in response to iron treatments, as shown with Tau1 antibody (Figure 3C). There are two generic reactions in which transition metals are related with some relevance to neurodegenerative disorders. First, a metal-protein association leading to protein aggregation; this reaction can involve redox-inert metal ions such as Zn^{2+}, or redox-active metal ions such as Cu^{2+} and Fe^{3+}. Second, metal-catalyzed protein oxidation leading to protein damage: this reaction involves a redox-active metal ion such as Cu^{2+}, Fe^{3+} or Mn^{2+} (Smith *et al.* 1997; Perry *et al.* 1998).

In the search for a mechanistic approach for the analysis of oxidative stress effects on tau phosphorylation patterns

Several reports have indicated that an imbalance in the oxidative stress cellular responses could be responsible for the hyperphosphorylation of cytoskeleton proteins involved in neurodegenerative diseases. This has been extensively analyzed with respect to the role of the microtubule-associated protein tau in the etiology and pathogenesis of Alzheimer's disease (Smith *et al.* 1998; Maccioni *et al.* 2001b). Nevertheless, there are

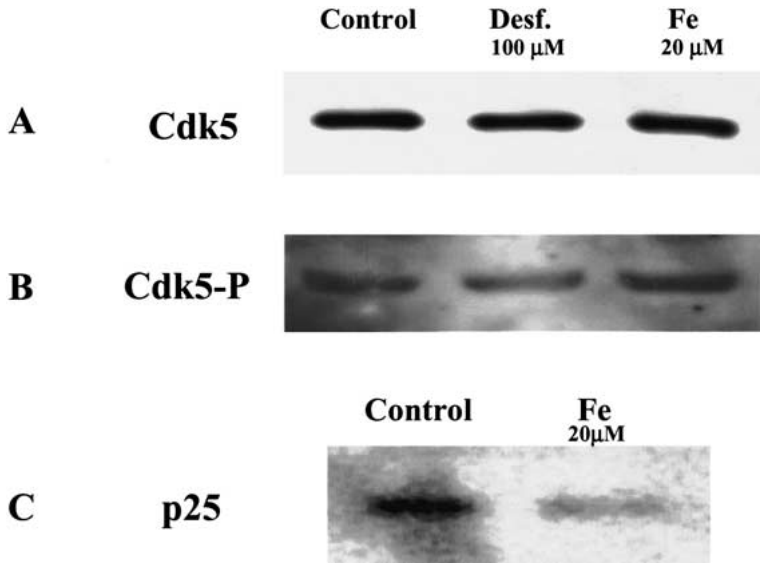

Fig. 2. Western blots of hippocampal extracts incubated in the presence or absence of iron. Neuronal cells were incubated in the absence of iron (control), and in the presence of 100 μM Deferal or 20 μM iron. A. Cells were homogenized and the high speed supernatants subjected to Western blots assays by using an anti-Cdk5 antibody clone C8 from Santa Cruz. B. Cell extracts were analyzed by using and anti-Cdk5 phosphorylated at Tyr15. C. Cell extracts of cells incubated in the presence and absence of iron were subjected to Western blot analysis for p25 using an anti-p25 monoclonal antibody.

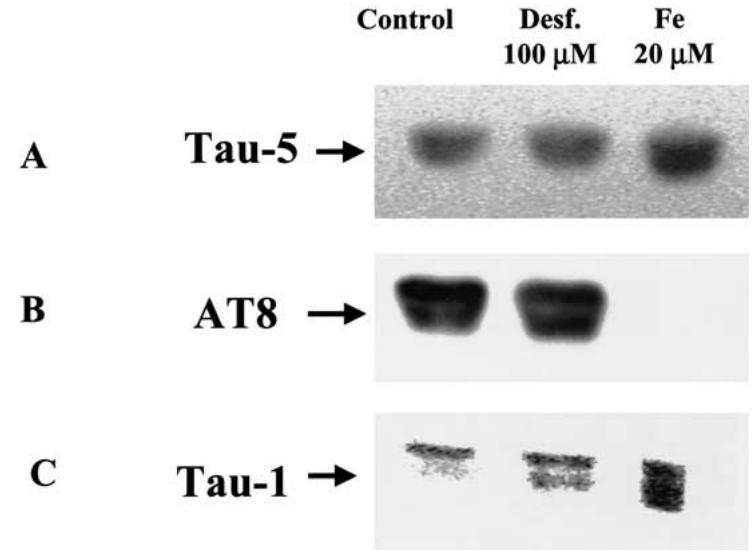

Fig. 3. Western blots of neuronal extracts incubated in the presence or absence of iron. Neuronal cells were incubated without iron (control), and in the presence of 100 μM Deferal or 20 μM iron. A. Hippocampal extracts treated and untreated with iron analyzed by Western blots using Tau-5 antibody which recognizes conformational epitopes for all tau variants. B. Cells were homogenized and the high speed supernatants subjected to Western blots assays by using the AT8 antibody that recognizes Azheimer's type tau epitopes. C. Cell extracts were analyzed by using Tau-1 antibody that recognizes unphosphorylated tau.

contradictory data on the phosphorylation state of tau protein in response to oxidative stress. Some reports point out an increase in the phosphorylation of tau in AD brains derived tissue (Takeda *et al.* 2000). On the other hand H_2O_2-induced oxidative stress has been shown to produce dephosphorylation of tau protein in rat primary neuronal cultures (Davis et al. 1997). An increase in the amount of dephosphorylated tau is also shown in neurons treated with glutamate (Anderton *et al.* 1995; Davis *et al.* 1995; Fleming & Johnson 1995) and ischaemia (Geddes *et al.* 1994; Schakelford & Nelson 1996). However the decrease of hyperphosphorylated tau protein is not dependent on an increase of GSK3β kinase levels (Davis *et al.* 1997). Thus, the increase of dephosphorylated tau isoforms upon oxidative stress effects is likely to depend on Cdk5, since AT8, PHF1 and Tau1 antibodies recognize mainly phosphoepitopes on tau that are catalyzed by kinases belonging to the proline directed protein kinases family. This could be the situation for the effects of iron observed in this study, since the depletion in p25 levels could account for the low levels of tau phosphorylation upon iron treatment. Therefore, the fine regulation and cross-talks of these kinases involved in the molecular events in the pathogenesis of Alzheimer's disease, needs further analysis (for review Maccioni *et al.* 2001b).

The fact that different oxidative stress treatments lead to contradictory results in the tau phosphorylation levels suggest that some of these differences could be related to variations of intracellular messengers in response to such treatments. Calcium ion could be one of the possible intracellular messengers involved in this response. Calcium is an important intracellular messenger for neuronal signaling pathways. Through variations in both the amplitude and frequency of intracellular calcium transients, the same calcium ion can elicit different responses. Alterations in intracellular calcium concentrations are clearly involved in modulating the phosphorylation state of tau protein *in situ*. However, results have been decidedly mixed, and there is little consensus as to the specific effects of elevating calcium intracellular concentration on tau phosphorylation. For example, acute treatment of primary neuronal cultures with calcium ionophores has been reported to increase (Mattson *et al.* 1991; Mattson *et al.* 1992) and decrease (Adamec *et al.* 1997) tau phosphorylation. The same mixed results have been found using human neuroblastoma cell lines, as ionophore treatment resulted in both increases (Shea *et al.* 1997) and decreases (Xie & Johnson 1998) in tau phosphorylation. Increasing intracellular calcium by activation of N-methyl-D-aspartate receptors has been shown to result in the dephosphorylation of tau in rat brain slices (Fleming & Johnson 1995) and cortical neuronal cultures (Adamec *et al.* 1997). In an elegant study, it was shown that the variations of the levels of tau phosphorylation were in fact dependent on the transient calcium concentration (Hartigan & Johnson 1999). Thus, a possible explanation for discrepancies between different studies dealing with the role of oxidative stress could be related with the overall modification of the intracellular calcium homeostasis. These differences could be mimicking some acute or chronic responses of the cellular machinery to oxidative stress (Figure 4).

What are the relationships between Aβ neurotoxicity and oxidative stress effects in neurodegeneration? In regard to β-amyloid effects in inducing deregulation of the Cdk5/p35 complex, Cdk5 activation increases hyperphosphorylated tau protein in neuronal cell cultures (Alvarez *et al.* 1999) and in a transgenic mice overexpressing tau protein (Gotz *et al.* 2001). Moreover, tau hyperphoshphorylation is also detected in a double mutant mouse overexpressing amyloid precursor protein and tau (Lewis *et al.* 2001). The effect of Aβ fibrils have been suggested to produce an increase in the oxidative stress (Behl *et al.* 1994; Harris *et al.*, 1995; Manelli & Puttfarcken 1995; Mattson 1995a, b; for review Maccioni *et al.* 2001b). Also, it has been reported that Aβ induced an increase in tau phosphorylation (Busciglio *et al.* 1995; Takashima *et al.* 1996 Alvarez *et al.* 1999).

Alzheimer's disease is characterized by the deposition of Aβ within the neocortex, associated with neuronal loss and oxidative stress. The deposition of Aβ is considered to be closely related to the primary pathogenesis of familial AD. Familial AD-linked mutations of amyloid precursor protein (APP), presenilin-1 and presenilin-2, increase both cerebral Aβ burden and Aβ1-42 production, underscoring the role that Aβ metabolism plays in AD pathogenesis. Furthermore, the deposition of Aβ in the neocortex of transgenic mice overexpressing Aβ is accompanied by many of other neuropathological features of AD including intraneuronal tau abnormalities and neuronal loss (Calhoun *et al.* 1998), as well as signs of oxidative damage similar to those observed in AD-affected brain (Smith *et al.* 1998). The length of the Aβ species is considered to be one important factor in AD pathogenesis as Aβ1-42, a minor free soluble species in biological fluids, is enriched in amyloid

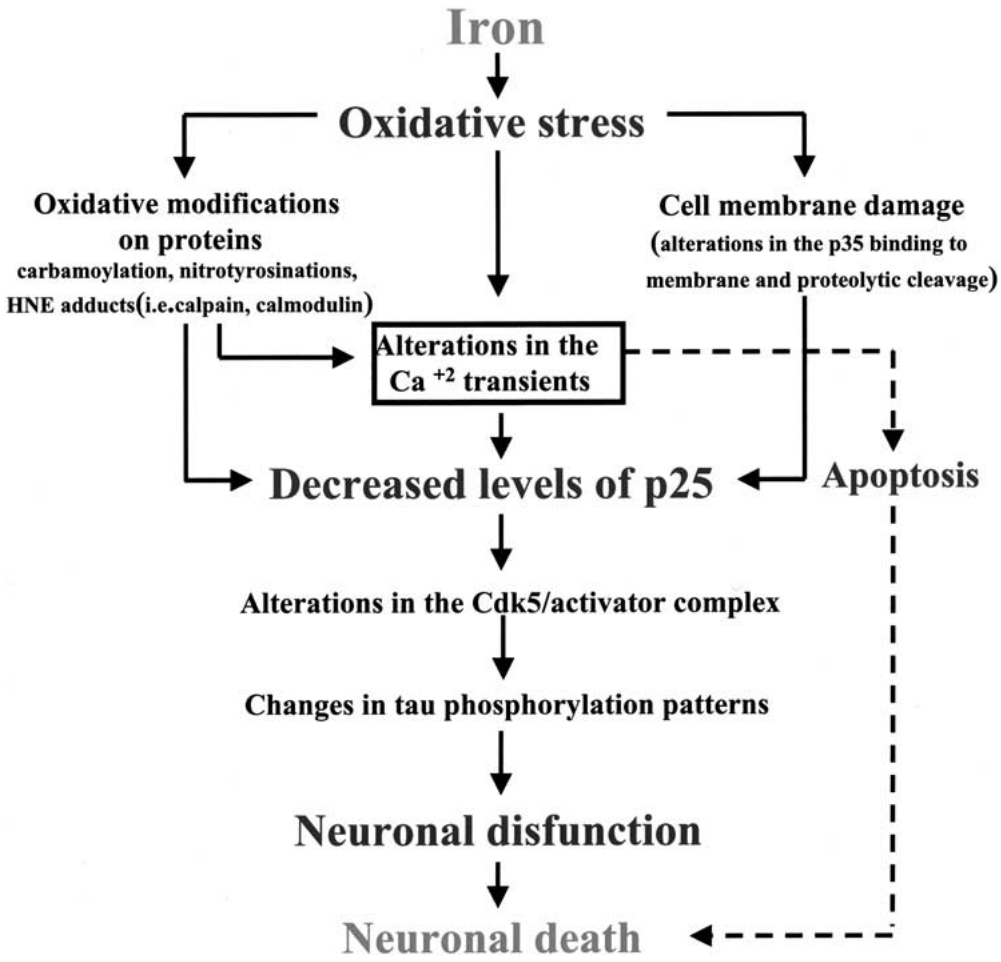

Fig. 4. Schematic representation of the action of oxidative stress via iron effects on neurodegeneration of hippocampal cells on the basis of data from these studies and previous investigations (for review Maccioni *et al.* 2001b). The mechanism considers the possible contribution of changes in calcium transient and the depletion in Cdk5 activators (i.e., p25) in the observed changes on phosphorylation patterns on brain tau protein.

deposits. Many studies have now confirmed that Aβ is neurotoxic in cell culture. Hence, there is a compelling argument to considered Aβ deposition as a therapeutic target in AD. As for transition metals role in Aβ-mediated neurotoxicity it has been suggested that Cu^{2+} and Fe^{3+}, unlike Zn^{2+} induce greater Aβ aggregation under mildly acidic conditions such as those believed to occur in AD brain (Atwood *et al.* 1998). Significantly, the solubility of rat or mouse Aβ1-40 is unaffected by Zn (II) or Cu (II) at low micromolar concentrations. Apolipoprotein E can also modulates the precipitation of Aβ by Cu^{2+} and Zn^{2+}, which is important because ApoE isoforms segregate with the genetic risk for AD; inheritance of the ApoE4 isoform carries the greatest risk. The ApoE4 isoform is poorest in maintaining Aβ in a soluble form, as compared with the others isoforms (ApoE2 and ApoE3), whether the precipitating metal is Cu^{2+} or Zn^{2+} (Moir *et al.* 1999). It has been also reported that some Cu/Zn selective chelators enhance the solubilization of Aβ deposits from post-mortem AD brain samples, supporting the possibility that these metals could play a role in the assembly of the deposits (Atwood *et al.* 1998). However, metals could be playing more than this role. It has been also reported that Aβ is redox active, and reduces Cu (II) or Fe (III) and then produces H_2O_2 by electron transfer to O_2. The metal-reducing activity and H_2O_2 production of Aβ species is enhanced in human Aβ42 as compared with human Aβ40. Considering that neurodegeneration is a multifactorial process, these investigations suggest that oxidative stress and Aβ amyloid, through its different

aggregation forms, could induce different alterations in the signaling pathways of neurons. This could be exerted by either independent mechanisms or by concerted actions, and the potentiation of these signals appears to be critical for neuronal degeneration.

Acknowledgements

The authors would like to thanks Drs Lester Binder for antibodies Tau1 and Tau5 and Peter Davies for the antibody PHF1. This work was supported by the Millennium Scientific Initiative (project PI031-F) and a grant 1020/55 from Fondecyt. J.T.E and C.Z were supported by the Millennium Institute for Advanced Studies in Cell Biology and Biotechnology Scholarships.

References

Adamec E, Mercken M, Beermann ML, Didier M, Nixon RA. 1997 Acute rise in the concentration of free cytoplasmic calcium leads to dephosphorylation of the microtubule-associated protein tau. *Brain Res* **757**, 93–101.

Alvarez A, Toro R, Cáceres A, Maccioni RB. 1999 Inhibition of tau phosphorylating protein kinase cdk5 prevents beta-amyloid induced neuronal death. *FEBS Lett* **459**, 421–426.

Alvarez, A, Muñoz JP, Maccioni RB. 2001 A cdk5-p35 stable complexis involved in the β-amyloid-induced deregulatim of cdk5 activity in hippocampal neurons. *Exp. Cell. Res.* **264**, 266–274.

Anderton BH, Brion JP, Couck AM, Davis DR, Gallo JM, Hanger DP *et al.* 1995 Modulation of PHF-like tau phosphorylation in cultured neurons and transfected cells. *Neurobiol. Aging* **16**, 389–397.

Atwood CS, Moir RD, Huang X, Bacarra NME, Scarpa RC, Romano DM *et al.* 1998 Dramatic aggregation of Alzheimer Aβ by Cu (II) is induced by conditions representing physiological acidosis. *J Biol Chem* 273, 12817–12826.

Banker GA, Cowan WM. 1977 Rat hippocampal neurons in dispersed cell culture. *Brain Res* **126**: 397–442.

Bradford MM. 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Behl C, Davis JB, Lesley R, Schubert D. 1994 Hydrogen peroxide mediates amyloid β toxicity. *Cell* 77, 817–828.

Benzi G, Moretti A. 1995. Are reactive oxygen species involved in Alzheimer's disease? *Neurobiol. Aging* **16**, 661–674.

Bottenstein JE, Sato GH. 1979 Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc Natl Acad Sci USA* 76, 514–517.

Brown DR. 2001 Prion and prejudice: normal protein and the synapse. *Trends Neurosci.* **24**, 85–90.

Busciglio J, Lorenzo A. Yeh J, Yankner BA. 1995 β-amyloid fibrils induce tau phosphorylation and loss of microtubule binding. *Neuron* **14**, 879–888.

Butterfield DA, Howard BJ, LaFontaine MA. 2001 Brain oxidative stress in animal models of accelerated aging and the age-related neurodegenerative disorders, Alzheimer's disease and Huntington's disease. *Curr Med Chem* **8**, 815–828.

Calhoun ME, Wiederhold KH, Abramowski D, Phinney AL, Probst A, Sturchler-Pierrat C *et al.* 1998 Neuron loss in APP transgenic mice. *Nature* **395**, 755–756.

Capote C, Maccioni RB 1998. The association of tau-like proteins with vimentin filaments in cultured cells. *Exp Cell Res* **239**, 202–213.

Christien Y. 2000 Oxidative stress and Alzheimer disease. *Am J Clin Nutr* **71**, 621S–629S

Davis DR, Brion JP, Couck AM, Gallo JM, Hanger DP, Ladhani K *et al.* 1995 The phosphorylation state of the microtubule-associated protein tau is affected by glutamate, colchicines and β-amyloid in rat cortical neuronal cultures. *Biochem J* **309**, 941–949.

Davis DR, Anderton BH, Brion JP, Hugh Reynolds C, Hanger DP. 1997 Oxidative stress induces dephosphorylation of τ in rat primary neuronal cultures. *J Neurochem* **68**, 1590–1597.

Deckel AW. 2001 Nitric oxide and nitric oxide synthase in Huntington's disease. *J Neurosci Res* **64**, 99–107.

Dotti CG, Sullivan CA, Banker GA. 1988 The establishment of polarity by hippocampal neurons in culture. *J Neurosci* **8**, 1454–68.

Estevez AG, Crow J, Sampson JB, Reiter C, Zhuang Y, Richardson G, Tarpey M, Barbeito L, Beckman J. 1999 Induction of nitric oxide dependent apoptosis in motor neurons by Zinc deficient superoxide dismutase. *Science* **286**, 2498–2500.

Fischer P, Gotz ME, Danielczyk W, Gsell W, Riederer P. 1997 Blood transferrin and ferritin in Alzheimer's disease. *Life Sci* **60**, 2273–2278.

Fleming LM, Johnson GVW. 1995 Modulation of the phosphorylation state of tau *in situ*: The roles of calcium and cyclic AMP. *Biochem J.* **309**, 41–47.

Geddes JW, Schwab C, Craddock S, Wilson JL, Pettigrew LC. 1994 Alterations in τ immunostaining in the rat hippocampus following transient cerebral ischemia. *J Cereb Blood Flow Metab* **14**, 554–564.

Goedert M. 2001 Alpha-synuclein and neurodegenerative diseases. *Nat Rev Neurosci* **2**, 492–501.

Goldstein LE, Leopold MC, Huang X, Atwood CS, Saunders AJ, Hartshorn M *et al.* 2000 3-Hydroxykynurenine and 3-hydroxyanthranilic acid generate hydrogen peroxide and promote alpha-crystalline cross-linking by metal ion reduction. *Biochemistry* **39**, 7266–7275.

Gotz J, Chen F, van Dorpe J, Nitsch RM. 2001 Formation of neurofibrillary tangles in P301L tau transgenic mice induced by Aβ42 fibrils. *Science* **293**, 1491–1495.

Harris ME, Hensley K, Butterfield DA, Leedle RA, Carney JM. 1995 Direct evidence of oxidative injury produced by the Alzheimer's β-amyloid peptide (1–40) in cultured hippocampal neurons. *Exp Neurol* **131**, 193–202.

Hartigan JA, Johnson GVW. 1999 Transient increases in intracellular calcium result in prolonged site-selective increases in tau phosphorylation through a glycogen synthase kinase 3β-dependent pathway. *J Biol Chem* **274**, 21395–21401.

Hazel JR, Williams EE. 1990 The role of alterations in membrane lipids composition in enabling physiological adaptation of organisms to their physical environment. *Prog Lip Res* **29**, 167–227.

Jellinger KA. 1999 Is there apoptosis in Lewy body disease? *Acta Neuropathol* **97**, 413–415.

Joseph J, Shukitt-Hale B, Denisova NA, Martin A, Perry G, Smith MA. 2001 Copernicus revisited: amyloid beta in Alzheimer's disease. *Neurobiol Aging* **22**, 131–46.

Julien JP. 2001 Amyotrophic lateral sclerosis. Unfolding the toxicity of the misfolded. *Cell* **104**, 581–91.

Laemmli UK. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G *et al.* 2001 Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science* **293**, 1487–1491.

Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR. 1998 Copper, iron and zinc in Alzheimer's disease senile plaques. *J Neurol Sci* **158**, 47–52.

Lynch T, Cherny RA, Bush AI. 2000 Oxidative processes in Alzheimer's disease: the role of Aβ-metal interactions. *Exp Gerontol* **35**, 445–451.

Maccioni RB, Otth C, Concha II, Muñoz JP. 2001a. The protein kinase cdk5. Structural aspects, roles in neurogenesis and involvement in Alzheimer's pathology. *Eur J Biochem* **268**, 1518–1527.

Maccioni RB, Muñoz JP, Barbeito L. 2001b The molecular bases of Alzheimer's disease and other neurodegenerative diseases. *Arch Med Res* **32**, 367–381.

Manelli AM, Puttfarcken PS. 1995 Beta-amyloid-induced toxicity in rat hippocampal cells: *in vitro* evidence for the involvement of free radicals. *Brain Res Bull* **38**, 569–576.

Mark RJ, Lovell MA, Markesbery WR, Uchida K, Mattson MP. 1997 A role for 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid beta-peptide. *J Neurochem* **68**, 255–264.

Markesbery WR. 1997 Oxidative stress hypothesis in Alzheimer's disease. *Free Radic Biol Med* **23**, 134–147.

Mattson MP, Engle MG, Rychlik B. 1991 Effects of elevated intracellular calcium levels on the cytoskeleton and tau in cultured human cortical neurons. *Mol Chem Neuropathol* **15**, 117–142.

Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE 1992 Beta-amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J Neurosci* **12**, 376–389.

Mattson MP. 1995a Free radicals and disruption of neuronal ion homeostasis in AD: A role for amyloid β-peptide. *Neurobiol Aging* **16**, 679–682

Mattson MP. 1995b Degenerative and protective signals mechanisms in the neurofibrillary pathology of AD. *Neurobiol Aging* **16**, 447–457.

Moir RD, Atwood CS, Romano DM, Laurans MH, Huang X, Bush AI *et al.* 1999 Differential effects of apolipoprotein E isoforms on metal-induced aggregation of A-beta using physiological concentrations. *Biochemistry* **38**, 4595–4603.

Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH. 1999 Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* **402**, 615–622.

Perez M, Valpuesta JM, de Garcini EM, Quintana C, Arrasate JL, Lopez Carrascosa JL *et al.* 1998. Ferritin is associated with the aberrant tau filaments present in progressive supranuclear palsy. *Am J Pathol* **152**, 1531–1539.

Perry G, Castellani RJ, Hirai K, Smith M. 1998 Reactive oxygen species mediate cellular damage in Alzheimer's disease. *J Alz Disease* **1**, 45–55.

Qian ZM & Shen X. 2001 Brain iron transport and neurodegeneration. *Trends Mol Med* **7**, 103–108.

Quintana C, Lancin M, Marhic C, Perez M, Martin-Benito J, Avila J, Carrascosa JL. 2000 Initial studies with high resolution TEM and electron energy loss spectroscopy studies of ferritin cores extracted from brains of patients with progressive supranuclear palsy and Alzheimer disease. *Cell Mol Biol* (Noisy-le-Grand) **46**, 807–820.

Sagara Y, Dargusch R, Klier FG, Schubert D, Behl C. 1996 Increased antioxidant enzyme activity in amyloid β protein-resistant cells. *J Neurosci* **16**, 487–505.

Sayre LM Perry G, Smith MA. 1999 Redox metals and neurodegenerative disease. *Curr Opin Chem Biol* **3**, 220–225.

Sayre LM, Smith MA, Perry G. 2001 Chemistry and biochemistry of oxidative stress in neurodegenerative disease. *Curr Med Chem* **8**, 721–738.

Schakelford DA, Nelson KE. 1996 Changes in phosphorylation of τ during ischemia and reperfusion in the rabbit spinal cord. *J Neurochem* **66**, 286–295.

Shea TB, Prabhakar S, Ekinci FJ. 1997 Beta-amyloid and ionophore A23187 evoke tau hyperphosphorylation by distinct intracellular pathways: Differential involvement of the calpain/protein kinase C system. *J Neurosci Res* **49**, 759–768.

Smith MA, Harris P, Sayre LM, Perry G. 1997 Iron accumulation in Alzheimer's disease is a source of redox-generated free radicals. *Proc Natl Acad Sci* **94**, 9866–9868.

Smith MA, Hirai K, Hsiao K, Papolla MA, Harris P, Siedlak S *et al.* 1998. Amyloid beta deposition in Alzheimer transgenic mice is associated with oxidative stress. *J Neurochem* **70**, 2212–2215.

Smith MA, Nunomura A, Zhu X, Takeda A, Perry G. 2000 Metabolic, metallic, and mitotic sources of oxidative stress in Alzheimer disease. *Antioxid Redox Signal* **2**, 413–420.

Takashima A, Noguchi K, Michel G, Mercken M, Hoshi M, Ishiguro K *et al.* 1996 Exposure of rat hippocampal neurons to amyloid β peptide (25–35) induces the inactivation of phosphatidylinositol 3-kinase and the activation of tau protein kinase I glycogen synthase kinase 3β. *Neurosci Lett* **203**, 33–36.

Takeda A, Smith M, Avila J, Nunomura A, Siedlak S, Zhu X, Perry G, Sayre LM. 2000. In Alzheimer's disease, heme oxugenase is coincident with Alz50, an epitope of tau induced by 4-hydroxynonenal modification. *J Neurochem* **75**, 1234–1241.

Varadarajan S, Yatin S, Aksenova M, Butterfield DA. 2000 Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. *J Struct Biol* **130**, 184–208.

Xie HQ, Johnson GV. 1998 Calcineurin inhibition prevents calpain-mediated proteolysis of tau in differentiated PC12 cells. *J Neurosci Res* **53**, 153–164.

Zuckerberg, LR, Patrick GN, Nikolic M, Humbert S, Wu CL, Lanier LM, Gertler FB, Vidal M, Etten RA, Tsai LH. 2000 Cables links Cdk5 and c-Ab1 and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation and neurite outgrowth. *Neuron* **26**, 633–646.