OXIDATIVE STRESS PROMOTES τ DEPHOSPHORYLATION IN NEURONAL CELLS: THE ROLES OF Cdk5 AND PP1

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Abstract—Oxidative stress has been demonstrated to produce modifications in several intracellular proteins that lead to alterations in their activities. Alzheimer's disease is related to an increase of oxidative stress markers, which may be an early event in the progression of the disease and neurofibrillary tangles formation. Abnormal phosphorylation of τ has been implicated in the etiopathogenesis of Alzheimer's disease. By using phospho-specific antibodies, we analyzed the changes in τ phosphorylation patterns after treatment of rat hippocampal and SHSY5Y human neuroblastoma cells with H_2O_2. We found that τ isoforms were hypophosphorylated at the Tau1 epitope after 2 h in the presence of H_2O_2. The decrease in the phosphorylation levels of τ protein were prevented by pretreatment with N-acetyl-L-cysteine. These changes were shown to depend on the activity of the cdk5/p35 complex, since a 3-fold increase in substrate phosphorylation and a 2-fold increase for the complex association were observed. Also, a decrease in the amount of inhibitor-2 bound to phosphatase PP1 was found in SHSY5Y cells under oxidative stress conditions. This decrease of inhibitor-2 bound to PP1 is due to an increased phosphorylation of the inhibitor-2 protein, thus leading to increased PP1 activity. Therefore, we propose that oxidative stress-induced activation of cdk5 leads to inhibitor-2 phosphorylation, relieving its inhibitory effect on PP1.

Keywords—Tau phosphorylation, Oxidative stress, Cyclin-dependent kinase 5, Protein phosphatase 1, Hydrogen peroxide, Alzheimer's disease, Free radicals

INTRODUCTION

Oxidative stress is a major pathological aspect in several neurodegenerative conditions such as stroke [1], spinal cord injury [2], multiple sclerosis [3], amyotrophic lateral sclerosis [4], Parkinson's disease [5], frontotemporal dementia [6], and Alzheimer's disease (AD) [7]. Oxidative stress results from the generation of reactive oxygen species (ROS) by the electron transport chain in the mitochondrion or another enzymatic system such as NADPH oxidase. AD is an aging-related pathology that is characterized by the presence of oxidative stress markers such as 8-hydroxyguanosine and hydroxynonenal adducts at early stages of the pathology [8]. Hydrogen peroxide (H_2O_2) is particularly toxic because it is considered to be a relatively stable compound among free radical species that can diffuse freely inside cells [9]. It can be detected in the rat brain at micromolar concentrations after ischemia/reperfusion [10]. Once inside the cell, it reacts with Fe^{2+} to give rise, via the Fenton reaction, to the highly reactive hydroxyl radical [11].

AD is characterized by the accumulation of senile plaques largely composed of the Aβ peptide within the neocortex. This deposition has been implicated in neuronal death and is related to oxidative stress. Also, the abnormal accumulation of the aberrantly phosphorylated microtubule-associated protein τ is another feature of the disease. Many efforts have been made to establish clear and univocal relationships between these two pathological hallmarks. In this regard, it has been indicated that

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Aβ can produce an increase in oxidative stress [7,12–18], and it has been reported that Aβ induces an increase in τ phosphorylation, indicating that Aβ neurotoxicity is mediated by τ modifications [19–22]. These effects of Aβ are overcome by inhibiting two proline-directed protein kinases, namely gsk3 and cdk5. Thus, Aβ neurotoxicity is diminished in neuronal cells incubated with butyrolactone I or cdk5 antisense oligonucleotides [21,22] and with lithium chloride [23]. On the other hand, the role of phosphatases in the regulation of τ phosphorylation patterns has been focused on PP1, PP2A, and PP2B (calcineurin). It has been shown that cdk5 is involved in the phosphorylation of the inhibitor-2 (I-2) of phosphatase PP1 at Thr^{72}, leading to its dissociation from the phosphatase and ultimately to its activation [24]. Despite some controversial results regarding the effect of oxidative stress on the phosphorylation levels of τ protein [25–27], it is clear that τ phosphorylation can be modified in response to such cellular insults. In this report, we describe the decrease of τ phosphorylation due to H_2O_2 being paralleled by an increase in the phosphorylation of I-2 and PP1 activity, most likely due to decreased association of I-2 to the enzyme. These changes depend on an increase in the cdk5 activity. Thus, at early stages of the oxidative stress exposure of neuronal cells, cdk5 could promote τ dephosphorylation of τ at the Tau1 epitope by activating PP1.

MATERIALS AND METHODS

Cell cultures

Hippocampal neuronal cell cultures were prepared from E18.5 rat embryos [28]. Briefly, hippocampi were dissected and then put into 0.25% trypsin-EDTA for 15 min at 37°C. After trypsin treatment, the tissue was washed with HBSS solution (Gibco BRL, Grand Island, NY, USA) and then disaggregated using a fire-polished Pasteur pipette. Neurons were plated on poly-L-lysine-coated coverslips at 5000 cells/cm^2 for immunofluorescence experiments and 15,000 cells/cm^2 for Western blot analyses. Cultures were maintained in 10% horse serum for 3 h after plating, when the culture medium was replaced with medium containing the N2 supplement (Gibco BRL) [29]. Cells were maintained in culture for 5 days, and the N2 medium was replaced every 48 h. Human neuroblastoma SHSY5Y cells were cultured in DMEM/10% FBS. Prior to the experiments with H_2O_2, the medium was replaced with DMEM without serum.

H_2O_2 treatment

H_2O_2 (Sigma Chemical Co., St. Louis, MO, USA) previously diluted with sterile PBS was added to cell cultures at 100 μM in serum-free medium containing an N2 supplement. Also, antioxidant treatments with N-acetyl-L-cysteine (NAC) were performed 30 min before the addition of H_2O_2 to prevent oxidative stress. All the treatments were done on neurons cultured during the 5 days. All reagents were prepared freshly before use.

Viability assays

Primary hippocampal neurons were seeded into 96-well culture plates coated with poly-lysine at 100 μg/ml. Different concentrations of H_2O_2, NAC, or a combination of both were added to the cells. MTT was added to all wells and further incubated overnight at room temperature in the dark [21]. The day after, neurons were lysed and absorbance at 590 nm was determined using a Metertech E960 spectrophotometer (Metertech Inc., Nankang, Taipei, Taiwan). MTT assays were performed in triplicate. Also, neuronal viability was assayed with the Mito-Capture kit (Calbiochem, San Diego, CA, USA) following the manufacturer's instructions.

Immunoblots

Neurons were homogenized in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μM leupeptin, and 1 μg/ml pepstatin] and the protein concentration was determined by the Bradford analysis [30]. Equal aliquots of each sample were separated in 12% PAGE-SDS gels [31]. After transfer to nitrocellulose membranes, samples were blocked in 5% nonfat dry milk and incubated with primary antibodies for 2 h at room temperature or overnight at 4°C. After three washing steps with PBS, membranes were incubated with peroxidase-conjugated secondary antibodies (Sigma Chemical Co.). Finally, detection was performed using a chemiluminescence system (Amersham, Arlington Heights, IL, USA) and samples were analyzed in a Molecular Imager FX (Bio-Rad, Richmond, CA, USA). We used the following primary antibodies: AT8, which recognizes phosphorylated epitopes Ser^{202} and Thr^{205} on τ protein; Tau1, which recognizes the same unphosphorylated epitopes of τ; PHF1, which recognizes phosphorylated epitopes Ser^{396} and Thr^{404}; and Tau5, which recognizes an independent phosphorylation epitope on τ protein. The antibody against Michael's adducts of 4-hydroxynonenal-L-lysine was purchased from Calbiochem. Monoclonal anti-cdk5 and polyclonal anti-p35 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used according to the manufacturer's instructions. Monoclonal antibodies generated from hu-

man PP1 α and I-2 were purchased from BD Transduction Laboratories (San Jose, CA, USA).

Immunofluorescence

Cell cultures were fixed in 4% paraformaldehyde/4% sucrose for 15 min at 37°C. Subsequently, fixation samples were permeabilized with 0.2% Triton X-100 at room temperature for 5 min. Samples were then washed three times in PBS and blocked with 5% BSA for 1 h at room temperature. Primary antibodies were diluted in 1% BSA, 0.1% Triton X-100, and incubated in a humid chamber for 2 h at room temperature or overnight at 4°C. After washing three times with PBS, preparations were incubated with fluorescein or rhodamine-conjugated secondary antibodies (Sigma Chemical Co.) for 1 h at room temperature. Finally, samples were washed with PBS and mounted with Prolong mounting media (Molecular Probes, Eugene, OR, USA). Images were acquired with a Zeiss LSM confocal microscope, model META (Carl Zeiss, Göttingen, Germany).

Immunoprecipitation and cdk5 activity assays

Primary cultured neurons were plated at 5×10^5 cells/cm^2 on polylysine-coated 60 mm dishes. After treatments, cells were lysed in RIPA buffer; 100 μg of total protein extract were used for immunoprecipitation with an anti-cdk5 antibody (C8 antibody; Santa Cruz Biotechnology) at a final dilution of 1:100. Then, the antigen-antibody complex was captured with either agarose-protein A for polyclonal antibodies or agarose-protein G for monoclonal antibodies. For in vitro kinase assays, the immunoprecipitates were rinsed three times with RIPA buffer and once with kinase buffer (50 mM Hepes, 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 1 mM DTT). The rinsed agarose beads were incubated with kinase buffer containing 2.5 μg histone H1 plus 5 μCi [γ-^{32}P] ATP, in a final volume of 30 μl, for 30 min at 30°C. After incubation, the samples were analyzed by SDS-PAGE and autoradiography with a Molecular Imager FX (Bio-Rad).

In vitro phosphatase assay

To analyze PP1 activity, the enzyme was immunoprecipitated from both control and H_2O_2-treated SHSY5Y cells. Immunopurified PP1 was washed with phosphatase buffer containing 37.5 mM HCl-Tris (pH 7.5), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.01% Tween 20, 1 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin protease inhibitors. The enzyme was then mixed with 50 μg/ml of purified τ from bovine brain in a final volume of 30 μl for 1 h at 30°C. The reaction was stopped by adding 4X Laemmli buffer and the samples were then analyzed for τ phosphorylation in the AT8 epitope.

Metabolic labeling of I-2 protein

SHSY5Y cells were incubated for 2 h with DMEM medium without phosphate, and then 200 μCi/ml of aqueous radiolabeled [^{32}P]-phosphate was added. After 1 h, 100 μM H_2O_2 was added to cells, while control cells were incubated with vehicle. Later on, the cells were collected for I-2 immunoprecipitation, separated by SDS-PAGE, and analyzed for $^{32}PO_4$ incorporation through a Molecular Imager FX (Bio-Rad).

RESULTS

Oxidative stress in hippocampal neurons and SHSY5Y cells

In the first set of experiments, we assessed the effect of oxidative stress induced by H_2O_2. For this purpose, we initially determined the number of live neurons after treatment with different concentrations of H_2O_2. We analyzed hippocampal neuronal viability for H_2O_2 concentrations ranging from 0 to 500 μM. At 100 μM H_2O_2, 84% of the cells were viable (Fig. 1B). Even at higher concentrations up to 500 μM, over 75% of the neurons were viable. For all subsequent experiments, we set the H_2O_2 concentration at 100 μM. To confirm that H_2O_2 treatment was not deleterious in our system, we analyzed cell viability by using the Mito-Capture assay. As indicated in Fig. 1A, there were no variations between control and treated cells. Finally, to confirm that oxidative stress response produced after H_2O_2 treatment leads to protein modifications implicated in the oxidative stress pathways, we determined the formation of 4-hydroxynonenal (4-HNE) adducts, a canonical post-translational modification of proteins after oxidative stress insults [32]. Thus, by using indirect immunofluorescence, we verified that hippocampal cells under oxidative stress showed an increase of 4-HNE adducts, particularly in those regions corresponding to the axon and minor processes (Fig. 1C). Taken together, these results indicate that the effects mediated by H_2O_2 indeed involve oxidative stress pathways. On the other hand, the concentration of H_2O_2 used was not deleterious for neuronal survival at working concentrations after 2 h of treatment. To discard the possibility that these effects were due to alterations in glial cells contaminating at our primary hippocampal cultures, we estimated the number of glial cells in our culture system. Using GFAP staining, we estimated that glial cells represented less than 5% of the total cell population (data not shown).

Tau phosphorylation is modified by oxidative stress in hippocampal cells

Oxidative stress has been related to the onset of AD [7], and one of the pathological hallmarks for AD is the

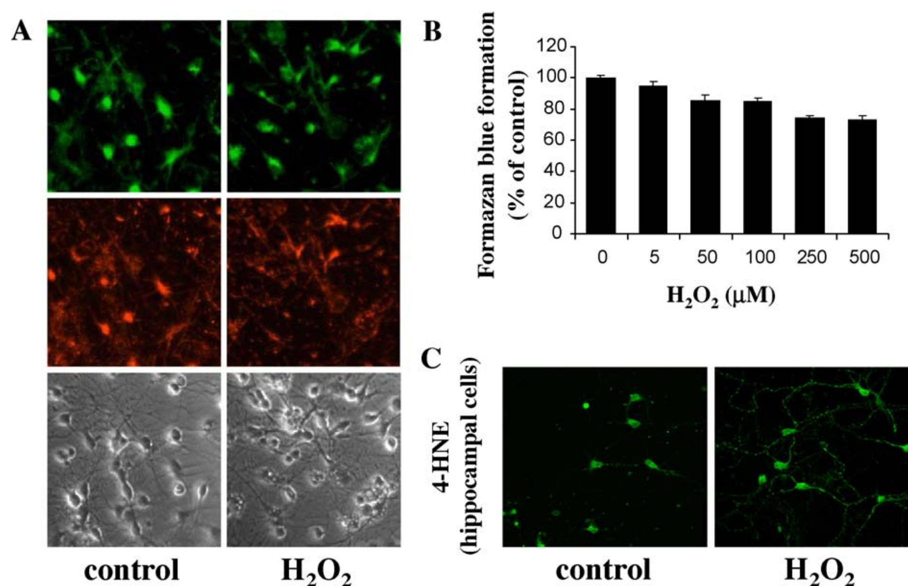

Fig. 1. Viability of hippocampal cells under oxidative stress conditions. (A) No differences in hippocampal neurons viability after treatment with H_2O_2 were evident, as assessed by the Mito-Capture assay. (B) The percentage of live hippocampal cells after treatment with different H_2O_2 concentrations, as assessed by the MTT assay. Based on these data, the working concentration for the subsequent experiments was set at 100 μM. (C) Immunofluorescence of 4-HNE adduct formation with ϵ-lysines in hippocampal cells treated with 100 μM H_2O_2 and the untreated controls (magnification ×40 in A and C).

accumulation of the abnormally hyperphosphorylated microtubule-associated protein τ. Previous findings from our laboratory indicated that Aβ peptide induced an imbalance in the amount of phosphorylated τ due to a deregulation of the cdk5/p35 complex [22]. Also, Aβ effects have been related to an increase in the oxidative stress response of neuronal cells [7,12–18]. Therefore, by using phosphospecific antibodies, we analyzed the relative amounts of phosphorylated and unphosphorylated τ epitopes AT8, PHF1, and Tau1 following H_2O_2 treatment. As indicated in Fig. 2A, hippocampal cells treated with 100 μM H_2O_2 exhibited a decrease in the amount of phosphorylated τ variants, as given by AT8 antibody. Consistent with this finding, a decrease in the PHF1 epitope was also verified (Fig. 2A). Concomitantly with these results, an increase in the amount of species recognized by Tau1 antibody, which reacts with unphosphorylated τ epitopes, was evidenced (Fig. 2A). These variations in the relative abundance of phosphorylated τ isoforms were not due to differences in the amount of total protein, since protein extracts incubated with the Tau5 were equivalent in all cases (Fig. 2A). Tau5 antibody recognizes conformational epitopes on total τ protein in a phosphorylation-independent way. These data were also confirmed in fixed neurons through indirect immunofluorescence. Thus, a decrease in the amount of phosphorylated τ forms was verified in H_2O_2-treated neurons, as given by AT8 antibody (Fig. 2B). In the same manner, an increase of unphosphorylated forms of τ protein was detected in H_2O_2-treated cells, as given by Tau1 antibody (Fig. 2B). No variations between control and treated cells were found with Tau5 (Fig. 2B) or by actin immunostaining of these cells (data not shown). Interestingly, the overall appearance of neurons seemed unchanged after H_2O_2 treatment. Altogether, these results indicate that H_2O_2 treatment produces a decrease in the phosphorylation levels of τ protein at epitopes that have been shown to be dependent on cdk5 activity.

Cdk5 activity is increased in neurons subjected to oxidative stress

Since AT8 and Tau1 recognize epitopes dependent on proline-directed protein kinases, a good candidate to assess such responses was the cdk5/p35 complex. We first analyzed the total levels of the kinase in protein extracts derived from control and H_2O_2-treated neurons. The levels of the protein kinase were unaltered in response to oxidative stress stimuli. Furthermore, the presence of the antioxidant NAC did not alter the total protein levels of cdk5 (Fig. 3A). Loading was verified by using actin as an internal control (Fig. 3A). We then estimated the amount of the kinase in the different experimental conditions and we found no variations between control and treated groups (Fig. 3B). As indicated above, our findings dealing with the levels of phosphorylated τ suggested that the activity of the cdk5/p35 complex could be decreased. Thus, we decided to analyze cdk5 activity in vitro. Surprisingly, immunoprecipitated cdk5 after H_2O_2 treatment was

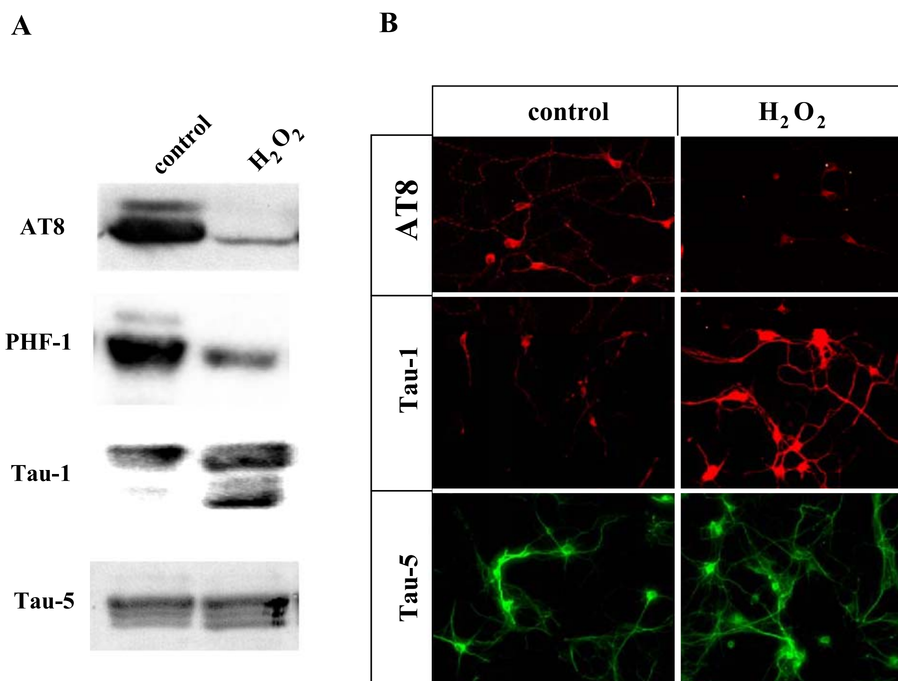

Fig. 2. Oxidative stress decreases τ phosphorylation. (A) Western blots of hippocampal neurons treated with 100 μM H_2O_2, showing that H_2O_2 induces a decrease in the phosphorylated forms of τ protein. AT8 (first panel), PHF-1 (second panel), and Tau1 (third panel) antibodies are phosphoepitope-specific antibodies used to detect either phosphorylated or unphosphorylated τ, respectively. Tau5 (fourth panel) recognizes total τ isoforms in a phosphorylation-independent manner. (B) Immunofluorescence of hippocampal neurons treated with 100 μM H_2O_2 reproduce the results obtained in the Western blot analyses. Thus, a decrease in AT8 (upper panel) staining concomitant with an increase in Tau1 (middle panel) staining is verified in hippocampal cells. In control experiments, no changes in Tau5 (lower panel) staining were detected.

found to be more active as shown by the phosphorylation of histone-1 (Fig. 3C). The activation of the kinase occurred even 1 h after H_2O_2 treatment (Fig. 3C). The presence of NAC again was able to prevent the cdk5 activation (Fig. 3C). We estimated the increase of cdk5 activity to be 3.5-fold compared to untreated controls (Fig. 3D). Since cdk5 activity depends on neurospecific activators binding, we then analyzed the expression profile of the neurospecific cdk5 activator p35. Overall, p35 and p25 protein levels were unchanged in neurons treated with H_2O_2, NAC, or both compounds (Fig. 3E). As in the previous experiments, actin was used as a loading control. The fact that we could not detect changes in the overall amount of p35 gave rise to the possibility that variations in the activity of the kinase was indeed due to changes in the affinity of the activator for the kinase after H_2O_2 treatment. To test this hypothesis, we performed cdk5 immunoprecipitation after H_2O_2 incubation, and then we estimated the amount of p35 bound to cdk5, leading to an active complex. Interestingly, an increase in the amount of p35 bound to cdk5 was verified in the H_2O_2 condition (Fig. 3F). This result suggests that H_2O_2 treatment induced an increase in the activity of cdk5 that depended on differential p35 binding to the enzyme.

Cdk5 activity is involved in the PP1-dependent dephosphorylation of τ

To evaluate the dependence of cdk5 activity on the response to oxidative stress in neural cells, we performed combined treatments of H_2O_2 and roscovitine, an inhibitor of cdk5. Hippocampal cells under this oxidative stress stimulus showed an increase in the unphosphorylated τ species at the Tau1 epitope (Figs. 2A and 4A). When hippocampal cells were exposed to H_2O_2 in combination with roscovitine, a decrease in the unphosphorylated forms of τ protein was observed, as given by Tau1 staining. These results suggest that, under these particular oxidative stress conditions, cdk5 might be mediating τ dephosphorylation rather than increasing τ phosphorylation (Fig. 4A). Quantification by densitometric analyses confirmed the Western blot data (Fig. 4C, $p < 0.05$). A possible explanation for this behavior might be that cdk5 plays a role in the control of a protein phosphatase activity responsible for τ dephosphorylation at the Tau1 epitope. To further examine this possibility, we combined treatment with H_2O_2 and 1 μM okadaic

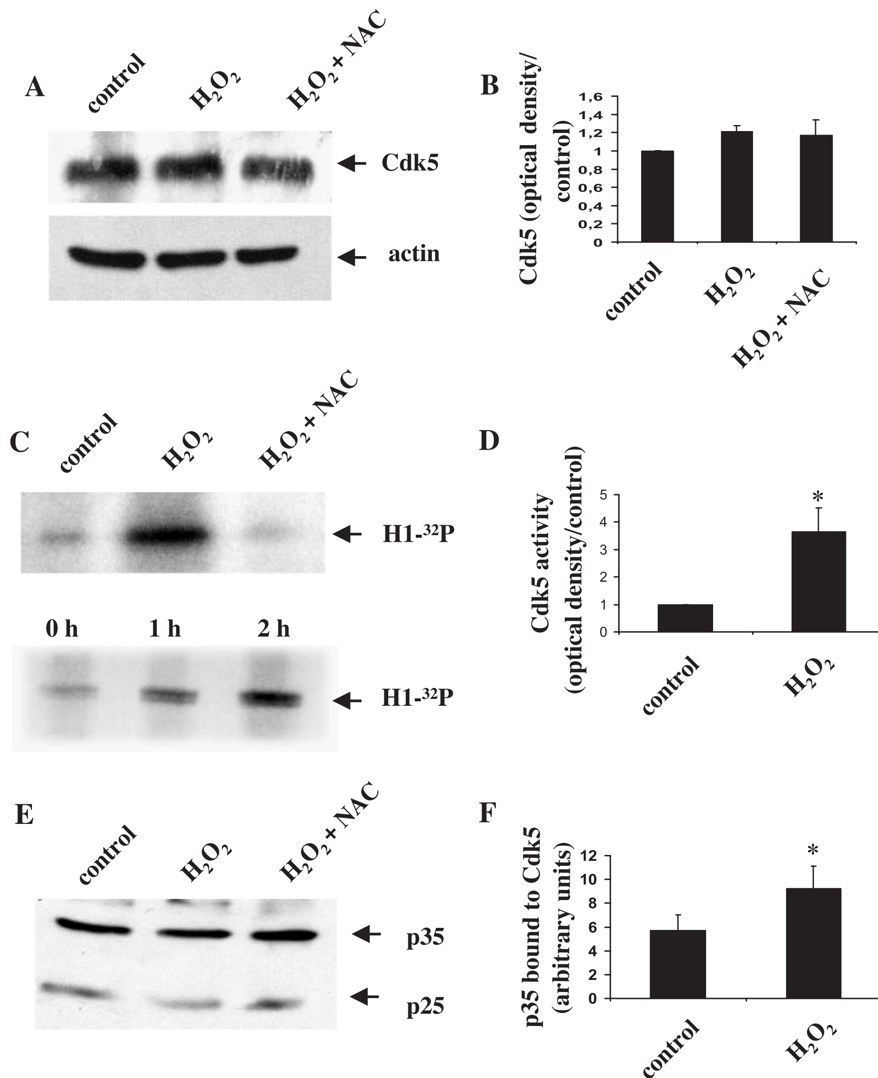

Fig. 3. Oxidative stress increases cdk5 activity. (A) Cdk5 protein levels were not modified in the presence of H_2O_2 or after the addition of H_2O_2 plus NAC, as assessed by Western blot analysis. Actin was used to normalize samples loading. (B) Densitometric analyses showing no significant variation in the overall levels of the kinase. (C) Cdk5 activity assay, using histone H1 as substrate, showed an increase in enzyme activity after 2 h of treatment with H_2O_2. The effect was further verified by autoradiography at shorter time intervals (1 h, lower panel). Also, the effect on cdk5 activity was reversed by adding NAC, as analyzed by autoradiography (upper panel). (D) Densitometric analyses showing a 3-fold induction in the cdk5 activity after H_2O_2 treatment ($n = 4$, $p < 0.05$). (E) p35 and p25 overall levels in neurons treated with H_2O_2. There were no variations in the amount of both p35 and p25 levels in control and treated cells. Also, treatment with H_2O_2 plus antioxidant NAC did not modify endogenous cdk5 activator levels. (F) Graph showing a variation in the amount of p35 bound to immunoprecipitated cdk5. Statistical analyses indicating that p35 binding to cdk5 is significantly increased after H_2O_2 treatment ($n = 4$, $p < 0.05$).

acid, an inhibitor of protein phosphatases PP1 and PP2A. Interestingly, okadaic acid treatment was able to reverse H_2O_2-induced dephosphorylation (Fig. 4B). Also, incubation of cells with 5 nM okadaic acid, a concentration described to be specific for PP2A inhibition, failed to reverse τ dephosphorylation, suggesting that PP1 could be the phosphatase responsible for τ dephosphorylation. Moreover, analogous treatment with cyclosporine A, a calcineurin inhibitor, did not affect τ dephosphorylation at the Tau1 epitope induced by H_2O_2 (Fig. 4B). These data indicate that τ dephosphorylation may be mediated by PP1 rather than PP2A and PP2B. Again, quantitative densitometric analyses clearly revealed the okadaic acid effects in reversing τ dephosphorylation at the Tau1 epitope (Fig. 4C).

It has been previously reported that cdk5 could be involved in the phosphorylation of the PP1 I-2, resulting in the loss of a negative regulation of the phosphatase due to dissociation of the inhibitor [24]. To confirm this possibility, we immunoprecipitated PP1 from human

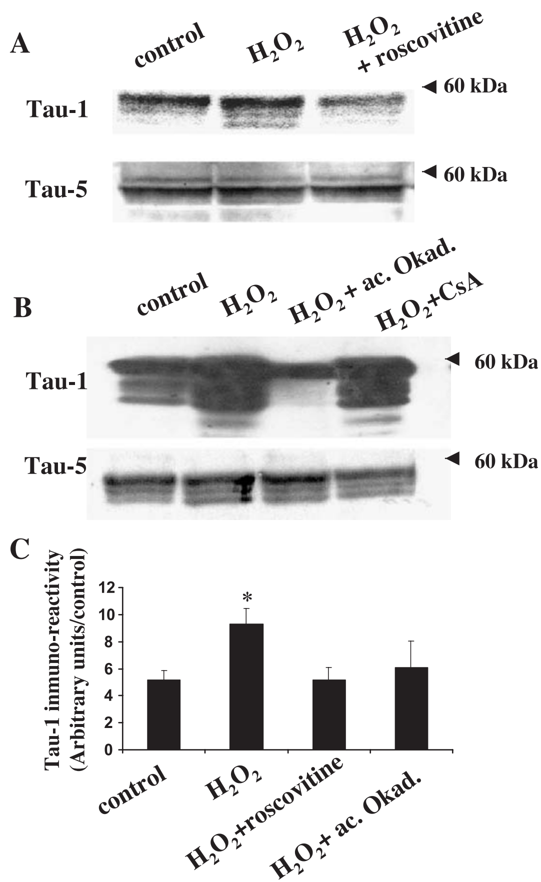

Fig. 4. Cdk5 and PP1 inhibitors prevent τ dephosphorylation. (A) Tau dephosphorylation induced by oxidative stress is reversed in the presence of the cdk5 inhibitor roscovitine. (B) Tau dephosphorylation induced by oxidative stress is also reversed in the presence of okadaic acid, an inhibitor of protein phosphatase PP1. On the other hand, the calcineurin inhibitor cyclosporine A did not reverse τ dephosphorylation. (C) Quantitative analyses indicating the significant increase of Tau1 staining in H_2O_2-treated neurons, and the reversion of the effect with both cdk5 and PP1 inhibitors ($p < 0.05$). Details are given in Materials and Methods.

SHSY5Y cells treated with H_2O_2 at different time intervals. Subsequently, we analyzed the amount of the I-2 pulled down by PP1 (Fig. 5). Notably, we found a significant decrease in the amount of I-2 associated with PP1 after H_2O_2 treatments. The time-dependent decrease occurred after 15 min of H_2O_2 treatment and continued for at least 2 h. Concomitant with a loss of PP1 inhibition, we verified an increase in the dephosphorylated forms of τ associated with the immunoprecipitated pellets containing PP1, as given by the Tau1 epitope antibody (Fig. 5A, Tau-1 P) and a decrease in the soluble forms of dephosphorylated τ (Fig. 5A, Tau-1 S). All the stated effects were verified without any substantial change in the PP1 levels (Fig. 5A). These results clearly suggest that PP1 activation in response to oxidative stress is most likely to depend on the release of I-2 from the I-2/PP1 complex. Quantitative analyses showed a 3-fold decrease in the amount of I-2 associated with PP1 after 2 h of H_2O_2 treatment (Fig. 5B).

To substantiate that activation of cdk5 after H_2O_2 treatment was paralleled by changes in the phosphorylation of I-2 and the increase in PP1 activity, we performed metabolic labeling experiments and an in vitro PP1 phosphatase assay. Fig. 6A shows that immunoprecipitated PP1 from H_2O_2-treated cells was more active, as given by a decrease in the phosphorylated τ at the AT8 epitope in an in vitro phosphatase assay. Concomitantly, this decrease of phosphorylated τ at the AT8 epitope was shown to be significant ($n = 3$, $p < 0.05$). An increase in PP1 activity should be paralleled by an increase in I-2 phosphorylation, leading to dissociation of the I-2/PP1 complex. Therefore, we estimated the changes in metabolic labeling of I-2 after H_2O_2 treatment. As shown in Fig. 6B, I-2 incorporated more $^{32}PO_4$ after H_2O_2 treatment, strengthening the idea that changes in the phosphorylation state of I-2 could be related to increased PP1 activity. Again, these results were confirmed to be significant when quantified ($n = 4$, $p < 0.05$). Altogether, these experiments clearly suggest that, after the addition of H_2O_2, cdk5 is activated and the activity of PP1 is increased. Thereaf-

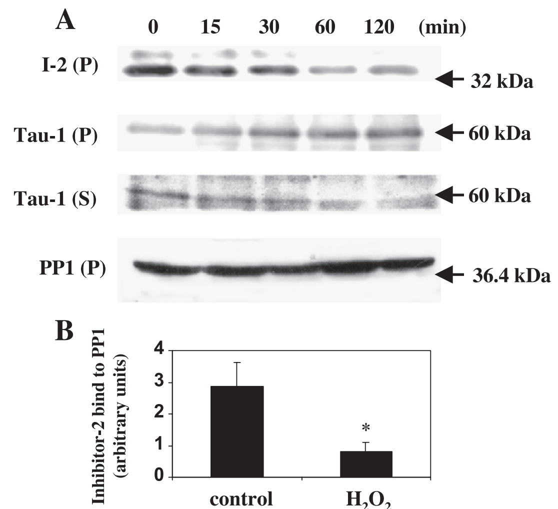

Fig. 5. I-2/PP1 complex was dissociated under oxidative stress stimuli. (A) Immunoprecipitated PP1 pellets were analyzed for the presence of either pulled-down I-2 or dephosphorylation at the Tau1 epitope. A time-dependent decrease in the I-2 association to PP1 was observed in neuronal cells treated with H_2O_2 (first panel). Concomitant with this, a time-dependent increase of dephosphorylated Tau1 epitope associated to PP1 was found (second panel). The increase of dephosphorylated τ (recognized by Tau1) associated with PP1 was paralleled by a decrease in the soluble fraction of dephosphorylated τ (third panel). As a control for the amount of PP1, Western blots using an antibody against catalytic subunit revealed no changes in the PP1 levels (fourth panel). (B) Quantitative analyses revealed that, after 2 h of H_2O_2 addition, there was a 3-fold reduction in the association of I-2 to PP1.

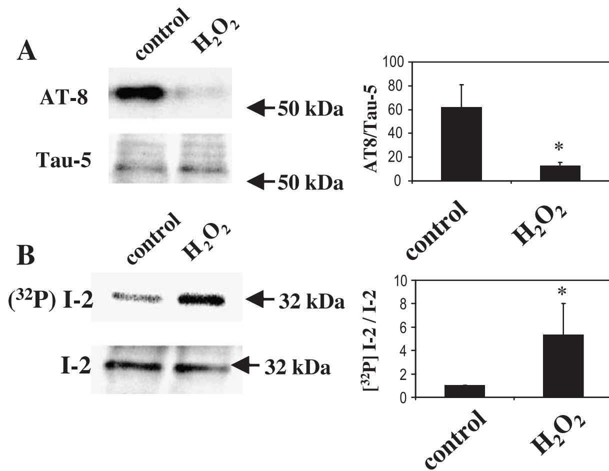

Fig. 6. In vitro phosphatase activity of PP1 and metabolic labeling of I-2 with $^{32}PO_4$ in SHSY5Y cells treated with H_2O_2. (A) In vitro phosphatase activity was performed by incubating immunopurified PP1 enzyme from both control and H_2O_2-soluble protein extracts. After immunopurification, the enzyme was mixed with pure τ protein derived from bovine brain, and the amount of phosphorylated τ at the AT8 epitope was determined (more details are given in Materials and Methods). The assay indicates that H_2O_2 treatment results in an increase in the activity of PP1 derived from cells, as evidenced by a decrease in AT8 labeling (left panel). Quantitative analyses showed the ratio between τ phosphorylated at the AT8 epitope and total τ levels, confirming Western blot results ($n = 3$, $p < 0.05$) (right panel). (B) Metabolic labeling of I-2 after H_2O_2 treatment, showing an increase in the amount of $^{32}PO_4$ incorporation to I-2 after oxidative stress stimuli. The difference was not due to changes in the protein overall levels (left panel). Quantitative analyses showed the ratio between [^{32}P] I-2 and total I-2, confirming an increase in the metabolic labeling of I-2 after H_2O_2 treatment ($n = 4$, $p < 0.05$) (right panel).

ter, the active phosphatase should be responsible for τ dephosphorylation at the Tau1 epitope.

DISCUSSION

In this study, we showed that neuronal oxidative stress induced by the addition of H_2O_2 produced a decrease in microtubule-associated protein τ phosphorylation at the AT8 and PHF1 epitopes. Oxidative stress induced by H_2O_2 also increased the amount of 4-HNE-modified proteins. The phosphorylation decrease in τ protein is most likely to be produced by an increased activity of PP1 triggered by the dissociation of its I-2. Cdk5 is a member of cyclin-dependent kinases that, among other activities, phosphorylates τ protein under Aβ stimulus in residues that are found abnormally phosphorylated in AD [21,22]. In our biological model, we observed an increase in the activity of cdk5 in neurons treated with H_2O_2 but a decrease in the phosphorylation of τ. Since no major changes were observed in p35 levels, we determined that the increase of cdk5 activity was produced by an increase in the amount of p35 bound to the enzyme. Alternatively, it might be possible that another activator could be participating in cdk5 activation [22]. According to the present data, the mechanism implicated in such a dephosphorylation considers an initial phosphorylation of the PP1 I-2. After its phosphorylation, the I-2 would be released from the complex, and PP1 could be then responsible for τ dephosphorylation.

Oxidative stress phenomena have been related to the onset of several neurodegenerative disorders including amyotrophic lateral sclerosis (ALS) [4], Parkinson's disease [33], and AD [7,34]. The effects of such oxidative damage can be evaluated in terms of modifications in proteins, lipids, and nucleic acids. It has been shown that, in ALS, the heavy neurofilament subunit is modified with 4-HNE [35]. Similarly, in AD, τ protein can be modified by 4-HNE in vitro [32] and in AD-derived brain sections [36]. Oxidative stress can be produced in AD by the extracellular deposition of Aβ peptide [19]. In previous findings by our laboratory, we demonstrated that Aβ peptide also induced a deregulation of the cdk5/p35 complex in cell cultures [22]. Increased τ phosphorylation at the AT8 epitope was verified in those cells, and this response was inhibited by using pharmacological inhibition of the kinase with butyrolactone I or roscovitine [21]. Furthermore, we have recently described a cdk5 imbalance in a transgenic model that overexpress AβPP [37]. Many efforts have been made to analyze the relationships between the amyloid pathway and τ phosphorylation. In this respect, it has been proposed that the Aβ effect on neurons could be elicited by triggering oxidative stress responses [12,38] in vitro and in vivo [39,40]. However, other stimuli leading to an increase of the oxidative stress responses have seeded controversy in the τ phosphorylation issue. Thus, H_2O_2 has been described to produce a decrease in τ phosphorylation in primary cortical neurons [41].

In this study, we have implicated some of the kinase and phosphatase systems responsible for the decreased τ phosphorylation. The increase of unphosphorylated τ isoforms is most likely to depend on cdk5, since AT8 and Tau1 antibodies recognize mainly phosphoepitopes on τ that depend on kinases belonging to the proline-directed protein kinase family, and this decrease of phosphorylated τ species recognized by the aforementioned antibodies was not dependent on the activity of gsk3β levels [41].

These changes have also been described for neurons treated with excitotoxic levels of glutamate [42–44] and under other conditions, such as ischemia [45,46]. Moreover, a decrease of Alzheimer's-type τ phosphorylation was detected in hippocampal cells suffering another oxidative insult, namely iron overload [47]. The fact that different oxidative stress treatments lead to opposite results on τ phosphorylation suggests that differences could be related to certain variations in the intracellular

levels of second messengers. Thus, dephosphorylation of τ protein could indicate an initial response of cells against oxidative insults. In oligodendrocytes, it has been demonstrated that τ is dephosphorylated in response to in vivo oxidative insults such as stroke and head injury in humans [48] and rodents [49]. Tau protein in its dephosphorylated forms binds more efficiently to microtubules [50] and cell membranes [51]. Interestingly, protein phosphatase 1 can be targeted to microtubules upon binding to τ protein, suggesting that the control of τ phosphorylation by PP1 in our system might have relevant biological effects [52]. Our results along with previous reports suggest that an initial response of neurons after H_2O_2 treatment (an acute effect) would lead to τ dephosphorylation at the AT8 epitope, due to an increased PP1 activity through a mechanism involving phosphorylation of I-2. However, τ phosphorylation results from the balance of kinases and phosphatase activities. Thus, later on, chronic and cumulative oxidative stress conditions in combination with other factors (i.e., amyloid β or inflammatory factors) should favor cdk5/p35 activity on τ protein that, together with other kinases, could lead to increased τ phosphorylation. Thus, in this report we suggest a novel mechanism by which acute treatments with H_2O_2 might induce τ dephosphorylation.

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REFERENCES

[1] Heim, C.; Zhang, J.; Lan, J.; Siekluckak, M.; Kurz, T.; Riederer, P.; Gerlach, M.; Sontag, K. H. Cerebral oligaemia episode triggers free radical formation and late cognitive deficiencies. *Eur. J. Neurosci.* **12:**715–725; 2000.

[2] Mautes, A. E.; Bergeron, M.; Sharp, F. R.; Panter, S. S.; Weinzierl, M.; Guenther, K.; Noble, L. J. Sustained induction of heme oxygenase-1 in the traumatized spinal cord. *Exp. Neurol.* **166:** 254–265; 2000.

[3] Shoham, S.; Youdim, M. B. Iron involvement in neural damage and microgliosis in models of neurodegenerative diseases. *Cell Mol. Biol. (Noisy-le-grand)* **46:**743–760; 2000.

[4] Julien, J. P. Amyotrophic lateral sclerosis. unfolding the toxicity of the misfolded. *Cell* **104:**581–591; 2001.

[5] Goedert, M. The significance of τ and and α-synuclein inclusions in neurodegenerative diseases. *Curr. Opin. Genet. Dev.* **11:** 343–351; 2001.

[6] Butterfield, D. A.; Kanski, J. Brain protein oxidation in age-related neurodegenerative disorders that are associated with aggregated proteins. *Mech. Ageing Dev.* **122:**945–962; 2001.

[7] Maccioni, R. B.; Munoz, J. P.; Barbeito, L. The molecular bases of Alzheimer's disease and other neurodegenerative disorders. *Arch. Med. Res.* **32:**367–381; 2001.

[8] Nunomura, A.; Perry, G.; Aliev, G.; Hirai, K.; Takeda, A.; Balraj, E. K.; Jones, P. K.; Ghanbari, H.; Wataya, T.; Shimohama, S.; Chiba, S.; Atwood, C. S.; Petersen, R. B.; Smith, M. A. Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **60:**759–767; 2001.

[9] Ohno, Y.; Gallin, J. I. Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. *J. Biol. Chem.* **260:**8438–8446; 1985.

[10] Hyslop, P. A.; Zhang, Z.; Pearson, D. V.; Phebus, L. A. Measurement of stratial H_2O_2 by microdialysis following global forebrain ischemia and reperfusion in the rat: correlation with the cytotoxic potential of H_2O_2 in vitro. *Brain Res.* **671:**181–186; 1995.

[11] Symmons, M. C. R.; Gutteridge, J. M. C. Free radicals and iron: chemistry, biology and medicine. Oxford University Press, New York, 1998.

[12] Behl, C.; Davis, J. B.; Lesley, R.; Schubert, D. Hydrogen peroxide mediates amyloid β protein toxicity. *Cell* **77:**817–827; 1994.

[13] Shearman, M. S.; Ragan, C. I.; Iversen, L. L. Inhibition of PC12 cell redox activity is a specific, early indicator of the mechanism of β-amyloid-mediated cell death. *Proc. Natl. Acad. Sci. USA* **91:**1470–1474; 1994.

[14] Harris, M. E.; Hensley, K.; Butterfield, D. A.; Leedle, R. A.; Carney, J. M. Direct evidence of oxidative injury produced by the Alzheimer's β-amyloid peptide (1-40) in cultured hippocampal neurons. *Exp. Neurol.* **131:**193–202; 1995.

[15] Manelli, A. M.; Puttfarcken, P. S. Beta-amyloid-induced toxicity in rat hippocampal cells: in vitro evidence for the involvement of free radicals. *Brain Res. Bull.* **38:**569–576; 1995.

[16] Mattson, M. P.; Goodman, Y. Different amyloidogenic peptides share a similar mechanism of neurotoxicity involving reactive oxygen species and calcium. *Brain Res.* **676:**219–224; 1995.

[17] Mattson, M. P.; Lovell, M. A.; Furukawa, K.; Markesberry, W. R. Neurotrophic factors attenuate glutamate-induced accumulation of peroxides, elevation of intracellular Ca^{2+} concentration, and neurotoxicity and increase antioxidant enzyme activities in hippocampal neurons. *J. Neurochem.* **65:**1740–1751; 1995.

[18] Café, C.; Torri, C.; Bertorelli, L.; Angeretti, N.; Lucca, E.; Forloni, G.; Marzatico, F. Oxidative stress after acute and chronic application of β-amyloid fragment 25–35 in cortical cultures. *Neurosci. Lett.* **203:**61–65; 1996.

[19] Busciglio, J.; Lorenzo, A.; Yeh, J.; Yankner, B. A. Beta-amyloid fibrils induce τ phosphorylation and loss of microtubule binding. *Neuron* **14:**879–888; 1995.

[20] Takashima, A.; Noguchi, K.; Michel, G.; Mercken, M.; Hoshi, M.; Ishiguro, K.; Imahori, K. Exposure of rat hippocampal neurons to amyloid β peptide (25–35) induces the inactivation of phosphatidyl inositol-3 kinase and the activation of τ protein kinase I/glycogen synthase kinase-3 β. *Neurosci. Lett.* **203:**33–36; 1996.

[21] Alvarez, A.; Toro, R.; Caceres, A.; Maccioni, R. B. Inhibition of τ phosphorylating protein kinase cdk5 prevents β-amyloid-induced neuronal death. *FEBS Lett.* **459:**421–426; 1999.

[22] Alvarez, A.; Munoz, J. P.; Maccioni, R. B. A Cdk5-p35 stable complex is involved in the β-amyloid-induced deregulation of Cdk5 activity in hippocampal neurons. *Exp. Cell Res.* **264:** 266–274; 2001.

[23] Alvarez, G.; Munoz-Montano, J. R.; Satrustegui, J.; Avila, J.; Bogonez, E.; Diaz-Nido, J. Lithium protects cultured neurons against β-amyloid-induced neurodegeneration. *FEBS Lett.* **453:** 260–264; 1999.

[24] Agarwal-Mawal, A.; Paudel, H. K. Neuronal Cdc2-like protein kinase (Cdk5/p25) is associated with protein phosphatase 1 and phosphorylates inhibitor-2. *J. Biol. Chem.* **276:**23712–23718; 2001.

[25] Shea, T. B.; Prabhakar, S.; Ekinci, F. J. Beta-amyloid and ionophore A23187 evoke and τ hyperphosphorylation by distinct intracellular pathways: differential involvement of the calpain/protein kinase C system. *J. Neurosci. Res.* **49:**759–768; 1997.

[26] Xie, H. Q.; Johnson, G. V. Calcineurin inhibition prevents calpain-mediated proteolysis of τ in differentiated PC12 cells. *J. Neurosci. Res.* **53:**153–164; 1998.

[27] Hartigan, J. A.; Johnson, G. V. Transient increases in intracellular calcium result in prolonged site-selective increases in τ phosphorylation through a glycogen synthase kinase 3 β-dependent pathway. *J. Biol. Chem.* **274:**21395–21401; 1999.

[28] Banker, G. A.; Cowan, W. M. Rat hippocampal neurons in dispersed cell culture. *Brain Res.* **126:**397–402; 1977.
[29] Bottenstein, J. E.; Sato, G. H. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* **76:**514–517; 1979.
[30] Bradford, M. M. 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; 1976.
[31] Laemmli, U. K.; Quittner, S. F. Maturation of the head of bacteriophage T4. IV. The proteins of the core of the tubular polyheads and in vitro cleavage of the head proteins. *Virology* **62:**483–499; 1974.
[32] Perez, M.; Cuadros, R.; Smith, M. A.; Perry, G.; Avila, J. Phosphorylated, but not native, τ protein assembles following reaction with the lipid peroxidation product, 4-hydroxy-2-nonenal. *FEBS Lett.* **486:**270–274; 2000.
[33] Castellani, R. J.; Siedlak, S. L.; Perry, G.; Smith, M. A. Sequestration of iron by Lewy bodies in Parkinson's disease. *Acta Neuropathol. (Berl.)* **100:**111–114; 2000.
[34] Campbell, A.; Smith, M. A.; Sayre, L. M.; Bondy, S. C.; Perry, G. Mechanisms by which metals promote events connected to neurodegenerative diseases. *Brain Res. Bull.* **55:**125–132; 2001.
[35] Wataya, T.; Nunomura, A.; Smith, M. A.; Siedlak, S. L.; Harris, P. L.; Shimohama, S.; Szweda, L. I.; Kaminski, M. A.; Avila, J.; Price, D. L.; Cleveland, D. W.; Sayre, L. M.; Perry, G. High molecular weight neurofilament proteins are physiological substrates of adduction by the lipid peroxidation product hydroxynonenal. *J. Biol. Chem.* **277:**4644–4648; 2002.
[36] Takeda, A.; Smith, M. A.; Avila, J.; Nunomura, A.; Siedlak, S. L.; Zhu, X.; Perry, G.; Sayre, L. M. In Alzheimer's disease, heme oxygenase is coincident with Alz50, an epitope of τ induced by 4-hydroxy-2-nonenal modification. *J. Neurochem.* **75:**1234–1241; 2000.
[37] Otth, C.; Concha, I. I.; Arendt, T.; Stieler, J.; Schliebs, R.; Gonzalez-Billault, C.; Maccioni, R. B. AβPP induces cdk5-dependent and τ hyperphosphorylation in transgenic mice Tg2576. *J. Alzheimers Dis.* **4:**417–430; 2002.
[38] Pappolla, M. A.; Chyan, Y. J.; Omar, R. A.; Hsiao, K.; Perry, G.; Smith, M. A.; Bozner, P. Evidence of oxidative stress and in vivo neurotoxicity of β-amyloid in a transgenic mouse model of Alzheimer's disease: a chronic oxidative paradigm for testing antioxidant therapies in vivo. *Am. J. Pathol.* **152:**871–877; 1998.
[39] Hensley, K.; Hall, N.; Subramaniam, R.; Cole, P.; Harris, M.; Aksenov, M.; Aksenova, M.; Gabbita, S. P.; Wu, J. F.; Carney, J. M.; Lovell, M.; Markesbery, W. R.; Butterfield, D. A. Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. *J. Neurochem.* **65:**2146–2156; 1995.
[40] Yatin, S. M.; Varadarajan, S.; Link, C. D.; Butterfield, D. A. In vitro and in vivo oxidative stress associated with Alzheimer's amyloid β-peptide (1–42). *Neurobiol. Aging* **20:**325–330; 1999.
[41] Davis, D. R.; Anderton, B. H.; Brion, J. P.; Reynolds, C. H.; Hanger, D. P. Oxidative stress induces dephosphorylation of τ in rat brain primary neuronal cultures. *J. Neurochem.* **68:**1590–1597; 1997.
[42] Anderton, B. H.; Brion, J. P.; Couck, A. M.; Davis, D. R.; Gallo, J. M.; Hanger, D. P.; Ladhani, K.; Latimer, D. A.; Lewis, C.; Lovestone, S. Modulation of PHF-like τ phosphorylation in cultured neurones and transfected cells. *Neurobiol. Aging* **16:**389–397; 1995.
[43] Davis, D. R.; Brion, J. P.; Couck, A. M.; Gallo, J. M.; Hanger, D. P.; Ladhani, K.; Lewis, C.; Miller, C. C.; Rupniak, T.; Smith, C. The phosphorylation state of the microtubule-associated protein τ as affected by glutamate, colchicine and β-amyloid in primary rat cortical neuronal cultures. *Biochem. J.* **309:**941–949; 1995.
[44] Fleming, L. M.; Johnson, G. V. Modulation of the phosphorylation state of τ in situ: the roles of calcium and cyclic AMP. *Biochem. J.* **309:**41–47; 1995.
[45] Geddes, J. W.; Schwab, C.; Craddock, S.; Wilson, J. L.; Pettigrew, L. C. Alterations in τ immunostaining in the rat hippocampus following transient cerebral ischemia. *J. Cereb. Blood Flow Metab.* **14:**554–564; 1994.
[46] Shackelford, D. A.; Nelson, K. E. Changes in phosphorylation of τ during ischemia and reperfusion in the rabbit spinal cord. *J. Neurochem.* **66:**286–295; 1996.
[47] Egana, J. T.; Zambrano, C.; Nuñez, M. T.; Gonzalez-Billault, C.; Maccioni, R. B. Iron induced oxidative stress modify and τ phosphorylation patterns in hippocampal cell cultures. *Biometals* **16:**215–223; 2003.
[48] Irving, E. A.; Nicoll, J.; Graham, D. I.; Dewar, D. Increased τ immunoreactivity in oligodendrocytes following human stroke and head injury. *Neurosci. Lett.* **213:**189–192; 1996.
[49] Papasozomenos, S. C. Heat shock induces rapid dephosphorylation of τ in both female and male rats followed by hyperphosphorylation only in female rats: implications for Alzheimer's disease. *J. Neurochem.* **66:**1140–1149; 1996.
[50] Drewes, G.; Ebneth, A.; Mandelkow, E. M. MAPs, MARKs and microtubule dynamics. *Trends Biochem. Sci.* **23:**307–311; 1998.
[51] Arrasate, M.; Perez, M.; Avila, J. Tau dephosphorylation at tau-1 site correlates with its association to cell membrane. *Neurochem. Res.* **25:**43–50; 2000.
[52] Liao, H.; Li, Y.; Brautigan, D. L.; Gundersen, G. G. Protein phosphatase 1 is targeted to microtubules by the microtubule-associated protein τ. *J. Biol. Chem.* **273:**21901–21908; 1998.

ABBREVIATIONS

4-HNE — 4-hydroxy-2-nonenal
I-2 — protein phosphatase 1 inhibitor 2
NAC — N-acetyl-L-cysteine
PP1 — protein phosphatase 1