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Original Contribution

Heme oxygenase-1 posttranslational modifications in the brain of subjects with Alzheimer disease and mild cognitive impairment

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ABSTRACT

Alzheimer disease (AD) is a neurodegenerative disorder characterized by progressive cognitive impairment and neuropathology. Oxidative and nitrosative stress plays a principal role in the pathogenesis of AD. The induction of the heme oxygenase-1/biliverdin reductase-A (HO-1/BVR-A) system in the brain represents one of the earliest mechanisms activated by cells to counteract the noxious effects of increased reactive oxygen species and reactive nitrogen species. Although initially proposed as a neuroprotective system in AD brain, the HO-1/BVR-A pathophysiological features are under debate. We previously reported alterations in BVR activity along with decreased phosphorylation and increased oxidative/nitrosative posttranslational modifications in the brain of subjects with AD and those with mild cognitive impairment (MCI). Furthermore, other groups proposed the observed increase in HO-1 in AD brain as a possible neurotoxic mechanism. Here we provide new insights about HO-1 in the brain of subjects with AD and MCI, the latter condition being the transitional phase between normal aging and early AD. HO-1 protein levels were significantly increased in the hippocampus of AD subjects, whereas HO-2 protein levels were significantly decreased in both AD and MCI hippocampi. In addition, significant increases in Ser-residue phosphorylation together with increased oxidative posttranslational modifications were found in the hippocampus of AD subjects. Interestingly, despite the lack of oxidative stress-induced AD neuropathology in cerebellum, HO-1 demonstrated increased Ser-residue phosphorylation and oxidative posttranslational modifications in this brain area, suggesting HO-1 as a target of oxidative damage even in the cerebellum. The significance of these findings is profound and opens new avenues into the comprehension of the role of HO-1 in the pathogenesis of AD.

Introduction

Increased oxidative and nitrosative stress represents one of the main mechanisms involved in the pathogenesis of neurodegenerative disorders such as Alzheimer disease (AD), which exhibits a large impairment of neuronal structure and molecular pathways due to oxidative stress-induced posttranslational modifications on both proteins and lipids [1,2].

AD is an age-related neurodegenerative disorder characterized histopathologically by the presence of senile plaques, neurofibrillary tangles (NFTs), and synapse loss in selected brain regions [3,4] and clinically by memory loss and dementia [5]. The main component of senile plaques is amyloid β-peptide (Aβ), a 40- to 42-amino-acid peptide derived by the proteolytic cleavage of amyloid precursor protein through the activity of β- and γ-secretases [4]. Although Aβ(1–42) is a neurotoxic peptide that exists in both soluble (monomers, oligomers, and protofibrils) and insoluble (fibrils) forms [6], recent studies suggested that the small oligomers, rather than Aβ fibrils, are the actual toxic species of this peptide [7–10], being responsible for oxidative/nitrosative-induced damage in the brain [1,11–13]. Amnestic mild cognitive impairment (MCI) is considered the transitional phase between normal aging and early AD [14]. MCI shares pathological features with AD, such as Aβ and NFT accumulation in the neocortex and medial temporal lobe [14,15], which leads to elevated pro-oxidant status [16] and clinical aspects including memory loss [16]. However, MCI subjects are not characterized by dementia, with subjects being able to perform normal activities of daily living [17].

Abbreviations: 3-NT, 3-nitrotyrosine; AD, Alzheimer disease; HNE, 4-hydroxy-2-nonenal; HO, heme oxygenase; MCI, mild cognitive impairment; PC, protein carbonyls; pSer, phosphoserine; pThr, phosphothreonine; pTyr, phosphotyrosine

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Under conditions of prolonged oxidative and nitrosative stress the brain reacts by upregulating genes involved in cell stress response processes to limit neuronal damage [18,19]. The heme oxygenase/biliverdin reductase (HO/BVR) system, whose upregulation is one of the earlier events in AD, plays a crucial role in the adaptive response to stress [20]. Heme oxygenase is a microsomal enzyme that exists in two main isoforms: the inducible HO-1 and the constitutive HO-2 [21]. Heme oxygenase-1, also known as heat shock protein-32, is induced by various stimuli, including reactive oxygen and nitrogen species, ischemia, heat shock, bacterial lipopolysaccharide, hemin, and the neuroprotective agent leteprinim potassium (Neotrofin), and is primarily involved in the cell stress response [21–23]. Conversely HO-2 is responsive to developmental factors and adrenal glucocorticoids and works as an intracellular sensor of oxygen, carbon monoxide, and nitric oxide [21,23]. Furthermore, our group demonstrated an upregulation of both HO-1 and HO-2 in the brain of aged dogs after atorvastatin treatment [24]. Heme oxygenase catalyzes the oxidation of the α-*meso*-carbon bridge of heme moieties, resulting in equimolar amounts of the pleiotropic gaseous neuromodulator carbon monoxide (CO), ferrous iron, and biliverdin-IXα. Biliverdin-IXα is further reduced by the cytosolic enzyme biliverdin reductase-A (BVR-A) into the powerful antioxidant bilirubin-IXα, the final product of heme catabolism [25–27]. It is noteworthy that the activity of both HO-1 and BVR-A was demonstrated to be regulated by the phosphorylation of serine/threonine/tyrosine residues [28,29].

In the central nervous system HO-2 is expressed in neuronal populations in almost all brain areas [21], whereas the inducible isoform is present at low levels in scattered groups of neurons, including the ventromedial and paraventricular nuclei of the hypothalamus [21,23]. HO-1 is also found in glial cells, where its expression can be induced by oxidative stress [30]. Similarly, BVR-A is coexpressed with HO-1 and/or HO-2 in cells of the rat brain that express these enzymes under normal conditions. BVR-A is also found in regions and cell types that can express heat-shock-inducible HO-1 [31].

Recent studies raised questions about the activation of the HO-1/BVR-A system in neurodegenerative disorders, opening a debate on its real pathophysiological and clinical significance. In particular, lately, our group has reported alterations in BVR activity related to decreased phosphorylation and increased oxidative/nitrosative posttranslational modifications in the brain of AD and MCI subjects [32,33]. Furthermore, Hui et al., in a recent work, provided a potential pathway to explain tau aggregation, through a mechanism involving excessive iron production mediated by HO-1 overexpression, which in turn induces tau phosphorylation [34]. In addition, Schipper et al. showed that targeted suppression of glial HO-1 hyperactivity may prove to be a rational and effective neurotherapeutic intervention in AD [35]. In this scenario, a deeper level of analysis is required to elucidate the contribution of the HO/BVR system to neurodegenerative disorders.

Based on the evidence that, despite an upregulation of the HO-1/BVR-A system, a substantial protection against oxidative and nitrosative stress is not observed in AD brain, we hypothesized that, as for BVR-A, even HO-1 could be a target of oxidative/nitrosative stress. The aim of this study was to investigate HO-1 protein levels along with (i) phosphorylation- and (ii) oxidative/nitrosative stress-induced posttranslational modifications in both hippocampus and cerebellum of subjects with AD or MCI.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Nitrocellulose membranes and the electrophoresis transfer system Trans-Blot semidry transfer cell were obtained from Bio-Rad (Hercules, CA, USA). Anti-mouse and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody and ECL Plus Western blot detection reagents were obtained from GE Healthcare Bio-Sciences (Piscataway, NJ, USA).

Subjects

Frozen hippocampal and cerebellar samples ($n=6$ each) from well-characterized subjects with AD and MCI and respective age-matched controls (Table 1A and B) were obtained from the University of Kentucky Rapid Autopsy Program of the Alzheimer's Disease Clinical Center (UK ADC) with a postmortem interval within the range 1.75–5.75 h for AD and MCI patients and age-matched control subjects. All the subjects were longitudinally followed and underwent annual neuropsychological testing and neurological and physical examinations. Control subjects were without history of dementia or other neurological disorders and with intact activities of daily living (ADLs), and they underwent annual mental status testing and semiannual physical and neurological exams as part of the UK ADC normal volunteer

Table 1

(A) Demographic information on amnestic MCI subjects and their respective age-matched controls

Subject demographics	Controls	MCI
Number of subjects	6	6
Sex	2 M, 4 F	2 M, 4 F
Age (years (range))	82 (74–93)	89 (82–99)
Brain weight (g (range))	1204 (1080–1315)	1102 (930–1200)
Postmortem interval (h)	1.75–4.00	2.00–5.00
Braak stage	I–II	III–V

(B) Demographic information on AD subjects and their respective age-matched controls

Subject demographics	Control (AD)	AD
Number of subjects	6	6
Sex	5 M, 1 F	2 M, 4 F
Age (years (range))	81 (72–87)	85 (80–92)
Brain weight (g (range))	1219 (1020–1410)	1104 (835–1260)
Postmortem interval (h)	2.00–3.75	2.00–5.75
Braak stage	I–III	V–VI

The values reported are the averages of six samples.

longitudinal aging study. The control subjects showed no significant histopathological alterations and the Braak score was within the range I–II for the MCI age-matched controls and I–III for the AD age-matched controls. Patients diagnosed with MCI met the criteria described by Petersen [14], which included a memory complaint supported by an informant, objective memory test impairment (age- and education-adjusted), general normal global intellectual function, intact ADLs, Clinical Dementia Rating score of 0.0–0.5, no dementia, and a clinical evaluation that revealed no other cause for memory decline. AD patient diagnosis was made according to criteria developed by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association [36]. All AD patients displayed progressive intellectual decline. The Braak scores were within the range III–V and V–VI for MCI and AD patients, respectively.

Sample preparation

Brain tissue samples (hippocampus and cerebellum) from control, MCI, and AD subjects were sonicated in Media 1 lysis buffer (pH 7.4) containing 0.32 M sucrose, 0.10 mM Tris–HCl (pH 8.8), 0.10 mM $MgCl_2$, 0.08 mM EDTA, proteinase inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), aprotinin (0.5 mg/ml), and phenylmethanesulfonyl fluoride (40 μg/ml) and phosphatase inhibitor cocktail. Because the phosphorylation of serine residues by specific kinases (e.g., Akt/PKB) is involved in HO-1 activity [28], kinase inhibitors could interfere with such activity. For this reason, kinase inhibitors were not included in Media 1 lysis buffer. Homogenates were centrifuged at 14,000*g* for 10 min to remove debris. Protein concentration in the supernatant was determined by the Pierce BCA method (Pierce, Rockford, IL, USA).

Western blot analysis

For the evaluation of HO-1 and HO-2 protein levels, 50 μg of total protein of brain homogenate was denatured in sample buffer for 5 min at 100 °C and then separated on 12% precast Criterion gels (Bio-Rad) by electrophoresis at 100 mA for 2 h in Mops buffer (Bio-Rad) in a Bio-Rad apparatus. For the evaluation of HO-1 posttranslational modifications, 150 μg of total protein of brain homogenate was used, as described below. The proteins from the gels were then transferred to a nitrocellulose membrane using the Trans-Blot semi-dry transfer cell at 20 mA for 2 h. Subsequently, the membranes were blocked at 4 °C for 1 h with fresh blocking buffer made of 3% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST). The membranes were incubated at room temperature in PBST for 2 h with the following primary antibodies, as separate experiments: anti-HO-1 (Stressgen, Ann Arbor, MI, USA, dilution 1:1000), anti-HO-2 (Stressgen, dilution 1:1000), anti-phosphoserine (Zymed, Invitrogen, Camarillo, CA, USA, dilution 1:250), anti-phosphothreonine (Zymed, dilution 1:250), anti-phosphotyrosine (Zymed, dilution 1:1000), anti-nitrotyrosine (3-NT; Sigma-Aldrich, dilution 1:100), anti-dinitrophenylhydrazone (DNP) protein adducts (Millipore, Billerica, MA, USA, dilution 1:100), anti-4-hydroxy-2-nonenal (HNE; Alpha Diagnostic International, San Antonio, TX, USA, dilution 1:100), and anti-β-actin (Sigma-Aldrich, dilution 1:2000). The membranes were then washed three times for 5 min with PBST followed by incubation with anti-mouse alkaline phosphatase or horseradish peroxidase-conjugated secondary antibody (1:3000) in PBST for 2 h at room temperature. Membranes were then washed three times in PBST for 5 min and developed using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium color-developing reagent for alkaline phosphatase secondary antibody or the ECL Plus WB detection reagents for horseradish peroxidase-conjugated secondary antibody. Blots were dried and scanned in TIF format using Adobe Photoshop on a Canoscan 8800F (Canon) or STORM UV transilluminator (λex 470 nm, λem 618 nm; Molecular Dynamics, Sunnyvale, CA, USA) for chemiluminescence. The images were quantified with ImageQuant TL 1D version 7.0 software (GE Healthcare). The optical density of the bands was calculated as volume (optical density × area) adjusted for the background.

Immunoprecipitation

The immunoprecipitation procedure was performed as previously described [37] with modifications. Briefly, 150 μg of protein extracts was dissolved in 500 μl of RIPA buffer (10 mM Tris, pH 7.6; 140 mM NaCl; 0.5% NP-40 including protease inhibitors) and then incubated with 1 μg of anti-HO-1 antibody at 4 °C overnight. Immunocomplexes were collected by using protein A/G suspension for 2 h at 4 °C and washed five times with immunoprecipitation buffer. Immunoprecipitated HO-1 was recovered by resuspending the pellets in reducing SDS buffers and subjected to electrophoresis on 12% gels followed by Western blot analysis. The membranes were then stripped and reprobed using an anti-HO-1 antibody as described above. Total HO-1 was used as loading control according to [38,39].

Postderivatization of protein

Samples were postderivatized with dinitrophenylhydrazine (DNPH) on the membrane and probed with anti-DNP antibody to identify the carbonylated proteins. The nitrocellulose membranes were equilibrated in solution A (20% (v/v) methanol and 80% (v/v) wash blot buffer (PBS solution containing 0.04% (v/v) Tween 20 and 0.10 M NaCl)) for 5 min, followed by incubation of membranes in 2 N HCl for 5 min. The proteins on blots were then derivatized in solution B (0.5 mM DNPH in 2 N HCl) for 10 min as described in [40]. The membranes were successively washed, 5 min each time, in 2 N HCl three times, wash blot buffer/methanol (50/50) five times, and finally wash blot buffer two times. The DNP adducts were detected immunochemically as described above.

Statistical analysis

All statistical analysis was performed using a two-tailed Student *t* test. $P < 0.05$ was considered significantly different from control.

Results

HO-1 and HO-2 protein levels in hippocampus and cerebellum of subjects with AD or MCI

In 1995 Schipper and colleagues observed intense immunoreactivity of HO-1 in neurons of the hippocampus and temporal cortex of AD brain relative to age-matched control specimens [41]. In addition, we previously observed an increased expression of HO-1 together with a decreased expression of HO-2 in the inferior parietal lobule of AD brains, a region that showed elevated oxidative and nitrosative stress [42].

Before proceeding with the analysis of posttranslational modifications, we first analyzed HO-1 and HO-2 protein levels in our samples. In the hippocampus of AD subjects, HO-1 protein levels were significantly increased by 119% with respect to age-matched controls (Fig. 1a). Conversely, HO-2 protein levels were significantly decreased by 35% in the same brain area (Fig. 1b). With respect to MCI, a previous study from our group performed by

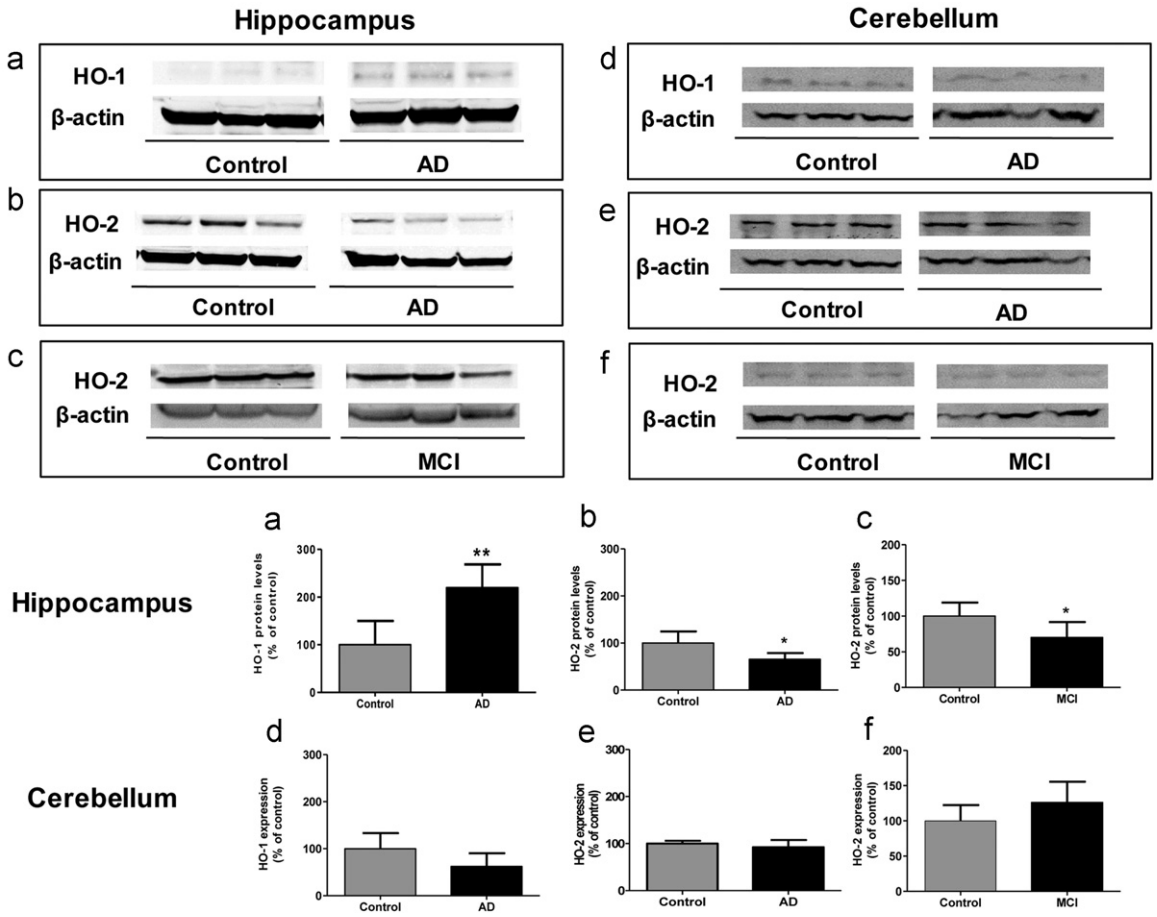

Fig. 1. Heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2) protein levels in the hippocampus and cerebellum of subjects with Alzheimer disease (AD) and mild cognitive impairment (MCI). HO-1 protein levels in the hippocampus of (a) AD subjects; HO-2 protein levels in the hippocampus of (b) AD and (c) MCI subjects; HO-1 protein levels in the cerebellum of (d) AD subjects; HO-2 protein levels in the cerebellum of (e) AD and (f) MCI subjects. Representative gels are shown. Data are expressed as means ± SD ($n=6$ individual samples per group). $^{*}P<0.05$ and $^{**}P<0.01$ versus control.

using the same set of samples showed that HO-1 protein levels, in both hippocampus and cerebellum, were not significantly different from those observed in age-matched controls [43]. Here we extended the analysis to HO-2 protein levels, which showed a significant decrease by about 30% in the hippocampus of subjects with MCI (Fig. 1c). In cerebellum, HO-1 protein levels were decreased (~38%) in AD subjects with respect to the matched controls (Fig. 1d), although this value did not reach statistical significance. No significant differences were found for HO-2 in the same brain area (Fig. 1e). Finally, no changes were observed in HO-2 protein levels in the cerebellum of MCI subjects with respect to the matched controls (Fig. 1f).

HO-1 phosphorylation modifications in the hippocampus and cerebellum of subjects with AD or MCI

In 2004 Salinas et al. demonstrated that human HO-1 can be phosphorylated at specific serine residues and such phosphorylation regulates the interaction with BVR as well as its own activity [28]. In contrast HO-2 has a nonphosphorylatable arginine instead of Ser [28]. For this reason, and because of the significant decrease in protein levels observed in AD and MCI hippocampi, HO-2 was not taken into account for the analysis of posttranslational modifications. To clarify if HO-1 undergoes phosphorylation modifications in the hippocampus and cerebellum of subjects with AD or MCI, we analyzed the levels of phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) of HO-1. As shown in Fig. 2a, pSer-HO-1 is significantly increased in the hippocampus of subjects with AD, whereas no changes were observed for pThr-HO-1 (Fig. 2b). Interestingly, no signal was obtained for pTyr-HO-1 (Fig. 2c), probably because of the lower number of Tyr residues in HO-1 with respect to Ser and Thr residues [44] or a lower involvement of Tyr phosphorylation. In the hippocampus of subjects with MCI no significant differences were observed for both pSer-HO-1 (Fig. 2d) and pThr-HO-1 (Fig. 2e) with respect to the matched controls. As for AD, no signal was obtained with regard to the analysis of pTyr-HO-1 in the hippocampus of subjects with MCI (data not shown). Finally, to evaluate whether changes observed for pSer-HO-1 levels were specific for hippocampal tissue, the same experiments were performed in the cerebellum. pSer-HO-1 is significantly increased by 21% (Fig. 3a) or 24% (Fig. 3b) in the cerebellum of subjects with AD or MCI, respectively.

Protein carbonyls (PC), protein-bound HNE, and 3-NT levels on HO-1 in the hippocampus and cerebellum of subjects with AD or MCI

To evaluate if HO-1 is a target for oxidative and nitrosative stress-induced posttranslational modifications, the levels of PC, protein-bound HNE, and 3-NT on HO-1 were evaluated in the hippocampus and cerebellum of subjects with AD or MCI. PC (Fig. 4a) and protein-bound HNE (Fig. 4b) on HO-1 were significantly increased by 30% and 52%, respectively, whereas no signal detection was observed for 3-NT (Fig. 4c) in the hippocampus of subjects with

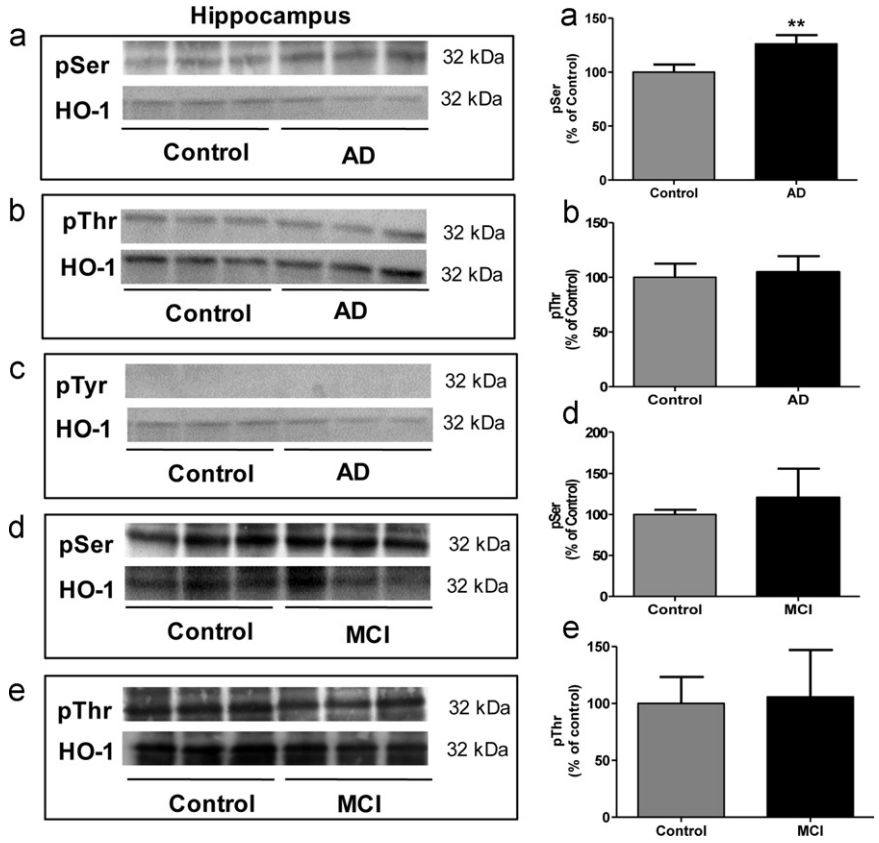

Fig. 2. HO-1 phosphorylation on serine (pSer), threonine (pThr), and tyrosine (pTyr) residues in the hippocampus of subjects with AD and MCI. (a) pSer, (b) pThr, and (c) pTyr levels on HO-1 in the hippocampus of AD subjects. (d) pSer and (e) pThr levels on HO-1 in the hippocampus of MCI subjects. Representative gels are shown. Data are expressed as means ± SD ($n=6$ individual samples per group). $^{**}P<0.01$ versus control.

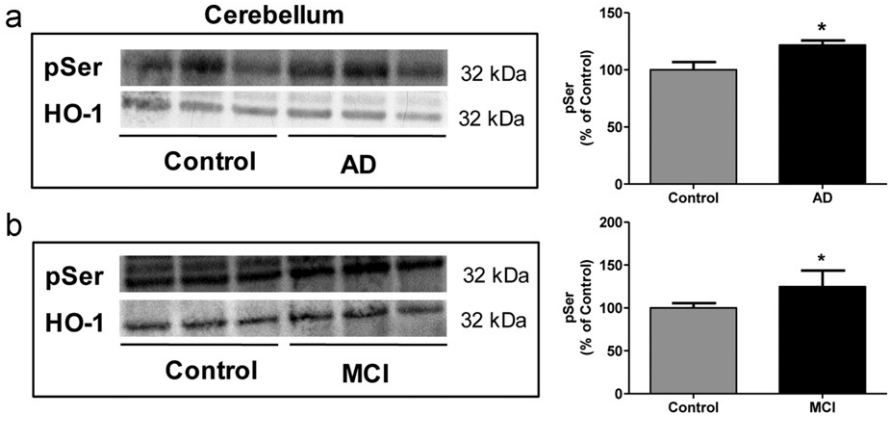

Fig. 3. HO-1 phosphorylation on serine (pSer) residues in the cerebellum of subjects with AD and MCI. pSer levels on HO-1 in the cerebellum of (a) AD and (b) MCI subjects. Representative gels are shown. Data are expressed as means ± SD ($n=6$ individual samples per group). $^{*}P<0.05$ versus control.

AD compared to age-matched controls. Together with the absence of pTyr-HO-1 signal detection, this last evidence probably results from the smaller contribution of Tyr residues in HO-1 posttranslational modifications. Interestingly, in the hippocampus of subjects with MCI no changes were found for PC (Fig. 4d), whereas a significant increase of 85% was observed in the levels of HNE-bound HO-1 (Fig. 4e). The analysis of cerebellar samples revealed no changes in the levels of PC in HO-1 on AD subjects with respect to the matched controls (Fig. 5a), whereas a significant increase of 36% in HNE-bound HO-1 (Fig. 5b) was observed. In contrast, in the cerebellum from subjects with MCI, we observed significantly increased levels of PC (about 32%) in HO-1 (Fig. 5c), whereas no changes were demonstrated for levels of HNE-bound HO-1 (Fig. 5d).

Discussion

Since it was discovered, the HO-1/BVR-A system has been considered a useful mechanism through which cells respond to oxidative/nitrosative stress insults, to prevent the impairment of cellular homeostasis. However, the effective contribution of HO-1/BVR-A system induction to cellular antioxidant defense is

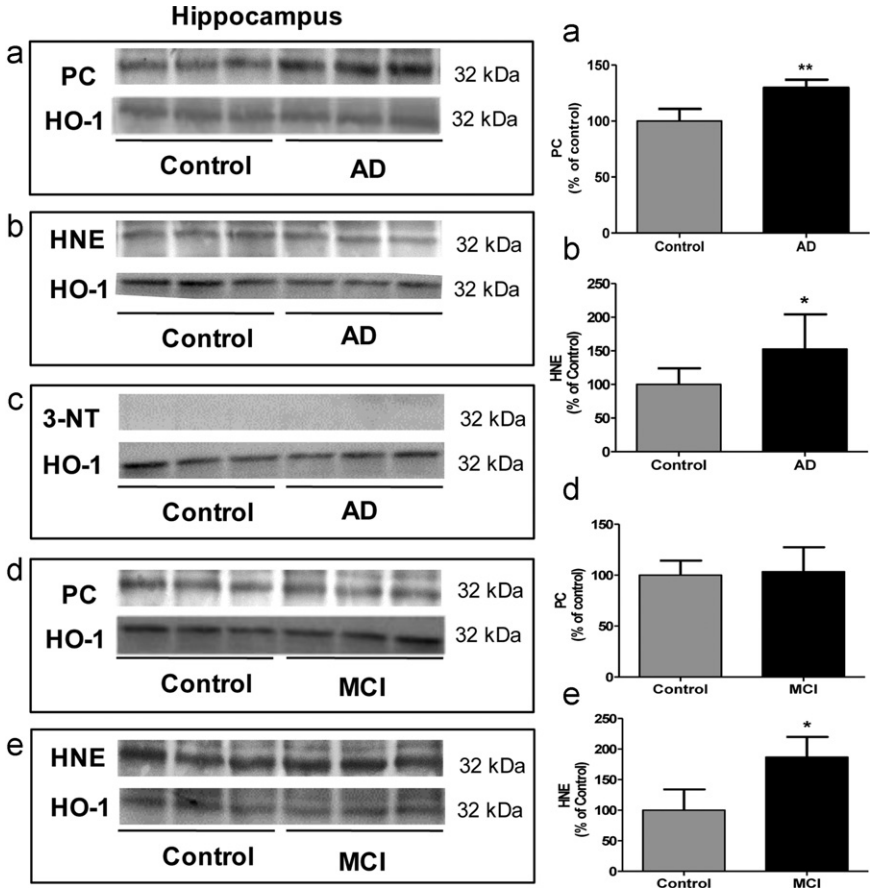

Fig. 4. HO-1 oxidative and nitrosative posttranslational modifications in the hippocampus of subjects with AD and MCI. (a) Protein carbonyls (PC), (b) 4-hydroxy-2-nonenal (HNE), and (c) 3-nitrotyrosine (3-NT) levels on HO-1 in the hippocampus of AD subjects. (d) PC and (e) HNE levels on HO-1 in the hippocampus of MCI subjects. Representative gels are shown. Data are expressed as means ± SD ($n=6$ individual samples per group). $^{*}P<0.05$ and $^{**}P<0.01$ versus control.

currently under debate because a growing amount of evidence has questioned its protective role in neurodegenerative disorders.

In this paper our primary goal was to determine if evidence existed regarding HO-1 posttranslational modifications in the brain of AD and MCI subjects. We chose to investigate the hippocampus and cerebellum brain areas for this study because of their differential involvement in free radical-induced injury and pathology. Indeed, the hippocampus is broadly recognized as a main target of neurodegenerative damage during AD progression, presenting increased levels of oxidative stress, neuronal loss, and marked atrophy with respect to the whole brain. Conversely, the cerebellum is largely devoid of pathology and oxidative stress [45–47].

The first novel result provided by this study is the different profiles of HO-1 and HO-2 in the brain of subjects with AD or MCI. Previous data from Premkumar and colleagues have already shown different patterns of expression for HO-1 and HO-2 among neocortex, cerebellum, and cerebral vessel from AD subjects [48]. Here we extend the neurobiological behavior of HO-1 and HO-2 in the brain of AD and MCI subjects to include the following: (i) increase in HO-1 protein levels in another well-known brain area involved in AD pathology such as hippocampus, (ii) decrease in HO-2 protein levels in the same brain area, and (iii) no observed changes in HO-2 protein levels in cerebellum of MCI subjects. The increase in HO-1 in the hippocampus of AD subjects (Fig. 1a) is in good agreement with previous results [41,48] and could be easily explained by the elevated levels of oxidative stress observed in this brain area [21], whereas the decrease in HO-2 protein levels (Fig. 1b and c) is less obvious and represents an intriguing finding. Very few lines of evidence exist about the possibility of modulating HO-2 expression [24,49,50]. In the present case we speculate that one possible explanation could come from the link between HO-2 and glucocorticoids. As previously demonstrated, the HO-2 gene possesses in its promoter region a consensus sequence for the glucocorticoid response element, which plays a main role in glucocorticoid-induced changes in HO-2 protein levels [51]. In 1994, Weber and colleagues demonstrated that corticosterone treatment decreased HO-2 protein levels in the hippocampus [49]. Furthermore, more recently, Chen et al. demonstrated that chronic restraint stress decreased HO-2 protein levels in hippocampal neurons and this stress-induced decrease in HO-2 protein levels may result from high corticosterone levels [50]. In addition, an impairment of the hypothalamic–pituitary–adrenal axis, which triggers the adrenal cortex to release glucocorticoids (cortisol in primates, corticosterone in mice and rats), was associated with AD and MCI pathogenesis [52–54]. In fact, glucocorticoids not only have been suggested to contribute to age-related loss of neurons in the hippocampus of rats [55], but also were shown to potentiate hippocampal damage induced by various noxious insults [56], including Aβ peptide [57]. Thus, a conceivable justification for the significant decrease in HO-2 protein levels observed in the hippocampus of both AD and MCI subjects could be represented by corticosterone-induced decrease in the protein in this brain area [49].

With regard to HO-1, an interesting aspect of our work is the observed significant increase in Ser-residue phosphorylation along with oxidative posttranslational modifications in the hippocampus of AD subjects (Figs. 2a, 4b and c). Because HO-1 is a stress-inducible protein, and phosphorylation on Ser residues seems to be important for its activation [28], the increase in oxidative stress levels in the hippocampus of AD subjects could

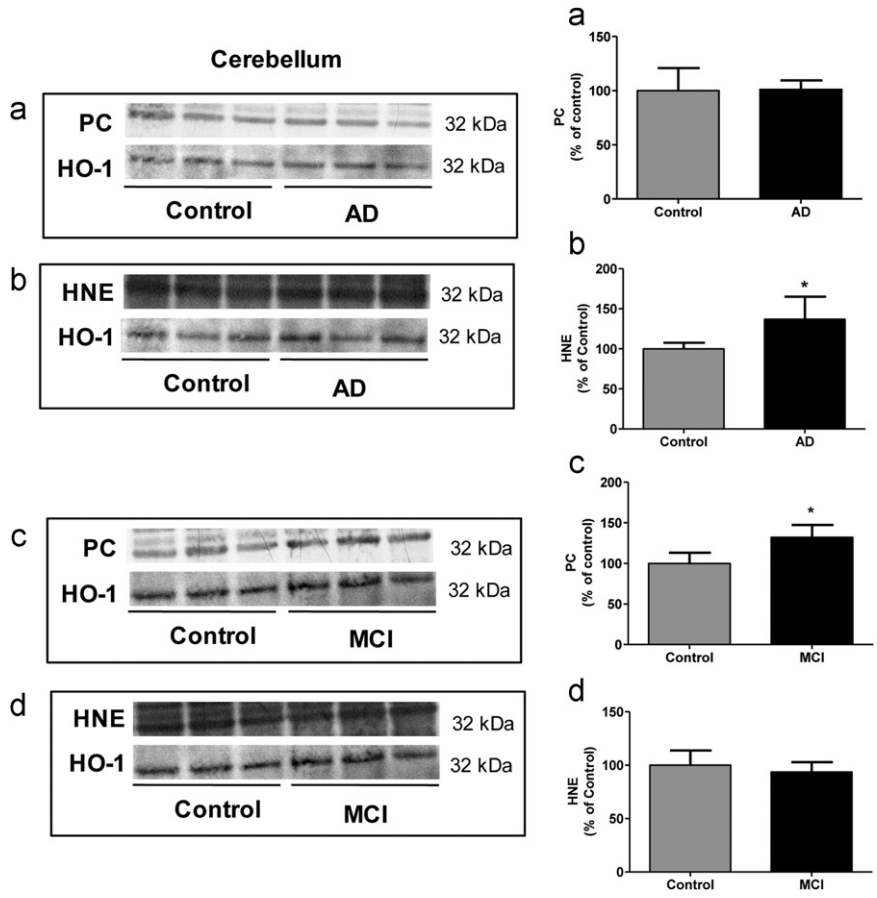

Fig. 5. HO-1 oxidative and nitrosative posttranslational modifications in the cerebellum of subjects with AD and MCI. Protein carbonyls (a) PC and (b) HNE levels on HO-1 in the cerebellum of AD subjects. (c) PC and (d) HNE levels on HO-1 in the cerebellum of MCI subjects. Representative gels are shown. Data are expressed as means ± SD ($n=6$ individual samples per group). *$P < 0.05$ versus control.

lead to an increase in HO-1 protein levels and phosphorylation to promote its activity and its interaction with BVR [28]. At the same time, the increased oxidative stress could be responsible for the observed rise in PC and HNE adducts, as was demonstrated for other proteins in AD [16], including BVR-A [32,33], leading to altered protein structure and function impairment [11,58–60]. Based on our experimental model, it is difficult to state which posttranslational modification precedes the other between phosphorylation and oxidative modification, and at least two interpretations could be conceivable: (1) oxidative stress promotes the increase in HO-1 oxidative damage (e.g., increased PC and HNE adducts on its structure). Consequently, the cell tries to restore the functionality of the protein by increasing Ser-residue phosphorylation. (2) Oxidative stress promotes the increase in Ser-residue phosphorylation to activate protein functions, but HO-1 quickly becomes a target for oxidative posttranslational modifications, which in turn could impair its function (Fig. 6). The finding of the presence of oxidative (HNE) and phosphorylative (pSer) modifications on HO-1 brings to mind the past results by Takeda et al. on tau conformational changes in the hippocampus of AD subjects [61]. In particular, these authors showed that the increases in both HNE production and tau phosphorylation are two events needed for the promotion of tau conformational changes, which in turn are associated with the induction of HO-1 in the same neurons [61]. These data, together with those from Hui et al. showing that overexpression of HO-1 promotes tau phosphorylation and aggregation [34], provide new insights for understanding the mechanisms involved in tau pathology. In our opinion, they refer to a tight link between HO-1 and tau, in which, as in a vicious circle, HO-1 could be induced in response to tau

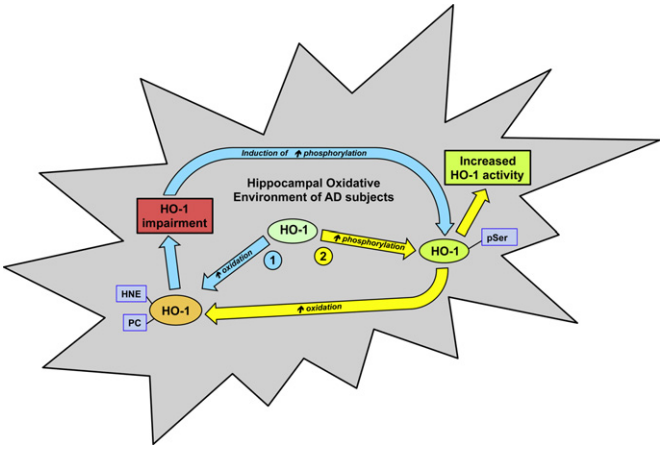

Fig. 6. Putative scenario of the mechanisms leading to HO-1 posttranslational modifications due to increased oxidative stress environment in AD hippocampus. (1) Blue arrows: the increase in oxidative stress levels observed in the hippocampus of AD subjects promotes the increase in HO-1 oxidative damage (e.g., increase in PC and HNE adducts on its structure). Consequently, the cell tries to restore the functionality of HO-1 by increasing Ser-residue phosphorylation. (2) Yellow arrows: the increase in oxidative stress promotes the increase in Ser phosphorylation to activate protein functions, then HO-1 quickly becomes a target of oxidative posttranslational modifications, which in turn could impair its function. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phosphorylation and oxidative stress to protect the neurons [61], but at the same time, if overexpressed, it could trigger tau aggregation and toxicity [34]. However, in the above-cited reports, the only

parameter taken into account was the expression level of HO-1; thus it is not completely clear if the mechanisms proposed are linked to a specific signal-transduction pathway mediated by HO-1. It should be very interesting in the future to evaluate if, based on our results, the posttranslational modifications on HO-1 observed in AD and MCI brain could affect tau pathology positively or negatively.

With regard to MCI, the results from hippocampus add new elements to the comprehension of the contribution of the HO-1/BVR-A system to AD pathogenesis. Unlike BVR-A, whose expression levels were significantly increased even in the hippocampus of subjects with MCI [33], HO-1 protein levels did not present any differences [43]. This result could mean that the induction of each member of the HO-1/BVR-A system is not concomitant with the other, and probably the threshold levels of oxidative/nitrosative stress needed to induce HO-1 and BVR-A are different. Because of the pleiotropic functions of BVR-A in the maintenance of cellular homeostasis [29], it is plausible that the induction of BVR-A precedes that of HO-1. In contrast, the formation of HNE adducts on HO-1 (Fig. 4e), along with BVR-A nitration [32], is already evident in the hippocampus of subjects with MCI. In this light, despite the progressive increase in HO-1/BVR-A protein levels observed from MCI to AD [62], the impairment of the system probably is an early event in the pathogenesis and progression of the disease. A different behavior is shown for protein phosphorylation. We did not observe any significant difference in pSer-HO-1 in the hippocampus of MCI subjects with respect to age-matched controls (Fig. 2d), suggesting that the increase in the phosphorylation process occurs at a later stage of the disease.

We can speculate that increased Ser-residue phosphorylation along with increased protein levels could act as a compensatory mechanism to overcome the inactivation of HO-1 by oxidative damage. However, whether HO-1 functionality is in part restored after Ser-residue phosphorylation remains an unsolved question. To complete this intricate puzzle, the measure of HO-1 activity should be considered. However, it is not possible to single out the differential contributions of HO-1 and HO-2 to the generation of their products (i.e., CO, ferrous iron, and biliverdin) because of a lack of reliable selective inhibitors [27].

Another problem involves the possible repercussions of HO-1 upregulation on cell metabolism. As noted earlier, with respect to metabolism, HO activity is something of a double-edged sword [63,64]. The reduced availability of heme after HO-catalyzed degradation may provide useful antioxidant effects, but prolonged HO activation could produce low enough intracellular heme levels to be incapable of meeting the cell's metabolic requirements [63,64]. In fact, heme is the prosthetic group of hemoglobin, myoglobin, cytochromes, and several other important proteins, and excessively low concentrations can impair mitochondrial function and cell respiration. Furthermore, both CO and iron, which have physiological effects when produced under basal conditions, may become toxic if produced in excess [65–67].

In the scenario described above, the following considerations are in good agreement with the concerns about the neuroprotective or neurotoxic role of HO-1 in AD: (i) the failure to protect neurons against the deleterious effects of oxidative/nitrosative stress could be due to an impairment of HO-1, together with BVR-A, as suggested by our group [32,33], and (ii) phosphorylation is able to restore HO-1 functionality and as a consequence the sustained activation of HO-1 could be responsible, at least in part, for the observed increased oxidative stress, as well as tau phosphorylation, in the hippocampus of AD subjects, as suggested by other groups [34,35]. Thus, we suggest that the neuroprotective effects mediated by the HO/BVR-A system can be obtained only if the fine balance between the activity of HO-1 or HO-2 and that of BVR-A is maintained.

Another novel finding of this study regards the cerebellum, which has long been considered an internal control because of minimal neuropathology [46]. Our results show a significant increase in HO-1 oxidation in the cerebellum of both MCI and AD subjects (Fig. 5b and c), although the elevations in HO-1 oxidation levels in a brain region with limited neuropathology may seem contradictory. However, recent studies suggest that disease-related changes could occur even in this brain area [68–70]. Furthermore, an increase in neurotoxic markers of lipid peroxidation (HNE or acrolein) and iron dyshomeostasis were found at an early stage of the disease, such as MCI or preclinical AD [46,70]. Thus, HO-1 could represent a likely target of oxidative damage even in the cerebellum. Finally, the observed increase in Ser-residue phosphorylation (Fig. 3a and b) along with oxidative modification in the cerebellum of MCI and AD subjects is in agreement with the explanation given for the hippocampus.

Conclusion

In conclusion, our previous studies coupled with the current investigation show that the HO-1/BVR-A system is impaired in AD and MCI brain. In our opinion, it is no longer correct to measure only total HO-1 or BVR-A protein levels as an index to evaluate the involvement of these enzymes in the cell stress response because posttranslational modifications appear to play a main role in the regulation of the neuroprotective and/or metabolic activities of these proteins. The significance of these lines of evidence is profound, and ad hoc research to clarify the mechanisms involved in the regulation of HO-1/BVR-A system in AD and MCI is ongoing in our laboratory.

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