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Oxidative and Nitrosative Modifications of Biliverdin Reductase-A in the Brain of Subjects with Alzheimer's Disease and Amnestic Mild Cognitive Impairment

Eugenio Barone[a,b,#], Fabio Di Domenico[a,c,#], Giovanna Cenini[a], Rukhsana Sultana[a], Raffaella Coccia[c], Paolo Preziosi[b], Marzia Perluigi[c], Cesare Mancuso[b] and D. Allan Butterfield [a,*]
[a]*Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY, USA*
[b]*Institute of Pharmacology, Catholic University School of Medicine, Roma, Italy*
[c]*Department of Biochemical Sciences, Sapienza University of Rome, Rome, Italy*

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Abstract. Biliverdin reductase-A (BVR-A) is a pleiotropic enzyme and plays pivotal role in the antioxidant defense against free radicals as well as in cell homeostasis. Together with heme oxygenase, BVR-A forms a powerful system involved in the cell stress response during neurodegenerative disorders including Alzheimer's disease (AD), whereas due to the serine/threonine/tyrosine kinase activity the enzyme regulates glucose metabolism and cell proliferation. In this paper, we report results that demonstrate BVR-A undergoes post-translational oxidative and nitrosative modifications in the hippocampus, but not cerebellum, of subjects with AD and amnestic mild cognitive impairment (MCI). A significant increase of nitrated BVR-A was demonstrated only in AD and MCI hippocampi, whereas no significant modifications were found in cerebellar tissue. In addition, a significant reduction in protein carbonyl-derivatives of BVR-A was found in both AD and MCI hippocampi (15% and 18%, respectively). Biliverdin reductase-bound 4-hydroxynonenals were not modified in hippocampi and cerebella from AD and MCI subjects. These results supported the hypothesis of a prevalence of nitrosative stress-induced modifications on BVR-A structure, and this evidence was confirmed by a significant upregulation of inducible nitric oxide synthase in hippocampal tissue of subjects with AD and MCI that was not present in cerebellum. In conclusion, nitrosative stress-induced modifications on hippocampal BVR-A are an early event in the pathogenesis of AD since they appear also in MCI subjects and could contribute to the antioxidant and metabolic derangement characteristic of these neurodegenerative disorders.

Keywords: Alzheimer's disease, biliverdin reductase, heme oxygenase, mild cognitive impairment, neurodegenerative disorders, oxidative stress

INTRODUCTION

Heme oxygenase (HO) catalyzes the oxidation of the alpha-meso-carbon bridge of heme moieties, resulting in equimolar amount of ferrous iron, carbon monoxide, and biliverdin-IX-alpha (thereafter BV). This latter is further reduced by the cytosolic enzyme biliverdin reductase (BVR) into bilirubin (BR), the

# Both the authors contributed equally.
*Correspondence to: Prof. D. Allan Butterfield, Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506-0055, USA. Tel.: 859-257-3184, Fax: 859-259-5876; E-mail: dabcns@uky.edu.

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final product of heme catabolism [1]. Heme oxygenase exists in two main isoforms, HO-1 and HO-2. Heme oxygenase-1 (HO-1), also known as heat shock protein (Hsp)-32, is induced by various stimuli, including reactive oxygen and nitrogen species (ROS and RNS, respectively), ischemia, heat shock, bacterial lipopolysaccharide (LPS), hemin, and the neuroprotective agent leteprinim potassium (Neotrofin) [2–4], whereas HO-2, the constitutive isoform, is responsive to developmental factors and adrenal glucocorticoids [2, 4]. The central nervous system (CNS) is characterized by measurable HO activity under basal conditions. Most can be attributed to HO-2, which is expressed in neuronal populations in almost all brain areas [2], whereas the inducible isoform is present at low levels in scattered groups of neurons, including the ventromedial and paraventricular nuclei of the hypothalamus [2, 4]. HO-1 is also found in glial cells where its expression can be induced by oxidative stress [5]. Similarly, two isoforms of BVR were described and named BVR-A and BVR-B [6–8]. Both these enzymes generate bilirubin (BR), but only BVR-A reduces BV into the powerful antioxidant molecule BR-IX-alpha (thereafter BR), whereas BVR-B prefers the other BV isoforms, such as BV-β, BV-γ, and BV-δ [6–8]. Biliverdin reductase is co-expressed with HO-1 and/or HO-2 in cells of the rat brain that express these enzymes under normal conditions. It is also found in regions and cell types that can express heat shock-inducible HO-1 [9].

The up-regulation of the HO/BVR-A system is considered an antioxidant response because: (i) the intracellular levels of pro-oxidant heme are reduced [4, 10], (ii) CO, the gaseous compound formed during the two-step reaction, has antinflammatory and antiapoptotic effects in some tissues [10–18], and (iii) bilirubin is an efficient scavenger of ROS, RNS and nitric oxide [19–26]. Indeed, the sustained activation of the HO-1 could result in a cytotoxic response since it could originate a significant increase in both carbon monoxide, which becomes toxic if produced in excess due to its binding to hemoprotein, and ferrous iron deposition which triggers lipid and protein oxidation through the Fenton reaction or by altering the ferrous/ferric iron balance [27, 28] In support of this dual effect of HO-1 on neurodegenerative disorders, Schipper and colleagues, first demonstrated co-localization of HO-1 to senile plaques in Alzheimer's disease (AD) brain [29] as well as the upregulation of HO-1 protein levels in amnestic mild cognitive impairment (MCI) and AD astrocytes [30], whereas other studies lend support to the hypothesis that HO-1/BVR-A overexpression is a useful mechanism for the brain tissue to counteract the oxidative/nitrosative damage that is a common feature of neurodegenerative disorders, including AD [31–36].

The activation of the HO/BVR-A system was then demonstrated in the inferior parietal lobule, plasma and lymphocytes of AD patients [33]. In addition, Kimpara and colleagues showed that AD subjects have an increased concentration of BR in cerebrospinal fluid with respect to control [37]. Although these lines of evidence demonstrate the activation of the HO/BVR-A axis in AD, its pathophysiological and clinical significance is still under debate. An intriguing aspect is to understand whether or not the pro-oxidant conditions in AD could modify the structure of members of the HO-1/BVR-A system thus affecting the antioxidant potential. Early studies by Maines and coworkers demonstrated that post-translational modification in the BVR-A structure, such as the autophosphorylation of selected serine-residues, regulates its own reductase activity [6, 38–40]. Very recently, our group reported on changes in BVR activity secondary to differential phosphorylation on serine/threonine/tyrosine residues in brains of subjects with AD or MCI [41]. These findings prompted us to explore the possibility that oxidative and nitrosative modifications could alter BVR-A function in AD. This hypothesis is further corroborated by several reports which demonstrated that many proteins involved in the regulation of important cellular functions became dysfunctional in AD brain due to oxidative and nitrosative modifications [42–50].

The aim of this study was to investigate the post-translational oxidative and nitrosative modifications of BVR-A in both hippocampal and cerebellum samples of subjects affected by AD or arguably its earliest form, MCI, the latter being the transitional phase between normal aging and early AD [51]. MCI shares with AD both pathological features, such as Aβ accumulation in the neocortex and numerous neurofibrillary tangles in the medial temporal lobe [51, 52], which generate pro-oxidant status, and clinical aspects of both disorders including memory loss. As indices of oxidative modifications of BVR-A both protein carbonyls and protein-bound 4-hydroxynonenal (HNE) were assayed whereas 3-nitrotyrosine (3-NT) was evaluated as a marker of nitrosative stress.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Nitrocellulose membranes and electrophoresis transfer system Trans-blot semi-dry Transfer Cell were

obtained from Bio-Rad (Hercules, CA, USA). Anti-mouse and anti-rabbit IgG horseradish peroxidase conjugate secondary antibody and ECL plus Western blot detection reagents were obtained from GE Healthcare Bio-Sciences corp. (Piscataway, NJ, USA).

Subjects

Frozen hippocampal and cerebellar samples from well-characterized subjects with AD, MCI and respective age-matched controls (n = 6 for each group, see also Table 1) 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 subjects and relative 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 semi-annual physical and neurological exams as part of the UK ADC normal volunteer 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 suffering from MCI met the criteria described by Petersen [51], which include: 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 to 0.5, no dementia, and a clinical evaluation that revealed no other cause for memory decline. AD patients diagnosis was made according to criteria developed by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's disease and Related Disorders Association (ADRDA) [53]. All AD patients displayed progressive intellectual decline. The Braak scores were within the range 3–5 and 5–6 for MCI and AD patients, respectively.

Table 1
Demographic information of AD and amnestic mild cognitive impairment (MCI) subjects and their respective age-matched controls. The values reported are the average of 6 samples

A) Subjects demographics data	Control (MCI)	MCI
Number of subjects	6	6
Gender	2 M, 4F	2 M, 4F
Age (years)	82 (74–93)	89 (82–99)
Brain weight (g)	1204 (1080–1315)	1102 (930–1200)
PMI (hours)	1.75–4.00	2.00-5.00
Braak stage	I-II	III-V
B) Subjects demographics data	**Control (AD)**	**AD**
Number of subjects	6	6
Gender	5 M, 1F	2 M, 4F
Age (years)	81 (72–87)	85 (80–92)
Brain weight (g)	1219 (1020–1410)	1104 (835–1260)
PMI (hours)	2.00–3.75	2.00–5.75
Braak stage	I–III	V–VI

Sample preparation

Brain tissues samples (hippocampus and cerebellum) from control, MCI and AD subjects were sonicated in Media 1 lysis buffer (pH 7.4) containing 320 mM Sucrose, 1% of 990 mM Tris-HCl (pH = 8.8), 0.098 mM $MgCl_2$, 0.076 mM EDTA, the proteinase inhibitors leupeptin (0.5 mg/mL), pepstatin (0.7 μg/mL), aprotinin (0.5 mg/mL), and PMSF (40 μg/mL) and phosphatase inhibitor cocktail (Sigma-Aldrich). Homogenates were centrifuged at 14,000 $\times g$ for 10 min to remove debris. Protein concentration in the supernatant was determined by the Pierce BCA method (Pierce, Rockford, IL, USA).

Immunoprecipitation

The immunoprecipitation procedure was performed as previously described [50] with modifications. Briefly, 150 μg of protein extracts were dissolved in 500 μl of RIPA buffer (10 mM Tris, pH 7.6; 140 mM NaCl; 0.5% NP40 including protease inhibitors) and then incubated with 1 μg of anti-BVR-A antibody at 4°C overnight. Immunocomplexes were collected by using protein A/G beads suspension for 2 h at 4°C and washed five times with immunoprecipitation buffer. Immunoprecipitated biliverdin reductase was recovered by resuspending the pellets in reducing SDS buffers and subjected to electrophoresis on 12% gels followed by Western blot analysis to identify BVR-A's protein carbonyls, HNE or 3-NT adducts (see below). In these experiments, total BVR-A was used as loading control [38, 40] and it was detected on the same membranes after a stripping/reprobing procedure by using a specific anti-BVR-A antibody (see below).

Western blot analysis

For Western blot analyses, 50 μg of protein were denatured in sample buffer for 5 min at 100°C, and proteins separated on 12% precast Criterion gels (Bio-Rad) by electrophoresis at 100 mA for 2 h in

MOPS buffer (Bio-Rad) into the Bio-Rad apparatus. The proteins from the gels were then transferred to nitrocellulose membrane using the Transblot-Blot SD 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 (BSA) 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: polyclonal anti-rabbit BVR-A (Sigma-Aldrich, dilution 1:1000) which recognizes a single band at 35 KDa; polyclonal anti-rabbit nitrotyrosine antibody (3-NT); (Sigma-Aldrich dilution 1:100 in wash blot); polyclonal anti-rabbit DNP protein adducts antibody (Millipore, Billerica, MA, USA, dilution 1:100); polyclonal anti-rabbit HNE antibody (Alpha Diagnostic International, San Antonio, TX, USA, dilution 1:100); polyclonal anti-rabbit iNOS antibody (LifeSpan BioSciences, Seattle, WA, USA, dilution 1:500), and polyclonal anti-rabbit β-actin (Sigma-Aldrich, dilution 1:2000).

The respective membranes were then washed three times for 5 min with PBST followed by incubation with anti-mouse alkaline phosphatase or horseradish peroxidase conjugate 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 or 5-bromo-4-chloro-3- indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) color developing reagent for alkaline phosphatase secondary antibody or ECL plus WB detection reagents for horseradish peroxidase conjugate secondary antibody. Blots were dried, scanned in TIF format using Adobe Photoshop on a Canoscan 8800F (Canon) or STORM UV transilluminator ($\lambda ex = 470$ nm, $\lambda em = 618$ nm, Molecular Dynamics, Sunnyvale, CA, USA) for chemiluminescence. The images were quantified with Image Quant TL 1D version 7.0 software (GE Healthcare). The optical density of bands was calculated as volume (optical density × area) adjusted for the background.

Post-derivatization of protein

Samples were post-derivatized with DNPH on the membrane and probed with anti-DNPH antibody to identify the carbonylated proteins. The nitrocellulose membranes where equilibrated in solution A (20% (v/v) methanol: 80% (v/v) wash blot buffer) for 5 min, followed by incubation of membranes in 2N HCl for 5 min. The proteins on blots were then derivatized in solution B (0.5 mM DNPH in 2N HCl) for exactly 10 min as described by Conrad et al. [54]. The membranes were washed three times in 2N HCl for 5 min each and then five times with 50% methanol and two times with wash blot each for 5 min. The 2,4-dinitrophenylhydrazone (DNP) adducts of the carbonyls of the brain proteins were detected immunochemically as described above.

Statistical analysis

All statistical analysis was performed using a two-tailed Student's t-test. $P < 0.05$ was considered as being significantly different from control.

RESULTS

BVR-A's protein carbonyls, HNE and 3-NT levels in brain of subjects with AD and MCI

Biliverdin reductase-A was immunodetected in the hippocampus and cerebellum of control and AD or MCI subjects [41]. BVR-A expression levels were markedly increased in hippocampi from subjects with AD and MCI (+45% and 44%, respectively, compared to control), whereas non-significant changes in levels of BVR-A were observed in cerebellum samples of AD and MCI subjects [41].

In the current study, following immunoprecipitation with specific anti-BVR-A antibody, the levels of protein carbonyls, HNE, and 3-NT on BVR-A were measured by Western Blot analyses. As shown in Figure 1, BVR-A's protein carbonyls (Panel A) were reduced by about 15%, whereas no significant changes were found for HNE levels (Panel B) in the hippocampus of AD subjects. On the contrary, AD subjects showed a significant increase (about 95%) in the levels of 3-NT-modified BVR-A (Panel C). Similarly, immunoprecipitated BVR-A from hippocampal samples of MCI subjects was characterized by a significant decrease (about 20%), in the levels of protein carbonyls (Figure 2, Panel A), whereas HNE levels did not change. With regard to 3-NT, levels were found increased by 30%, compared with the matched controls (Panel C). To evaluate whether or not oxidative and nitrosative modifications were specific for hippocampal tissue, the same experiments were performed in the cerebellum of AD and MCI subjects. As shown in Figure 3, no changes were observed for protein carbonyls or 3-NT levels in the cerebella samples from AD or MCI subjects.

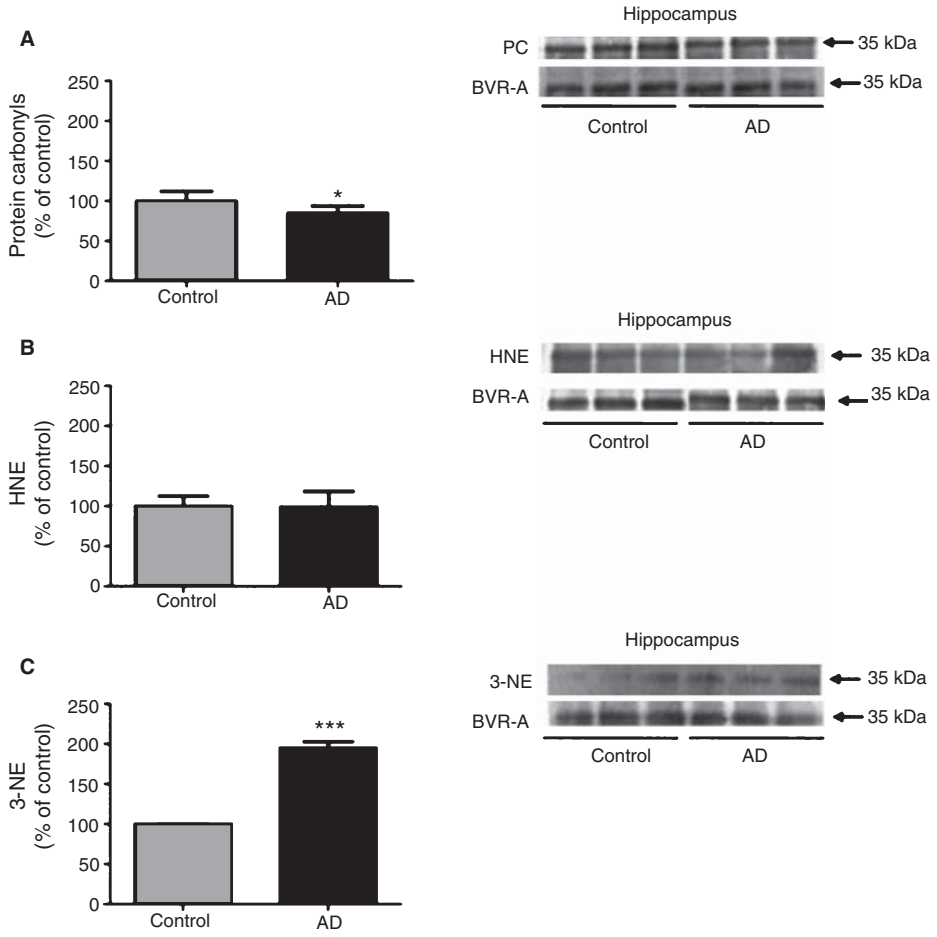

Fig. 1. Biliverdin reductase-A (BVR-A) protein carbonyls (PC), HNE and 3-NT levels in hippocampus of Alzheimer's disease (AD) subjects. Brain samples of hippocampus of subjects with AD were immunoprecipitated with anti-BVR-A antibody and probed with anti-DNP protein adducts polyclonal antibody (Panel A), anti-HNE polyclonal antibody (Panel B) and anti-nitrotyrosine polyclonal antibody (Panel C) by Western Blot as described under Materials and Methods. Densitometric values shown in the histograms are given as percentage of control, set as 100%, and are the product of the band value of the levels of each marker normalized per total BVR-A as loading control. In panels A-C representative gels are shown. Data are expressed as mean ± SD of six individual samples per group. *$p < 0.05$ and ***$p < 0.001$ versus control (Student's *t*-test).

iNOS expression in brain of subjects with AD and MCI

The elevated levels of 3-NT on BVR-A in the hippocampus samples was paralleled by a significant increase in the expression of the inducible isoform of nitric oxide synthase (iNOS). As shown in Figure 4, a significant induction of iNOS was found in the hippocampi from both AD (Panel A) and MCI (Panel B) subjects, whereas no significant changes were observed in cerebella (Panels C-D).

DISCUSSION

In this paper, the first evidence of the post-translational oxidative and nitrosative modifications of BVR-A in the hippocampus, and lack thereof in cerebellum of AD and MCI subjects, has been provided. The reason why we decided to focus the attention on these two selected brain areas depended on their differential selectivity to free radical-induced injury and histopathological alterations [55]. Indeed, 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 in respect to whole brain [55–63]. Conversely, the cerebellum is largely devoid of pathology and oxidative stress [64–66], consistent with normal BVR levels, activity and phosphorylation state [41].

The first novel finding provided by this paper is the increased level of nitrated BVR-A and the decreased formation of protein carbonyls in the hippocampi of

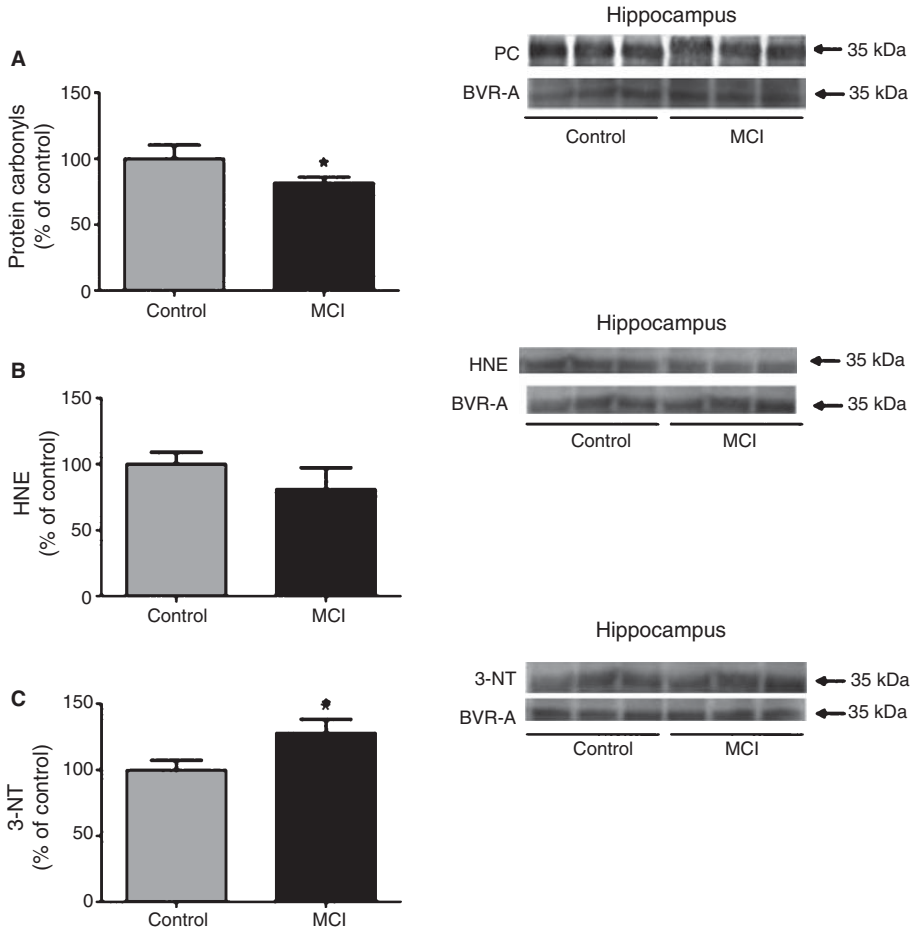

Fig. 2. Biliverdin reductase-A (BVR-A) protein carbonyls (PC), HNE and 3-NT levels in hippocampus of amnestic mild cognitive impairment (MCI) subjects. Brain samples of hippocampus of subjects with MCI were immunoprecipitated with anti-BVR-A antibody and probed with anti-DNP protein adducts polyclonal antibody (Panel A), anti-HNE polyclonal antibody (Panel B) and anti-nitrotyrosine polyclonal antibody (Panel C) by Western Blot as described under Materials and Methods. Densitometric values shown in the histograms are given as percentage of control, set as 100%, and are the product of the band value of the levels of each marker normalized per total BVR-A as loading control. In panels A-C representative gels are shown. Data are expressed as mean ± SD of six individual samples per group. *$p < 0.05$ versus control (Student's *t*-test).

both AD and MCI subjects. These results are consistent with the significant reduction in BVR activity coincident with an increased protein BVR-A expression in hippocampi of subjects with AD and MCI [41]. The rationale for loss of activity of BVR while protein levels were elevated in AD and MCI hippocampus was, at least in part, the reduced level of phosphorylation of serine/threonine residues which made BVR-A dysfunctional [6, 38, 41]. In accordance with these previous results are those provided in the current study, which showed a marked increase in the nitration of BVR-A's tyrosine residues. It is well known that the nitration of tyrosine residues in proteins results in a marked decrease in their function, and this was corroborated by the significant reduction in BVR activity in hippocampi from subjects with AD and MCI [41]. Taken together, these findings underline the importance of post-translational modifications, such as phosphorylation and nitration, on BVR-A functions. Keeping this in mind, it is no longer correct to link only overexpression of total BVR-A, with the production of the antioxidant and anti-nitrosative molecule BR [20, 41] and, therefore, a direct neuroprotective effect.

An interesting aspect of the current results is the reduced formation of BVR-A's protein carbonyls together with the lack of production of HNE-adducts with respect to 3-NT. This finding was not surprising and resembles the results from previous studies, which showed a differential degree of protein oxidation and the prevalence of nitration over carbonyl

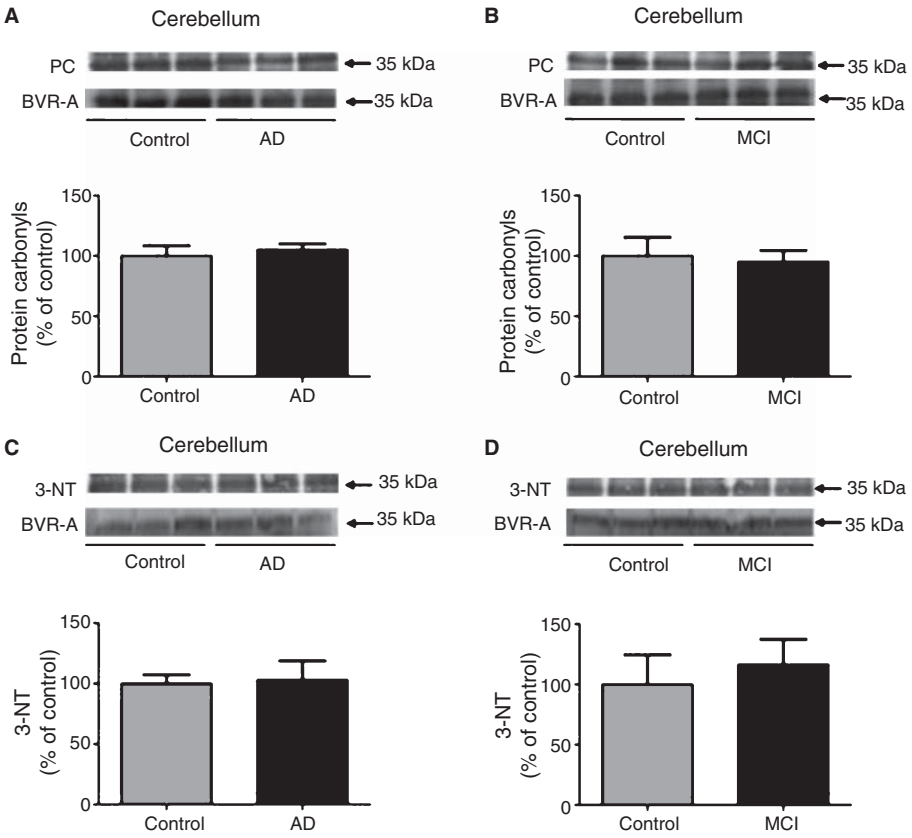

Fig. 3. Biliverdin reductase-A (BVR-A) protein carbonyls (PC) and 3-NT levels in cerebellum of Alzheimer disease (AD) (Panel A and C) or amnestic mild cognitive impairment (MCI) (Panel B and D) subjects. In order to evaluate whether or not oxidative and nitrosative modifications were specific for hippocampal tissue, brain samples of cerebellum of subjects with AD or MCI were immunoprecipitated with anti-BVR-A antibody and probed with anti-DNP protein adducts polyclonal antibody (Panel A and C), or anti-nitrotyrosine polyclonal antibody (Panel B and D) by Western Blot as described under Materials and Methods. Densitometric values shown in the histograms are given as percentage of control, set as 100%, and are the product of the band value of the levels of each marker normalized per total BVR-A as loading control. In panels A-D representative gels are shown. Data are expressed as mean ± SD of six individual samples per group.

formation in many dysfunctional proteins in AD [67, 68]. This hypothesis is also supported by the evidence of a significant induction of iNOS (Fig. 4) and NO formation, with consequent increase of nitrosative stress in the brain, plasma and lymphocytes of AD and MCI subjects (Fig. 4) [33, 46, 48, 49]. Considering that peroxynitrite formation occurs via the reaction between NO and superoxide anion (or sometimes hydrogen peroxide with the formation of intermediate nitrosonium) [69] and that this reaction proceeds very fast, it is plausible to conclude that in the presence of NO the formation of peroxynitrite prevails on ROS formation and the consequent protein oxidation [70, 71].

Another consequence of this study is the differential pattern of BVR-A's carbonyls and 3-NT formation in the hippocampus and cerebellum. A possible explanation for the lack of oxidative damage in cerebellum characteristic of AD subjects is the abundance of BVR-A and BR in this brain area [9, 72] . Our results demonstrated that there is no evidence for an increased degree of BVR-A oxidation or nitration in the cerebellum of AD or MCI subjects with respect to controls. These data are in good agreement with previous studies showing a lack of pathology and oxidative stress in cerebellum [55, 64–66]. That BVR-A did not undergo oxidative or nitrosative modifications in the cerebellum of AD or MCI subjects with respect to controls is consistent with the notion that the levels of bilirubin formed are still high, and this may contribute to the lack of oxidative stress in this brain region.

The HO-1/BVR-A system is not the only one involved in the cell stress response. Two other members of the heat shock protein family, such as Hsp60 and Hsp70, were shown to be involved in the adaptive stress response in AD. However, data from the litera-

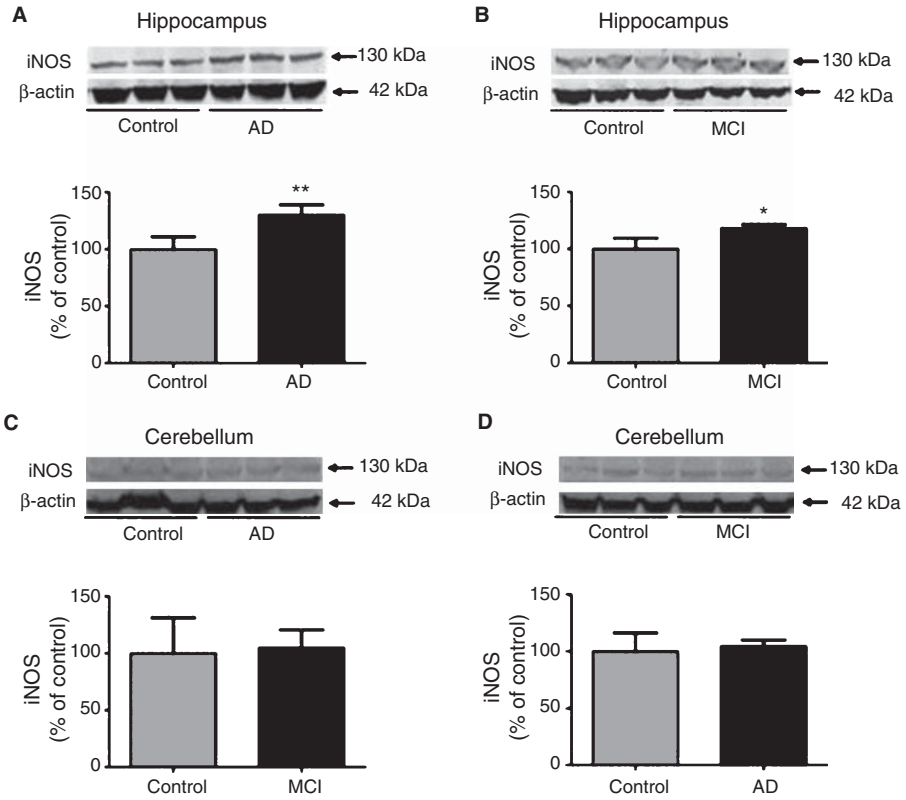

Fig. 4. Inducible nitric oxide synthase (iNOS) expression in hippocampus and cerebellum of Alzheimer's diseases (AD) and amnestic mild cognitive impairment (MCI) subjects. Brain samples of hippocampus (Panel A and B) and cerebellum (Panel C and D) of subjects with AD and MCI were assayed for iNOS by Western Blot as described under Materials and Methods. Densitometric values shown in the histograms are given as percentage of control, set as 100%, and are the product of the band value of the levels of iNOS normalized per β-actin as loading control. In panels A and B representative gels are shown. Data are expressed as mean ± SD of six individual samples per group. *$p < 0.05$ and **$p < 0.01$ versus control (Student's *t*-test).

ture failed to show any significant up-regulation of both Hsp60 and Hsp70, as well as HO-1, in the cerebellum of AD subjects [73–75].

In conclusion, this study represents the first report of oxidative and nitrosative differences in BVR-A in the hippocampus of subjects with AD and MCI. The evidence that BVR-A nitration occurred also in the hippocampus of MCI subjects, suggests that any modification in terms of cell stress response is an early event in the pathogenesis and progression of AD. The implications of these results for the adaptive responses to stress in AD are profound, and research on these subjects is ongoing in our laboratory.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=809).

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