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HO-1/BVR-A System Analysis in Plasma from Probable Alzheimer's Disease and Mild Cognitive Impairment Subjects: A Potential Biochemical Marker for the Prediction of the Disease

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Abstract. Several studies showed increased oxidative and nitrosative stress in plasma from patients with Alzheimer's disease (AD), however, little and controversial knowledge has emerged about the antioxidant functionality of the heme oxygenase-1/biliverdin reductase-A (HO-1/BVR-A) system in blood. The current study reports increased levels of both HO-1 and BVR-A in plasma from probable AD patients, as a result of the increased oxidative environment. However, the increase of oxidative stress in plasma result also in the increase of BVR-A 3-nitrotyrosine levels and the decrease of BVR-A phosphotyrosine levels and reductase activity, suggesting that nitrosative stress play the prominent oxidative role in plasma during AD. Our data on HO-1/BVR-A status in plasma closely correlate with recent reports in hippocampus of subjects with AD and arguably its early form, mild cognitive impairment. Moreover, we show that alterations on HO-1/BVR-A system are tightly connected with cognitive decline indexed by Mini-Mental Status Exam scores. We hypothesize that the HO-1/BVR-A system status in plasma might re ect the ongoing situation in the brain, offering an important biochemical tool for the potential prediction of AD at the earliest stages of the disease.

Keywords: Alzheimer disease's, bilirubin, biliverdin reductase-A, heme oxygenase-1, oxidative/nitrosative stress

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INTRODUCTION

Alzheimer's disease (AD) is one of the most disabling dementing disorders in the elderly affecting about 20 million people worldwide, a number that is expected to increase since the average human life span

the ongoing situation in the brain. A thorough analysis of the HO-1/BVR-A quantity and post-translational modi cations (PTMs) in blood-related bio uids might offer an important biochemical tool for the prediction of AD, even in the early stages of the disease, and might represent a potential window of pharmacological intervention.

MATERIALS AND METHODS

Subjects

For this study, 36 subjects (12 with probable AD (pAD), 12 with MCI, and 12 cognitively normal persons) were enrolled. Complete clinical and neuropsychological evaluation was performed at the Department of Gerontology and Geriatrics, University of Perugia. Diagnosis of pAD was according to the National Institute of Neurological and Communicative Disease and Stroke and Alzheimer's disease (NINCDS-ADRDA) criteria [35]. MCI diagnosis was made following components of Petersen criteria [36]. Cognitively normal subjects (CTR) were enrolled among relatives of patients and subjects admitted to the Day Hospital of the same department for routine evaluation of the health status. All subjects underwent a thorough clinical, neurological, and neuropsychological evaluation; the neuropsychological assessment included the Mini Mental State Examination (MMSE), while the Clinical Dementia Rating scale measured the severity of dementia. The functional ability of subjects enrolled was evaluated on the basis of the activities of daily living and the instrumental activities of daily living (IADL). Subjects anxiety or depression (evaluated by the Geriatric Depression Scale), Hachinski ischemic score, cholesterol, erythrocytes sedimentation, and C-reactive protein was taken in consideration. The investigation conforms to the principles outlined in the Declaration of Helsinki. All the subjects gave the written informed consent for blood donation. Blood was immediately centrifuged and plasma stored at Š80 C until analyses. Subjects' demographic and clinical data are reported in Table 1 and Supplementary Table 1.

Samples preparation

Plasma (100 l) samples from each subject were depleted of the major plasma proteins (albumin and Immunoglobulins G) using PROT-BA depletion kit according to manufacturer instructions (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration after albumin and Immunoglobulins G depletion was determined by using the Comassie protein assay (Pierce, Rock90(ceAr(j(Rock9ins)-32ock9ins)-331tx5dW6.2(alb)20Ik

nitrogen. The precipitate was re-suspended in 100 μl DMSO used for the investigation. A Waters 600 and a Waters 996 Photodiode (Waters Corporation, Milford, MA, USA) were used for HPLC analysis. The column used was a Hyper BDS column C18, 250 mm lengths and 4.6 mm diameter, 5 μm. Solution A was prepared with acetonitrile/water 80/20, while solution B was prepared with ammonium acetate 0.1 mM/acetonitrile 80/20. The column was equilibrated with A/B 95/5 for 5 min, followed by linear gradient of B increase from 5 to 80 in 20 min, and a final equilibration step with A/B 95/5. The flow rate was 1 ml/min. Bilirubin detection was conducted at 450 nm. A calibration curve, using bilirubin IX alpha standard, was built before the plasma analysis.

Statistical analysis

Data are expressed as mean ± SD of n independent samples. All statistical analyses were performed using a non-parametric one-way ANOVA with post hoc t-test. $p < 0.05$ was considered significantly different from control. Pearson correlations were calculated to test the linear association between cognitive test scores and markers of oxidative damage.

RESULTS

HO-1 protein quantitation and PTMs in plasma from pAD and MCI patients

The first parameter taken in consideration in the analysis of HO-1/BVR-A system in plasma of MCI and pAD patients was the measurement of HO-1 protein quantity. Previous studies from our laboratory showed an increase of HO-1 in hippocampus of MCI and pAD subjects in response to environmental increased oxidative/nitrosative stress [23–25]. Although several authors described increased oxidative stress at the systemic level (both in plasma/serum and CSF) in AD subjects [37–41], data about the systemic HO-1 expression are still controversial [20, 27–29].

In the current study, plasma samples from three groups of patients, namely pAD, MCI, and age-matched CTR (12 samples each) were assessed for the analysis of HO-1/BVR-A quantity. Each sample was previously depleted for the most abundant proteins, IgG and albumin. As shown in Fig. 1A, HO-1 quantity significantly increased in both MCI and pAD compared to CTR samples. Interestingly, the trend of increase paralleled the severity of the pathology with MCI having 70% and AD 130% increase compared to CTR (Fig. 1A). After the expression evaluation, each sample was tested for protein specific PTMs. Previous studies from the Butterfield laboratory revealed that increased quantity of HO-1-bound HNE and HO-1 protein carbonyls was present in hippocampi from AD and MCI subjects, respectively [26]. Furthermore, in the same samples, increased quantity of pSer/Thr-HO-1 was detected in AD, suggesting impaired functionality of the protein in pathological conditions. In the current study we found no significant alterations in either HO-1 oxidation (Supplementary Figure 1) or HO-1 Ser/Thr phosphorylation in pAD/MCI plasma. Our results indicated that, in contrast with the hippocampus, plasma HO-1 might preserve its activity under severe pathologic conditions.

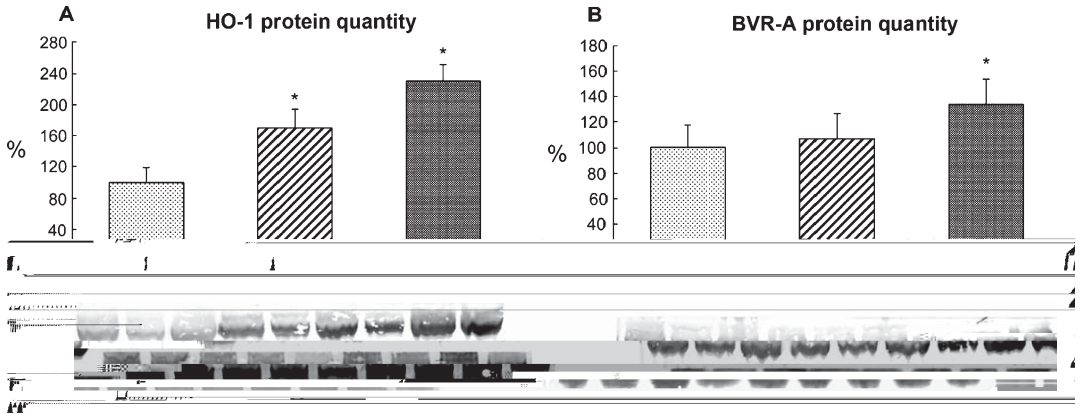

Fig. 1. HO-1 (A) and BVR-A (B) quantity in CTR, MCI, and pAD plasma samples. Densitometric values shown in the histograms are given as percentage of CTR, set as 100%. Data are expressed as mean ± SD of 12 individual samples per group. $p < 0.05$.

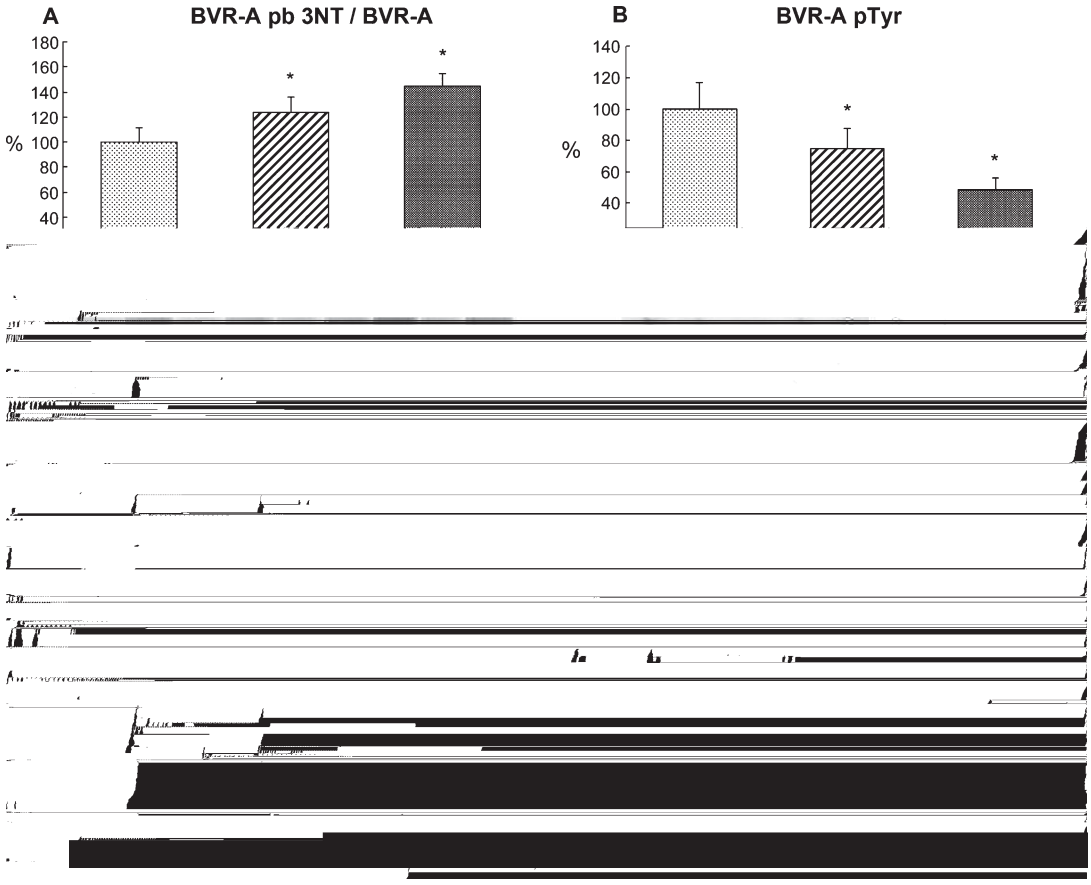

Fig. 2. BVR-A protein bound-3NT (A), phosphotyrosine (B), phsphoserine/threonine (C), and reductase activity (D) quantity in CTR, MCI, and pAD plasma samples. All the data from PTMs quantitation are normalized for BVR–A protein quantity. Densitometric values presented in the histograms are given as percentage of Control, set as 100%. Data are expressed as mean ± SD of 12 individual samples per group $p < 0.05$.

BVR-A protein quantitation, PTMs, and activity in plasma from pAD and MCI patients

Similarly to HO-1, BVR-A protein quantity increased in both pAD and MCI samples, but the induction of expression was slightly lower. Indeed, we observed an increase of 10% in MCI (not signi cant) and of 36% in pAD compared to CTR (Fig. 1B). Previous studies from the Butter eld laboratory showed increased BVR-A levels in the hippocampus of AD and MCI subjects, however, it was also shown that BVR-A underwent nitrosative stress modi cations during AD and MCI exhibiting altered quantity of protein carbonyls and protein nitration [24]. As a consequence of the increased oxidative and nitrosative environment in AD that resulted in increased BVR-A oxidation and nitration, were also found a decrease

Fig. 4. Correlation graphs I. A positive correlation ($p < 0.01$, $r = 0.53$) is shown for HO-1 and BVR-A protein quantity of pAD patients compared to CTR plasma (A) and for BVR-A protein quantity with BVR-A nitration in pAD compared to CTR ($p < 0.01$, $r = 0.63$) (B). Negative correlations were found for BVR-A protein quantity with BVR-A pTyr ($p < 0.01$, $r = -0.53$) (C) and BVR-A nitration with BVR-A pTyr ($p < 0.01$, $r = -0.73$) (D) in pAD versus CTR samples. A negative correlation ($p < 0.05$, $r = -0.48$) was found for BVR-A nitration with BVR-A pTyr in MCI compared to CTR plasma samples (E).

is corroborated by the parallelism between the changes occurring in plasma and those in the brain [23, 24, 26] and the correlation between plasma alteration and cognitive decline.

The main role of oxidative and nitrosative stress in the pathogenesis of AD has been largely investi-

An intriguing aspect of our previous research was the finding that the HO-1/BVR-A axis undergoes important PTMs in AD and MCI hippocampi [23–26]. This evidence obtained by studying an important brain area, such as the hippocampus, whose involvement in AD occurs at the earlier stages, prompted us to investigate whether or not the same PTMs were detected in

from the brain, passing through the blood brain barrier and released into the peripheral system (Scheme 1). With regard to BVR-A protein quantity, we previously stated that the investigation of only BVR-A expression might be ambiguous with regards to BVR-A functionality. Indeed, the analysis of BVR-A PTMs and reductase activity provide deeper and more detailed information about its real status during AD pathology [23, 24]. Consistent with the hippocampus results, our current ndings demonstrate increased amount of protein bound 3-NT in pAD and MCI plasma, while no alterations of protein carbonylation and protein bound-HNE were detected. These results con rm the prevalence of nitrosative stress-induced modi cations than protein carbonylation or protein bound-HNE on BVR-A structure during AD, as already observed in hippocampus [23].

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