Biochimica et Biophysica Acta 1852 (2015) 1428–1441

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Oxidative stress involving changes in Nrf2 and ER stress in early stages of Alzheimer's disease

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ARTICLE INFO

Article history:
Received 10 November 2014
Received in revised form 16 March 2015
Accepted 31 March 2015
Available online 6 April 2015

Keywords:
Alzheimer's disease
Peripheral blood mononuclear cell
Lymphocyte
Mild cognitive impairment
Oxidative stress
Calcium homeostasis

ABSTRACT

Oxidative stress and endoplasmic reticulum (ER) stress have been associated with Alzheimer's disease (AD) progression. In this study we analyzed whether oxidative stress involving changes in Nrf2 and ER stress may constitute early events in AD pathogenesis by using human peripheral blood cells and an AD transgenic mouse model at different disease stages. Increased oxidative stress and increased phosphorylated Nrf2 (p(Ser40)Nrf2) were observed in human peripheral blood mononuclear cells (PBMCs) isolated from individuals with mild cognitive impairment (MCI). Moreover, we observed impaired ER Ca^{2+} homeostasis and increased ER stress markers in PBMCs from MCI individuals and mild AD patients. Evidence of early oxidative stress defense mechanisms in AD was substantiated by increased p(Ser40)Nrf2 in 3 month-old 3xTg-AD male mice PBMCs, and also with increased nuclear Nrf2 levels in brain cortex. However, SOD1 protein levels were decreased in human MCI PBMCs and in 3xTg-AD mice brain cortex; the latter further correlated with reduced SOD1 mRNA levels. Increased ER stress was also detected in the brain cortex of young female and old male 3xTg-AD mice. We demonstrate oxidative stress and early Nrf2 activation in AD human and mouse models, which fails to regulate some of its targets, leading to repressed expression of antioxidant defenses (*e.g.*, SOD-1), and extending to ER stress. Results suggest markers of prodromal AD linked to oxidative stress associated with Nrf2 activation and ER stress that may be followed in human peripheral blood mononuclear cells.

1. Introduction

Alzheimer's disease (AD), the most prevalent form of dementia in the elderly, is characterized by memory deficits and cognitive decline that arise from synaptic and neuronal loss in the hippocampus and cerebral cortex ([50,74], for review). Main neuropathological hallmarks in AD brains are abnormal deposition of amyloid-beta peptide (Aβ) in extracellular senile plaques and intracellular neurofibrillary tangles formed by hyperphosphorylated tau. Mild cognitive impairment (MCI) is an intermediate stage between cognitively normal individuals and patients with AD [33]. About 10–20% of people aged 65 and older have MCI and approximately 15% of these individuals progress to dementia every year [83]. Therefore, alterations occurring in MCI individuals can be crucial to understand early basic mechanisms responsible for the neurodegenerative process in AD and develop effective disease-modifying strategies. Although not stopping AD progression, memantine (Mem) and acetylcholinesterase (AChE) inhibitors are presently the available therapies to ameliorate AD cognitive symptoms.

Oxidative stress, commonly linked to mitochondrial dysfunction, has been identified as an important mechanism leading to neuronal death in AD [10]. Similarly, other mechanisms of neurodegeneration have been described, namely impaired Ca^{2+} homeostasis and endoplasmic reticulum (ER) stress [27,28]. Indeed, we and other authors have shown that $A\beta_{1-42}$ oligomeric species cause disorganization of the cytoskeleton and neurite retraction [55], intracellular Ca^{2+} ($Ca^{2+}{}_i$) deregulation [25], ER stress and apoptosis [15,29,59]. Oxidative stress markers have been shown in MCI brains [4,65], in the plasma and erythrocytes of MCI and mild AD patients [3,4] and in

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http://dx.doi.org/10.1016/j.bbadis.2015.03.015
0925-4439/

the brain of transgenic AD mice before plaque deposition [68], suggesting that oxidative damage occurs in early stages of the disease and that progression to AD might be related to depletion of antioxidant defenses. Aβ was also demonstrated to induce oxidative stress in cultured hippocampal and cortical neurons [20,29].

There are evidences for modifications in transcription factors related with mitochondrial biogenesis and antioxidant defenses in AD [11]. In this respect, low levels of reactive oxygen species (ROS) induce nuclear factor erythroid derived 2-related (Nrf2) activation, a transcription factor that regulates the antioxidant response, in astrocytes from mixed neuron/astrocyte cultures, contributing to neuroprotection [6] and helping to delay AD-like pathology in APP/PS1 mice [41]. Decreased Nrf2 levels were detected in the nucleus of hippocampal neurons of human AD brains [66], suggesting diminished Nrf2-mediated transcription of antioxidant and detoxifying genes. Mild/moderate ER stress may be a trigger for nuclear translocation of Nrf2 and subsequent activation of an antioxidant response [19]. Recently, Glover-Cutter and colleagues (2013) demonstrated in the *Caenorhabditis elegans* that the inducible transcription factor SKN-1, a homologue of mammalian Nrf proteins, is regulated by the ER unfolded protein response (UPR), directly controls UPR signaling and transcription factor genes, binds to common downstream targets with XBP-1 and ATF-6, and is present at the ER. Moreover, SKN-1/Nrf was also found essential for resistance to ER stress and SKN-1/Nrf-mediated responses to oxidative stress were shown to depend upon signaling from the ER [14]. However, in severe and/or prolonged situations of ER stress, activation of protective mechanisms is not sufficient to restore normal ER function, and cells initiate autophagy or apoptosis [79]. Under these conditions, ER may be a source of oxidative stress [34]. Several studies established a correlation between abnormal ER function and AD progression [36,72] and Aβ has been shown to induce ER stress both *in vitro* and *in vivo*, subsequently leading to apoptotic cell death [16,30,80]. Furthermore, ER dysfunction can partially account for the perturbation of $Ca^{2+}{}_i$ homeostasis reported in AD patient's brain and peripheral cells [49]. Although these pathological mechanisms have been ascribed to AD progression, it is still unclear whether they constitute early events contributing to AD pathogenesis.

The long history of searching for human peripheral markers capable of reflecting AD pathology within the brain has prompted studies looking at changes occurring in peripheral blood samples, which can be easily accessible. Of relevance, decreases in antioxidant defenses were previously described in peripheral blood samples from MCI individuals and mild AD patients, indicating antioxidant depletion during progression to AD [3]. Studies in peripheral blood mononuclear cells (PBMCs) isolated from AD patients support that pro-apoptotic proteins may provide systemic markers for AD [77]. Eckert and colleagues (1998) found that apoptosis is induced in lymphocytes and neurons with similar oxidative stress inductors and that susceptibility to apoptosis is altered in AD lymphocytes relatively to controls. Furthermore, AD patient's lymphocytes showed significantly higher apoptosis *in vitro*[23,44]. Screening of cytokines produced by PBMCs also showed that alterations in immune response may precede clinical AD, since changes in cytokine production were observed in PBMCs from individuals with MCI [45].

In the present study we examined early formation of ROS involving changes in Nrf2 and ER stress in *in vivo* AD models, namely: i) human PBMCs obtained from MCI individuals and AD patients in mild and moderate plus severe stages, associated to progressive cognitive impairment, treated or not with Mem or AChE inhibitors, *versus* age-matched controls; ii) PBMCs and cortical brain extracts obtained from 3xTg-AD mice, a humanized animal model of AD exhibiting soluble Aβ in initial stages and a later temporal profile of extracellular Aβ aggregates and intracellular hyperphosphorylated tau (*e.g.*,[56,57]), *versus* wild-type (WT) mice. Data add to the knowledge that oxidative stress plays a relevant role in AD pathogenesis.

2. Materials and methods

2.1. Reagents

ECF reagentand anti-mouse and anti-rabbit IgG secondary antibodies were from Amersham (UK). Polyvinylidenedifluoride (PVDF) membrane, antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Hsp60 were purchased from Millipore Chemicon (Billerica, MA, USA). BioRad protein assay reagent as well as iScript™cDNA synthesis kit, SsoFast™EvaGreen®Supermix were purchased from BioRad (Hercules, CA, USA). RNeasy® mini kit was from Qiagen (Valencia, CA, USA). Antibodies against GADD153/CHOP, Bcl-2, CBP and PGC-1α (K-15) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against GRP78 and heparinized tubes for blood collection was from BD Bioscience (San Jose, CA, USA). Antibodies against Bax, pro-caspase 3, P(Thr980)PERK, PERK and CREB were from Cell Signalling(Danvers, MA, USA). Antibodies against Bak, XBP-1, Nrf2, p(Ser40)Nrf2, SOD1, GCLc and Lamin B1 were from Abcam (Cambridge, UK). Anti-mouse and anti-rabbit IgG were obtained from Amersham (Buckinghamshire, UK). Nuclear/cytosolic fractionation kit was obtained from BioVision (CA, USA). ELISA kits for detection of cerebrospinal fluid (CSF) Aβ42, tau and p-tau were purchased from Innotest/Innogenetics (Ghent, Belgium). Kit for DNA isolation for ApoE genotyping was from Roche Diagnostics GmbH (Manheim, Germany). Ficoll-Paque was obtained from GE Healthcare (Buc, France). Fluorescent probes Fura-2-AM and 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH2-DA) were purchased from Molecular Probes-Invitrogen (Eugene, OR, USA). Primers used for RT-PCR were obtained from EuroFins MWG Operon (Ebersberg, Germany). Glutamate, hydrogen peroxide (H_2O_2), thapsigargin, protease cocktail inhibitor, RPMI 1640, antibodies against β-actin and α-tubulin as well as other analytical grade reagents were obtained from Sigma Chemical and Co. (St. Louis, MO, USA).

2.2. Participants

A total of 104 subjects participated in this study, including 20 healthy controls, 24 clinically confirmed MCI, 27 mild AD and 33 moderate/severe AD patients from Portuguese families (Table 1). Patients were recruited at the dementia outpatient clinics at Coimbra University Hospital Center, in accordance with the Ethical Committee from this institution. Age-matched controls were volunteers, usually spouses or friends of patients who were requested and agreed to participate in the study. MCI and AD cases were subjected to clinical history, neurological examination, laboratorial evaluation and brain imaging (computed tomography or nuclear magnetic resonance scan). Inclusion criteria for AD were based on the 4th edition of The Diagnostic and Statistical Manual of Mental Disorders (DSM IV-TR) [2]. MCI criteria were those proposed by the European Alzheimer's Disease Consortium [64]. Cognitive impairment was also quantified using the Mini Mental State Evaluation (MMSE) [31]. Control subjects did not present evidence of cognitive deterioration or cognitive complaint had a MMSE above cut-off and their value in the Clinical Dementia Rating Scale (CDR) was zero. The exclusion criterion for all groups was the presence of other neurological, psychiatric or medical pathologies that could cause cognitive impairment, or a history of alcohol or drug abuse. All participants signed an informed consent before any study procedure. For AD patients, informed consents from respective caregivers were also obtained.

2.3. Quantification of soluble $A\beta_{1-42}$, tau and p-tau in the CSF and genotyping of ApoE allelic variants

Pre-analytical and analytical procedures were done in accordance with the Alzheimer's Association guidelines for CSF biomarker determination [51]. Briefly, CSF samples were collected in sterile polypropylene tubes, immediately centrifuged at 1800 ×*g* for 10 min at 4 °C, aliquoted

Table 1
Characterization of control, MCI and AD patients' sample population.

Groups	Number of individuals (n)	Gender		Age (years)	Education (years)	Disease onset (years)	Cognition (MMSE Score)	Mem-treated (%)	AChE inhibitors-treated (%)
		Male (n)	Female (n)						
Control (CDR = 0)	20	8	12	68 ± 6.5 (55–79)	5.7 ± 2.1 (3–9)	–	28.90 ± 0.12 (23–30)	–	–
MCI (CDR = 0.5)	24	12	12	71.8 ± 8.6 (48–93)	6.9 ± 4.6 (0–15)	67.3 ± 7.7 (46–78)	27.5 ± 0.63 (21–30)	4.2	16.7
Mild AD (CDR = 1)	27	11	16	72.5 ± 10.6 (53–92)	6.9 ± 4.0 (0–17)	68.2 ± 10.3 (51–89)	20.34 ± 0.89$^{***,\#\#\#}$ (10–28)	22.2	66.7
Moderate–Severe AD (CDR = 2 and 3)	33	12	21	74.4 ± 10.2 (55–90)	5.6 ± 3.8 (0–15)	67.9 ± 10.1 (50–85)	10.9 ± 1.02$^{***,\#\#\#,\$\$\$}$ (0–22)	30.3	33.3

Data represent the mean ± SEM of age, education, disease-onset and cognitive impairment (analyzed through MMSE, Mini Mental State Examination), as well as the percentage (%) of individuals under medication with Mem or acetylcholinesterase (AChE) inhibitors (donepezil, rivastigmine and galantamine) relatively to the number (n) of individuals per diagnostic group according to global staging (CDR = Clinical Dementia Rating); numbers in parentheses represent minimal and maximal values for each parameter. Statistical significance: $^{***}p < 0.001$ compared to control individuals, $^{\#\#\#}p < 0.001$ compared to MCI individuals, $^{\$\$\$}p < 0.001$ compared to mild individuals.

into polypropylene tubes and stored at −80 °C until analysis. CSF Aβ42, tau and p-tau were measured separately by sandwich ELISA kits, as previously described [5]. External quality control of the assays was performed under the scope of the Alzheimer's Association Quality Control Program for CSF Biomarkers [51]. Blood samples were also collected from MCI and AD patients for ApoE genotyping. DNA was isolated from whole EDTA-blood using a commercial kit and ApoE genotype was determined by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) assay, as previously described [18].

2.4. Isolation and culture of peripheral blood mononuclear cells (PBMCs)

Peripheral whole blood from MCI, AD or healthy age-matched individuals and from 3-month-old 3xTg-AD *versus* WT mice was drawn in heparinized tubes and mononuclear cells separated by gradient centrifugation in order to discard erythrocytes and granulocytes. Briefly, 10 ml or 450–700 μl of human or mice blood, respectively, were carefully layered onto Ficoll solution and tubes centrifuged at 1500 ×*g* for 20 min at 18 °C in a swing-out rotor, without break. After centrifugation, the ring containing mononuclear cells, mainly agranulocytes and more particularly lymphocytes, was carefully removed from the interface using a sterile Pasteur pipette and the harvested fraction diluted in 45 ml of sterilized phosphate saline buffer (PBS) containing (in mM): 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 $Na_2HPO_4 \cdot 2H_2O$, pH 7.4. Cells were pelleted by centrifugation at 530 ×*g* for 10 min at 18 °C, and resuspended in RPMI 1640 without serum. Cells were immediately extracted for Western blot analysis or alternatively cultured for 1 day in RPMI 1640 medium plus 10% (v/v) autologous serum in T25 culture flasks at a concentration of 2×10^6 cells/ml in a humidified incubator chamber with 95% air and 5% CO_2 at 37 °C, for $Ca^{2+}{}_i$ measurements or ROS production experiments. Nuclear fractions were not prepared from PBMCs due to the limitation in the amount of collected human blood samples. To avoid multiple blood collections from each participant, the number of samples assayed in the different experimental protocols was lower than the total number of samples per group.

2.5. Animals

3xTg-AD and WT strain (C57BL6/129S) mice (a generous gift from Dr. Frank Laferla, University of California, Irvine, USA) at young (3 month-old) or old (12 or 15 month-old) age were bred and maintained at CNC-Faculty of Medicine, University of Coimbra, animal house. Animals were housed under a constant temperature, humidity and a 12 h light/dark cycle. All procedures using animals were in accordance with the approved animal welfare guidelines and European legislation (European directive 2010/63/EU) and Portuguese legislation ("Decreto-Lei n°113/2013").

2.6. Measurement of reactive oxygen species

Analysis of ROS in PBMCs was performed with the fluorescent probe DCFH2-DA, which can be oxidized by hydroperoxides, peroxynitrite (ONOO—), hydroxyl radicals (•OH) or hypochlorous acid, among other oxidants, and redox-active metals such as iron [40]. PBMCs were washed in Na+ medium containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4, and then incubated with the non-specific fluorescent probe DCFH2-DA (20 μM) in Na+ medium for 30 min, at 37 °C. After a washing step, ROS production was measured (0.5×10^6 cells per experimental condition) for 5 min (basal values) and for further 30 min after adding 1 mM H_2O_2, 1 mM glutamate or 2.5 μM thapsigargin by using a microplate reader Spectrofluorometer Gemini EM (Molecular Devices, USA) (480 nm excitation, 550 nm emission). In order to evaluate the contribution of ER Ca^{2+} depletion and the role of ER homeostasis in regulating ROS production, experiments with 2.5 μM thapsigargin were performed in Na^+ medium without Ca^{2+}.

2.7. Intracellular free Ca^{2+} recording

PBMCs were washed in Na^+ medium (described in previous section) followed by spin down centrifugation and incubated with the Fura-2/AM ratiometric fluorescent probe (10 μM) for 40 min at 37 °C. After a washing step, Fura-2 fluorescence was analyzed (0.5×10^6 cells per experimental condition) using a Spectrofluorometer Gemini EM (Molecular Devices, USA) microplate reader, at a 340/380 nm excitation and 510 nm emission wavelengths. Fura-2 fluorescence was recorded for 5 min (basal values) and for further 15 min after exposure to H_2O_2 (1 mM), glutamate (1 mM) or thapsigargin (2.5 μM). All plotted values were normalized for baseline values. In experiments with thapsigargin, Ca^{2+} was omitted from Na^+ medium.

2.8. Sample preparation and Western blotting

In the case of both human and mice PBMCs, total protein extracts were obtained by resuspending cells in Ripa buffer, a strong lysis buffer normally used to study cytosolic, mitochondrial and nuclei proteins (http://docs.abcam.com/pdf/misc/abcam-protocols-book-2010.pdf) containing 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS, supplemented with 1 mM DTT, 1 mM PMSF, 25 mM NaF, 1 mM Na_3VO_4, 100 nM okadaic acid and 1 μg/ml protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin and antipain). 3xTg-AD and WT mice were sacrificed by cervical dislocation and brain cortices were isolated and homogenized with a potter at 280 rpm in ice cold supplemented Ripa buffer for preparation of total protein extracts. Homogenates were then centrifuged at 14,000 ×*g* for

10 min at 4 °C in order to obtain protein total extract. Nuclear fractions from mice cerebral cortices and cultured cerebral cortical cells were obtained using the Nuclear/Cytosolic fractionation kit. Protein content was determined using the BioRad protein assay reagent. Samples were treated with a denaturing buffer containing 50 mM Tris–HCl pH 6.8, 2% SDS, 5% glycerol, 100 mM DTT, 0.01% bromophenol blue, for 5 min, at 95 °C. Equivalent amounts of protein were separated by electrophoresis on a 6–15% SDS-PAGE gel and electroblotted onto PVDF membranes. The membranes were further blocked with 5% fat-free milk before incubation with the specific antibody against XBP-1 (1:1000), Grp78 (1:1000), GADD153 (1:500), Bcl-2 (1:500), Bax (1:1000), Bak (1:2500), pro-caspase 3 (1:1000), P(Thr980)PERK (1:1000), PERK (1:1000), GCLc (1:1000), Nrf2 (1:1000), P(Ser40)Nrf2 (1:500), CBP (1:200), CREB (1:1000), SOD1 (1:1000) or PGC-1α (1:500) overnight, at 4 °C. Furthermore, β-actin (1:20,000) or GAPDH (1:500) was used as a protein loading control. An anti-mouse IgG secondary antibody (1:20,000), anti-rabbit (1:20,000) or anti-goat (1:3000) was used. Immunoreactive bands were visualized by alkaline phosphatase activity after incubation for 5–10 min with ECF reagent on a BioRad Versa Doc 3000 Imaging System. Purity of nuclear extracts from mice cerebral cortices samples were also evaluated by Western blotting using antibodies against Lamin B1 (1:1000) specific for the nuclear fraction, Hsp60 (1:1000) specific for the mitochondrial fraction and α-tubulin (1:20,000) specific for the cytoplasmic fraction (data not shown).

2.9. RNA extraction, reverse transcription and quantitative real time PCR

Total RNAs were extracted using the Qiagen RNeasy mini kit, as described by the manufacturer and quantified with a spectrophotometer (NanoDrop 2000, from ThermoScientific). Reverse transcription was performed on each RNA sample (1 μg) using the cDNA synthesis kit in a final reaction volume of 20 μl, according to the manufacturer's instructions. All specific oligonucleotides were designed using the Primer3 and BLAST software. Gene specific primers used for real time PCR reactions are SOD1: forward 5′-CACTTCGAGCAGAAGGCAAG-3′ and reverse 5′-CCCCATACTGATGGACGTGG-3′; GCLc: forward 5′-ATTCCGCTGTCCAA GGTTGA-3′ and reverse 5′-AACATCCCCTGCAAGACAGC-3′; HO 1: forward 5′-TGCTAGCCTGGTGCAAGATAC-3′ and reverse 5′-TGTCTGGGAT GAGCTAGTGC-3′; Prdx 1: forward 5′-TATCAGATCCCAAGCGCACC-3′ and reverse 5′-AAGGCCCCTGAAAGAGATACC-3′; actin: forward 5′-GGA GAC GGG GTC ACC CAC AC-3′ and reverse 5′-AGC CTC AGG GCA TCG GAA CC-3′. Quantitative RT-PCR was performed with 10 ng of the cDNA, 300 nM of each primer, and SsoFast™ EvaGreen® Supermix. PCR cycles were proceeded as follows: Taq activation (30 s, 95 °C), denaturation (5 s, 95 °C), and annealing and extension (5 s, 57 °C) using the BioRad CFX 96 Real-time system, C1000 Thermal cycler. The melting-curve analysis showed the specificity of the amplifications. Threshold cycle, which inversely correlates with the target mRNA level, was measured as the cycle number at which the reporter fluorescent emission appears above the background threshold. The relative mRNA levels were estimated using the Bio-Rad CFX manager 2.1 software using actin as a reference gene.

2.10. Statistical analysis

Data were analyzed by using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) software and were expressed as mean ± SEM of the number of experiments indicated in the figure legends. Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA) followed by the Dunnett's post-hoc test. Unpaired two-tailed Student's *t*-test was also performed for comparison between two Gaussian populations, as described in the figure legends. Significance was defined as $p < 0.05$.

3. Results

3.1. Clinical and biochemical characterization of human subjects

The characteristics of the sample, by diagnostic group, are summarized in Table 1. Controls, MCI, mild and moderate to severe AD patients were similar in gender, age and educational level. A significant difference in the MMSE was observed between patients' groups ($p < 0.001$). The percentage of patients medicated with Mem, an uncompetitive antagonist of *N*-methyl-D-aspartate receptors (NMDARs), or AChE inhibitors (donepezil, rivastigmine and galantamine) are also indicated in Table 1. Data show that Mem was predominantly prescribed in AD cases (mild and moderate–severe AD), whereas AChE inhibitors were similarly prescribed in MCI and moderate–severe AD patients, and mainly prescribed to mild AD patients. Patients were also medicated with psychopharmaceuticals (67% of MCI individuals, 89% and 100% of mild and moderate–severe AD patients, respectively), cholesterol-lowering drugs (29.2% of MCI individuals, 3.7% and 21.2% of mild and moderate–severe AD patients, respectively) and anti-coagulating drugs (approximately 20% in all groups) (data not shown).

As depicted in Table 2, levels of $A\beta_{1-42}$ in the CSF are significantly decreased between MCI and mild ($p < 0.05$) or moderate/severe ($p < 0.01$) patients and tend to decrease with disease progression. Interestingly, AD patients could be distinguished from MCIs by detecting lower levels of $A\beta_{1-42}$ and higher t-tau and p-tau levels in the CSF, when compared to the reference values. Interestingly, increased total tau and p-tau were already detected in MCI individuals when compared with reference values. Moreover, ApoE genotype was positive (about 50%) both in MCI and AD patients.

3.2. ROS production in PBMCs from MCI and AD patients

Considering the relevance of oxidative stress in AD, production of ROS was evaluated in PBMCs obtained from MCI subjects or AD patients with different degrees of cognitive impairment (mild and moderate–severe stages, as depicted in Table 1) *versus* age-matched control individuals (Fig. 1). Measurements were performed under basal conditions or after incubation with H_2O_2, a stable and diffusible ROS, or high glutamate concentration, which inhibits cysteine uptake leading to a marked decrease in cellular GSH levels [62], both acting as oxidative stress inducers. Thapsigargin, a selective non-competitive inhibitor of ER Ca^{2+} ATPase that impairs Ca^{2+} homeostasis in this compartment [69] was also used. Detection of basal ROS production was significantly higher in MCI PBMCs, compared to controls (Fig. 1A, B), but no significant changes were observed in peripheral cells obtained from AD patients. Furthermore, exposure of PBMCs to H_2O_2 (Fig. 1C, D, E) or glutamate (Fig. 1F, G, H) induced a significant increase in ROS production in mild AD patient's PBMCs ($p < 0.05$), suggesting increased susceptibility to oxidative stress in early AD patients presenting mild symptoms. Exposure of MCI PBMCs to thapsigargin, which depletes ER Ca^{2+} levels, significantly increased ROS levels ($p < 0.05$) (Fig. 1I, J, K), implicating early ROS production possibly associated with ER dysfunction. No significant differences in ROS generation were observed following exposure to H_2O_2, glutamate or thapsigargin in cells from moderate–severe AD patients. Furthermore, no protective effects were observed in MCI or AD patients treated with Mem or AChE inhibitors (donepezil, rivastigmine and galantamine) (Fig. 1B, E, H, K). Additionally, no significant differences were observed between PBMCs obtained from men or women (data not shown).

3.3. Deregulation of $Ca^{2+}{}_i$ in PBMCs from MCI and AD patients

Taking into account the close relationship between ROS generation and Ca^{2+} rise in AD and that deregulated intracellular Ca^{2+} homeostasis has been described in brain [24] and peripheral blood cells [22] from AD patients, we evaluated free $Ca^{2+}{}_i$ levels in PBMCs from age-matched

Table 2
Analysis of CSF biomarkers in MCI and AD patients.

	MCI	Mild AD	Moderate/severe AD	Laboratory reference values
$A\beta_{1-42}$ (pg/mL)	634.8 ± 88.3 (n = 12)	415.5 ± 40.4 (n = 13)*	303.2 ± 38.5 (n = 7)**	>542
t-Tau (pg/mL)	353.0 ± 65.6 (n = 12)	564.8 ± 64.9 (n = 13)	453.0 ± 118.9 (n = 7)	<212
p-Tau (pg/mL)	46.5 ± 7.2 (n = 12)	68.2 ± 5.4 (n = 13)	56.8 ± 9.6 (n = 7)	<32
Biomarkers profile	1.2 ± 0.2	0.49 ± 0.09	0.50 ± 0.7	>1
ApoE (%ε4)	48% (n = 23)	52% (n = 50)		–

Data represent the mean ± SEM of $A\beta_{1-42}$, tau and phosphorylated-tau (p-tau) levels in CSF from MCI and AD patients (n = 12 for mild AD and n = 6 for moderate–severe AD). The biomarkers profile is calculated through the formula $A\beta_{1-42}/(240 + 1.18^{*}tau)$, considered typical of AD if <1. The ApoE genotype was also evaluated for MCI and AD patients (n = 23 for MCI and n = 50 for mild and moderate/severe AD). Statistical significance: *p < 0.05 and **p < 0.01 compared to MCI patients (Dunnett's *post-hoc* test).

controls, MCI and AD patients in basal conditions and following stimulation with the oxidative stress inducers, H_2O_2 and glutamate, or ER Ca^{2+} depletion with thapsigargin (Fig. 2). Basal free $Ca^{2+}{}_i$ levels were shown to be significantly higher in PBMCs obtained from mild AD patients (Fig. 2A, B), suggesting a deregulation of $Ca^{2+}{}_i$ homeostasis in human peripheral cells. Stimulation of cells with H_2O_2 or glutamate

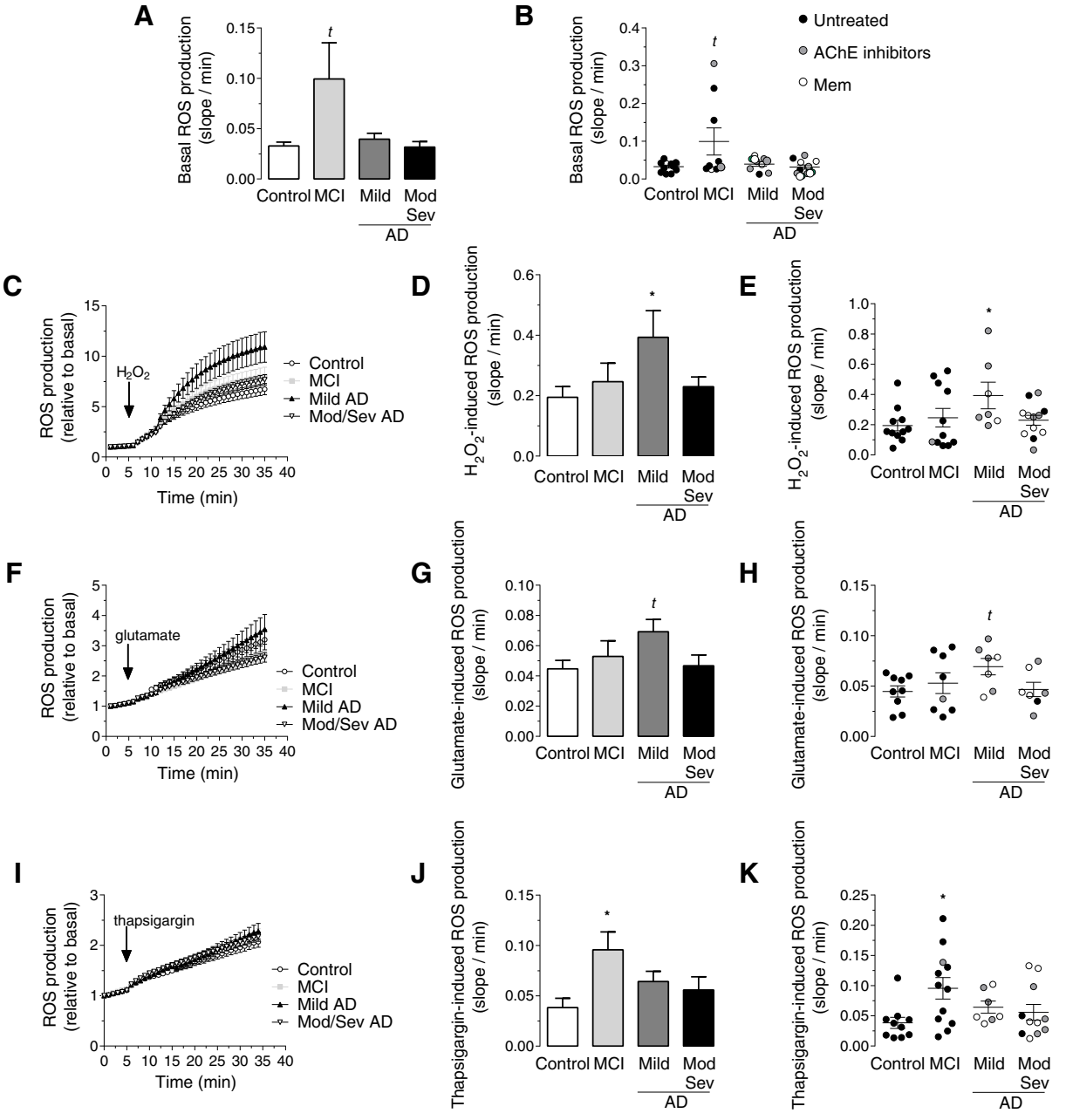

Fig. 1. ROS production in PBMCs of control, MCI and AD individuals. Basal DCF fluorescence was recorded for 5 min (A, B), cells were then stimulated with 1 mM H_2O_2 (C, D, E), 1 mM glutamate (F, G, H) or 2.5 μM thapsigargin (I, J, K), fluorescence recorded for further 30 min and slope per minute was calculated. In B, E, H and K, patients medicated with Mem, AChE or no medication (untreated) are represented with white, gray or black circles, respectively. Data are the mean ± SEM of triplicates from 8–12 individuals per group. Statistical analysis: *p < 0.05 significantly different when compared with control individuals (Dunnett's *post-hoc* test) and ^{t}p < 0.05 *versus* control (Student's *t*-test).

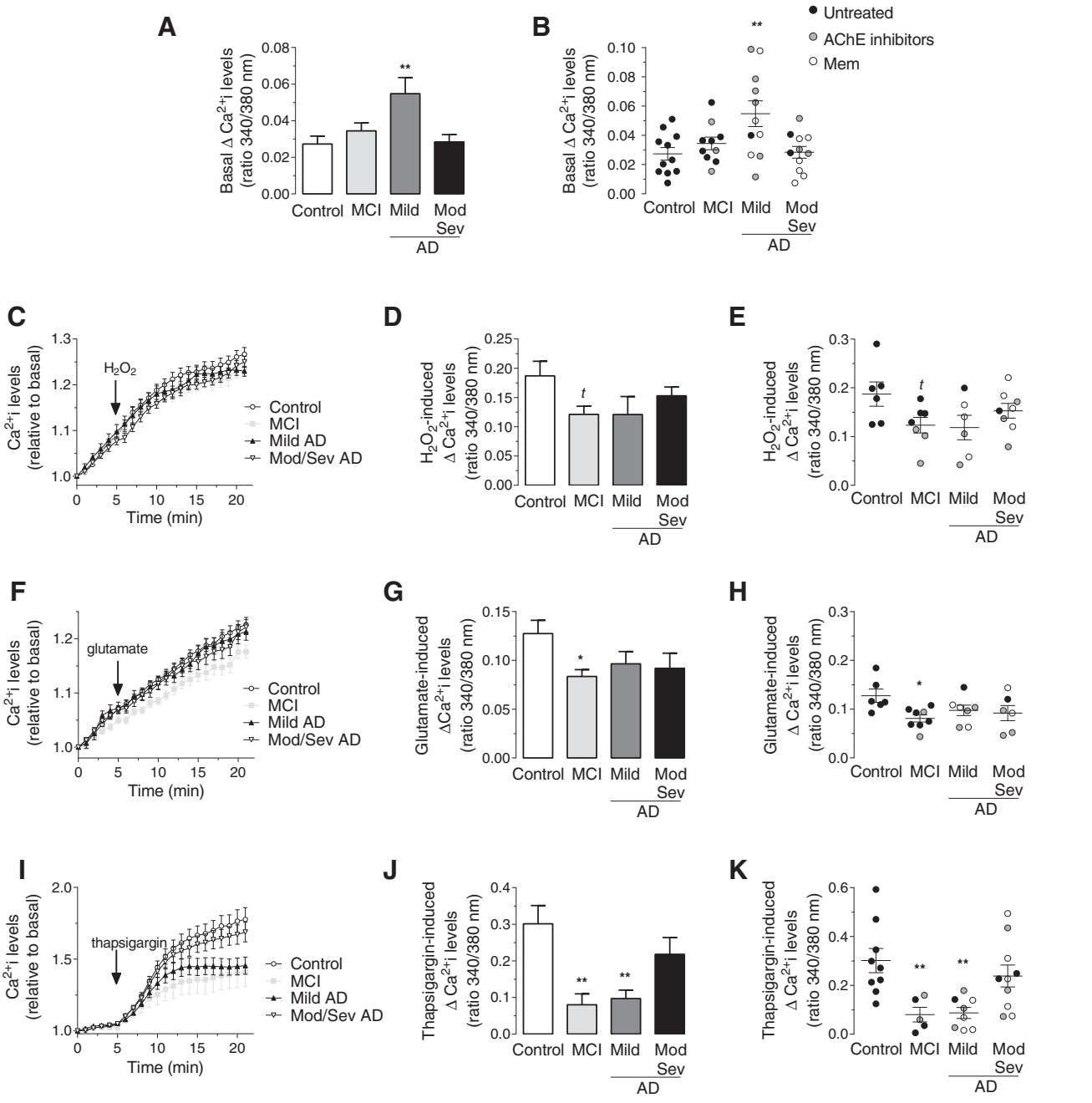

Fig. 2. Cytosolic and ER calcium levels in PBMCs of control individuals, MCI and AD patients. Levels of Ca^{2+} in the cytosol and in ER stores were evaluated by monitoring the fluorescence of Fura-2/AM in the presence or absence of external Ca^{2+}, respectively. (A, B) Basal $Ca^{2+}{}_i$ levels were recorded for 5 min. The effect of 1 mM H_2O_2 (C, D, E), 1 mM glutamate (F, G, H) or 2.5 μM thapsigargin (I, J, K) on free $Ca^{2+}{}_i$ levels were recorded for 15 min. Bars and dispersion graphics were obtained calculating the difference between the last recorded value after stimulus and the value immediately before the stimulus.In B, E, H and K, patients medicated with Mem, AChE or no medication (untreated) are represented with white, gray or black circles, respectively. Data are the mean ± SEM from 8–11 per group individuals performed in triplicates. Statistical analysis: $^{*}p < 0.05$ and $^{**}p < 0.01$ significantly different when compared with control individuals (Dunnett's *post-hoc* test) and $^{t}p < 0.05$ *versus* control (Student's *t*-test).

caused an increase in free $Ca^{2+}{}_i$ in all groups tested (Fig. 2C–H). However, the response was significantly lower in MCI ($p < 0.05$, Student's *t*-test), when compared to age-matched control human cells (Fig. 2D, E and G, H, respectively following H_2O_2 or glutamate exposure), reinforcing an inherent deficit in the regulation of $Ca^{2+}{}_i$. In order to evaluate the contribution of ER, PBMCs were also exposed to thapsigargin. In the presence of this classical disruptor of ER Ca^{2+} homeostasis and in the absence of external Ca^{2+}, the levels of free $Ca^{2+}{}_i$ significantly decreased in both MCI and mild AD PBMCs ($p < 0.01$), suggesting a decreased ER Ca^{2+} content in these cells (Fig. 2I, J, K). In contrast, no significant changes in free $Ca^{2+}{}_i$ levels were observed in PBMCs obtained from moderate–severe AD patients, as compared with controls. Moreover, no differences were observed in PBMCs obtained from Mem- or AChE inhibitors-treated and non-treated patients (Fig. 2B, E, H, K) or between genders (data not shown).

3.4. Activation of ER stress response in human PBMCs and 3xTg-AD mice brain cortex

Activation of ER stress sensors and downstream signaling pathways is a cellular response triggered in an attempt to restore ER homeostasis in cells submitted to several insults. Considering the large decrease in ER Ca^{2+}content in MCI and mild PBMCs (Fig. 2I, J, K), protein levels of ER stress markers were evaluated in PBMCs obtained from control, MCI

and AD subjects. In particular, levels of GRP78, an ER molecular chaperone, XBP-1, a transcription factor activated downstream of the IRE-1α ER stress sensor and a trigger for GRP78 gene expression, and GADD153/CHOP, a pro-apoptotic transcription factor which acts through downregulation of Bcl-2 and perturbation of redox status, were analyzed [52] (Fig. 3).

Significant changes in the levels of ER stress markers were found in peripheral cells obtained during pre-clinical or early stages of the disease. Indeed, we observed enhanced GRP78 and XBP1 levels. For GRP78, the trend increase observed in MCI individuals became statistically significant in mild AD patients (Fig. 3A, B; $p < 0.01$ by Student's *t*-test). The levels of XBP1, which is known to up-regulate GRP78, were significantly increased in PBMCs derived from MCI individuals, and remained high during disease progression (Fig. 3C, D; $p < 0.05$ by Student's *t*-test), indicating ER stress. These data are concordant with the depletion of ER Ca^{2+} stores, a well-known trigger of ER stress, in MCI and mild AD PBMCs.

In order to ascertain activation of ER stress in AD brain, levels of GRP78 and XBP-1 were also analyzed in cortical samples from 3xTg-AD mice brain at 3 and 12 months of age, in comparison with age-matched WT mice (Fig. 4). Since 3xTg-AD females were reported to exhibit different susceptibilities linked to differential expression of sex steroid hormones [12,35], we evaluated the levels of ER stress markers in the brain cortex of 3xTg-AD males and females. Results indicate

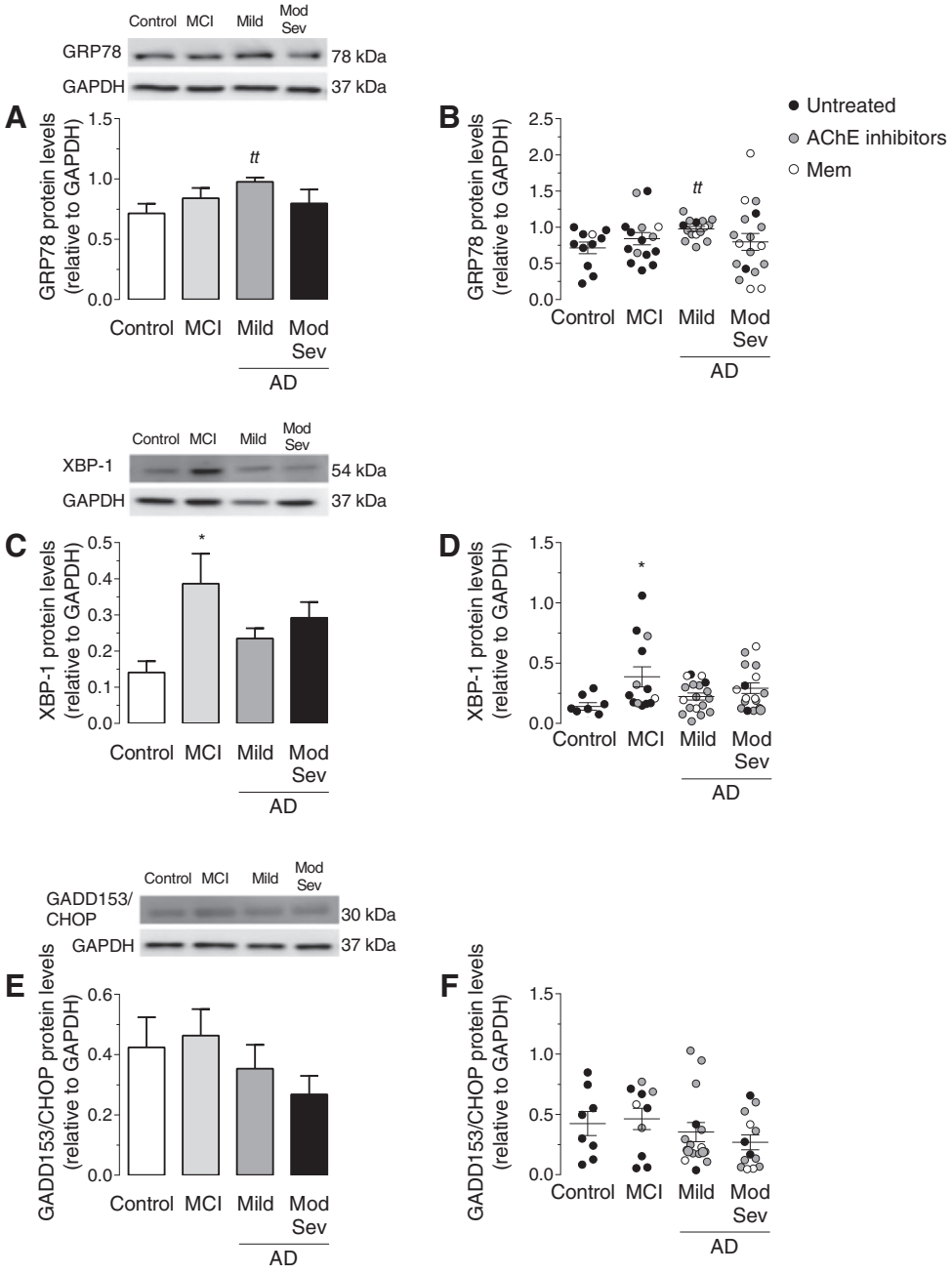

Fig. 3. ER stress markers in PBMCs of controls, MCI and AD patients. Levels of GRP78 (A, B), XBP-1 (C, D) and GADD153/CHOP (E, F) were analyzed by Western blotting. In B, D and E, patients medicated with Mem, AChE or no medication (untreated) are represented with white, gray or black circles, respectively. Results are expressed in arbitrary units relative to GAPDH as the mean ± SEM of 8–19 individuals per group. Statistical significance: $^{*}p < 0.05$ *versus* control (Dunnett's *post-hoc* test), $^{tt}p < 0.05$ *versus* control (Student's *t*-test).

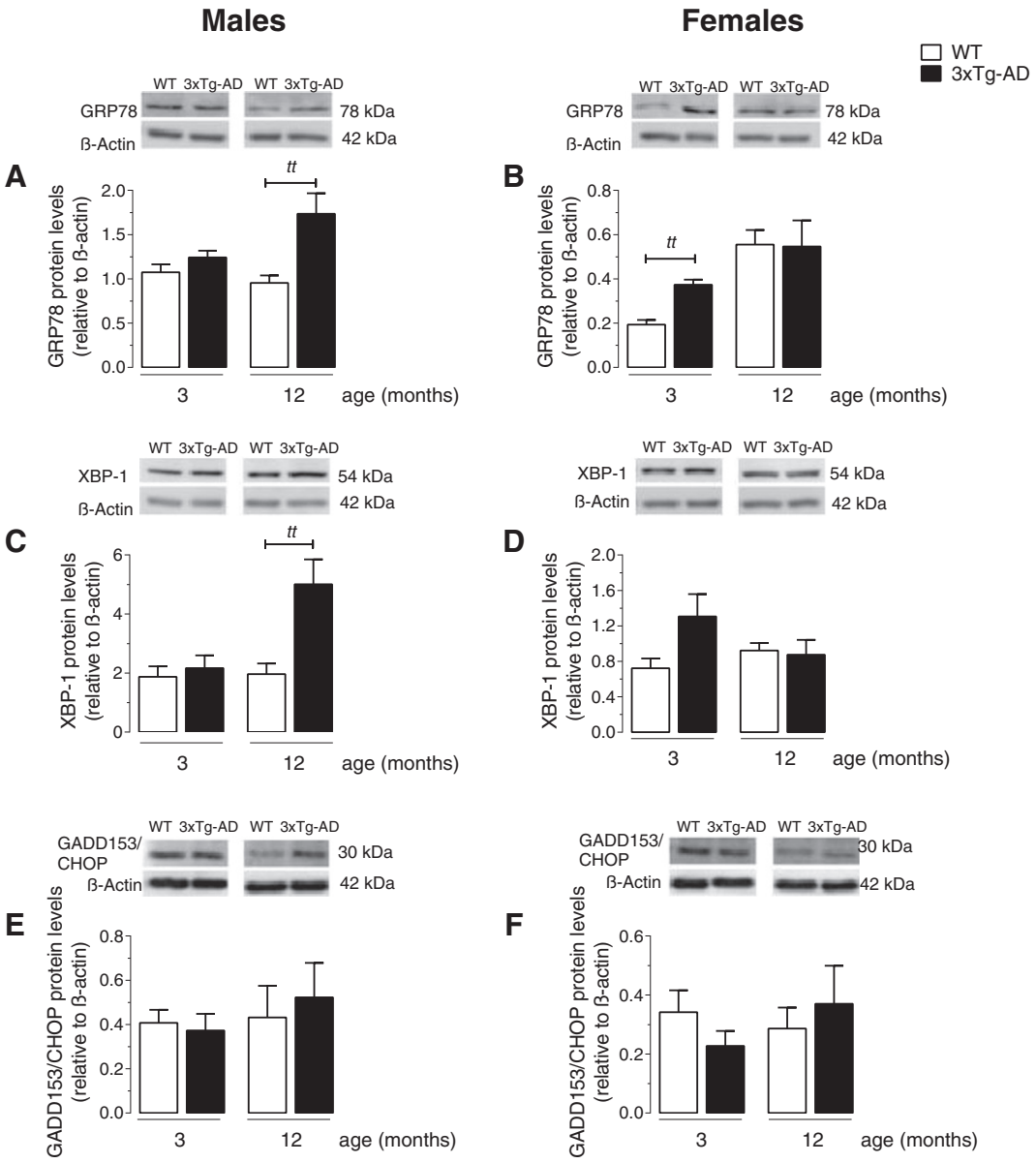

Fig. 4. ER stress markers in young and aged 3xTg-AD *versus* WT mice. Levels of GRP78 (A, B), XBP-1 (C, D) and GADD153/CHOP (E, F) in 3 and 12 months of age WT and 3xTg-AD males (A, C, E) and females (B, D, F) were analyzed by Western blotting. Results are expressed in arbitrary units relative to β-actin as the mean ± SEM of 6 animals per group. Statistical significance: $^{tt}p < 0.01$ *versus* control (Student's *t*-test).

that alterations in these proteins occur in an age- and gender-dependent manner in this AD model. In fact, the levels of the molecular chaperone GRP78 were significantly increased at 12 months of age in 3xTg-AD males, compared to age-matched WT (Fig. 4A). Conversely, in 3xTg-AD females, increased GRP78 levels, as compared to WT mice, were detected in younger animals, at 3 months of age (Fig. 4B). Regarding XBP-1 levels, results were similar to those obtained for GRP78: in 3xTg-AD mice females XBP-1 levels tend to increase at 3 months of age (although not significantly), whereas in males a significant increase was observed at 12 months of age (Fig. 4C, D). These results suggest a gender-dependent activation of ER stress pathways in the brain cortex of 3xTg-AD mice.

In contrast with GRP78 and XBP-1 ER stress markers, the levels of active p(Thr980)PERK [p(Thr980)PERK/PERK ratio] detected in the cerebral cortex of 3-month-old 3xTg-AD and WT mice males were not statistically significant (Supplementary Fig. 1S). Similarly, GADD153/CHOP, a transcription factor that acts as an important mediator of ER stress-induced cell death, was not upregulated in the peripheral human model, in PBMCs, along the progression of the disease (Fig. 3E, F) or in the cerebral cortex of 3xTg-AD males and females, at 3 and 12 months of age (Fig. 4E, F). Concordantly, the levels of the pro-apoptotic proteins Bax, Bak and pro-caspase 3, and also the levels of the anti-apoptotic protein Bcl-2, and thus the Bcl-2/Bax ratio, were not significantly changed in PBMCs from MCI and AD patients in comparison with age-matched controls (Supplementary Fig. 2S), largely suggesting that apoptotic cell death pathways are not activated in PBMCs during disease progression.

The above results indicate that ER stress occurs in PBMCs isolated from MCI individuals or mild AD patients, which involves upregulation of the XBP-1 stress sensor and the downstream GRP78 chaperone, two markers of UPR activation, but does not implicate ER stress-induced apoptotic cell death pathway triggered by the GAD153/CHOP transcription factor. In a similar way, in 3xTg-AD mice brain cortex, elevated levels of XBP-1 and GRP78 evidence ER stress occurring in a GAD153/CHOP-independent manner.

3.5. Modified levels of transcription factor linked to antioxidant profile in human and 3xTg-AD PBMCs and 3xTg-AD mice brain cortex

In order to investigate if the increase in ROS production in PBMCs (Fig. 1) was due to modified antioxidant pathways, we further investigated the levels of phosphorylated Nrf2 (p(Ser40)Nrf2), a transcription factor that regulates a broad spectrum of protective genes, including the superoxide dismutase 1 (SOD1) [60] and the glutamate-cysteine ligase catalytic subunit (GCLc), the rate limiting enzyme for the synthesis of glutathione [73]. Under basal conditions, Nrf2 is repressed by its binding to Keap1 (Kelch ECH associating protein 1, a repressor protein that binds to Nrf2 and promotes its degradation by the ubiquitin proteasome pathway) in the cytosol [38]; following oxidative stress, Nrf2 is released from Keap1 and phosphorylated at Ser40, allowing its translocation to the nucleus, although phosphorylation of Nrf2 is not required for the transcriptional activity of Nrf2 [8]; within the nucleus, Nrf2 binds to antioxidant response element (ARE) and regulates transcriptional activity of its target genes.

Importantly, a significant increase in p(Ser40)Nrf2 levels was observed in MCI PBMCs (Fig. 5A, B; $p < 0.05$ by Student's *t*-test). The increase in p(Ser40)Nrf2 was not accompanied by changes in other transcription factors or co-regulators, namely peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a transcriptional co-activator that mediates antioxidant responses and plays a central role in the regulation of cellular energy metabolism (*e.g.*, [76]), and two transcription factors linked to cell survival pathways, namely cAMP response element-binding (CREB) and its co-activator CREB-binding protein (CBP) (*e.g.*, [21]) (Fig. 3S).

Unexpectedly, a significant decrease in SOD1 levels was detected in MCI PBMCs (Fig. 6A, B; $p < 0.05$ by Student's *t*-test), while no significant changes were observed on GCLc protein levels (Fig. 6C, D). These results suggest a failure of the Nrf2 pathway in regulating its targets, leading to decreased antioxidant defenses in PBMCs from MCI individuals, which may underlie enhanced production of ROS. Peripheral cells isolated from mild AD patients showed enhanced levels of SOD1, although not statistically significant in comparison with controls. As previously described for other parameters, treatment with Mem or AChE inhibitors did not alter the levels of transcription factors (Fig. 5B; Fig. 3S B, D, F) or the Nrf2-regulated targets SOD1 and GCLc (Fig. 6B, D).

The deregulation of antioxidant pathways was also studied in the 3xTg-AD mice model. Results depicted in Fig. 7A show a tendency for an increase ($p = 0.0544$) in p(Ser40)Nrf2 levels in 3 month-old (pre-symptomatic) 3xTg-AD PBMCs, when compared to age-matched WT PBMCs, suggesting the activation of Nrf2, similarly as observed in MCI PBMCs (Fig. 5). Notably, the volume of blood collected from each animal was low (450–700 μL), yielding a limited number of PBMCs for these experiments. Nrf2 levels were also evaluated in nuclear fractions from brain cortical samples obtained from 3xTg-AD and WT mice, namely young (3 month-old) and old (15 month-old) males and females. We observed an increase in the levels of Nrf2 in nuclear fractions obtained from the cerebral cortex of 3 month-old 3xTg-AD males (Fig. 7B). Interestingly, similar to data obtained in human PBMCs (Fig. 6), brain cortical samples derived from young (pre-symptomatic) 3xTg-AD males exhibited decreased SOD1 and unaltered GCLc protein levels (Fig. 8A, B), suggesting a probable failure in the activation of the Nrf2 pathway. Moreover, in old 3xTg-AD mouse male brain cortex (at 15 months of age), the decrease in nuclear levels of Nrf2 (Fig. 7C) was not accompanied by alterations in GCLc or SOD1 protein levels (Fig. 8A, B). No significant alterations in nuclear Nrf2 (Fig. 7C), SOD1 or GCLc (Fig. 4S) levels were observed in 3xTg-AD females cerebral cortex at 3 or 15 months of age.

mRNA was further isolated from 3 month-old 3xTg-AD mouse male brain cortex in order to evaluate the gene expression of different Nrf2 targets, namely GCLc, SOD1, heme oxygenase (HO)-1 and peroxiredoxin (Prdx)-1, and to verify the failure of Nrf2 to activate its transcription targets. Interestingly, SOD1 and GCLc protein levels in young 3xTg-AD males (Fig. 8A, B) correlated with reduced SOD1 and unaltered GCLc mRNA levels (Fig. 9A, B). Moreover, a significant reduction in HO-1 mRNA and a trend for decreased Prdx-1 mRNA were detected in 3xTg-AD males at 3 months of age (Fig. 9C, D).

Our results support that alterations observed in PBMCs of 3xTg-AD mice may reflect changes observed in brain cortex. Moreover, increased Nrf2 activation, resulting from increased nuclear Nrf2 levels in the cerebral cortex of young/pre-symptomatic (3 month-old) 3xTg-AD mice males, may not be sufficient to activate the Nrf2 pathway through activation of transcription of its target genes, namely SOD1 or GCLc. Interestingly, early alterations in oxidative defenses in the PBMCs and the brain cortex of this AD mouse model are apparently similar to those observed in PBMCs from MCI patients.

4. Discussion

Investigation of cell injury in different stages of cognitive deterioration in AD peripheral human cells evidenced prodromal p(Ser40)Nrf2 upregulation, which correlated with heightened ROS production, along with decreased SOD1 and, as observed in PBMCs from individuals with MCI already showing increased tau and p-tau but normal $A\beta_{1-42}$ in the CSF. Furthermore, ER stress-mediated Ca^{2+} dyshomeostasis and ER-associated ROS generation, in the absence of apoptotic activation, were found in PBMCs obtained from MCIs and mild AD patients, constituting early events in AD. Interestingly, data obtained in human PBMCs were similar to that obtained from pre-symptomatic 3xTg-AD mouse PBMCs regarding p(Ser40)Nrf2 levels, which apparently correlated with increased nuclear Nrf2 in the cerebral cortex; interestingly, this was accompanied by decreased SOD1 and unaltered GCLc protein and mRNA levels, as well as increased ER stress markers.

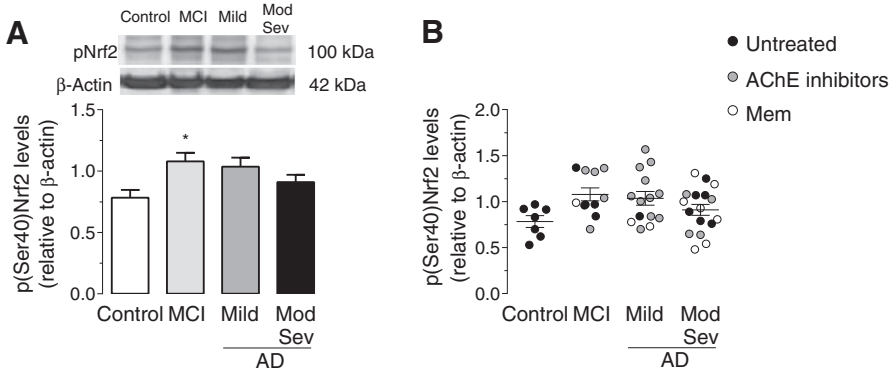

Fig. 5. Levels of phosphorylated Nrf2 in PBMCs of controls, MCI individuals and AD patients. Levels of p(Ser40)Nrf2 were analyzed by Western blotting. In B, patients medicated with Mem, AChE or no medication (untreated) are represented with white, gray or black circles, respectively. Results are expressed in arbitrary units relative to β-actin as the mean ± SEM of 7–17 individuals per group. Statistical analysis: $^{*}p < 0.05$ significantly different when compared with control individuals (Dunnett's *post-hoc* test).

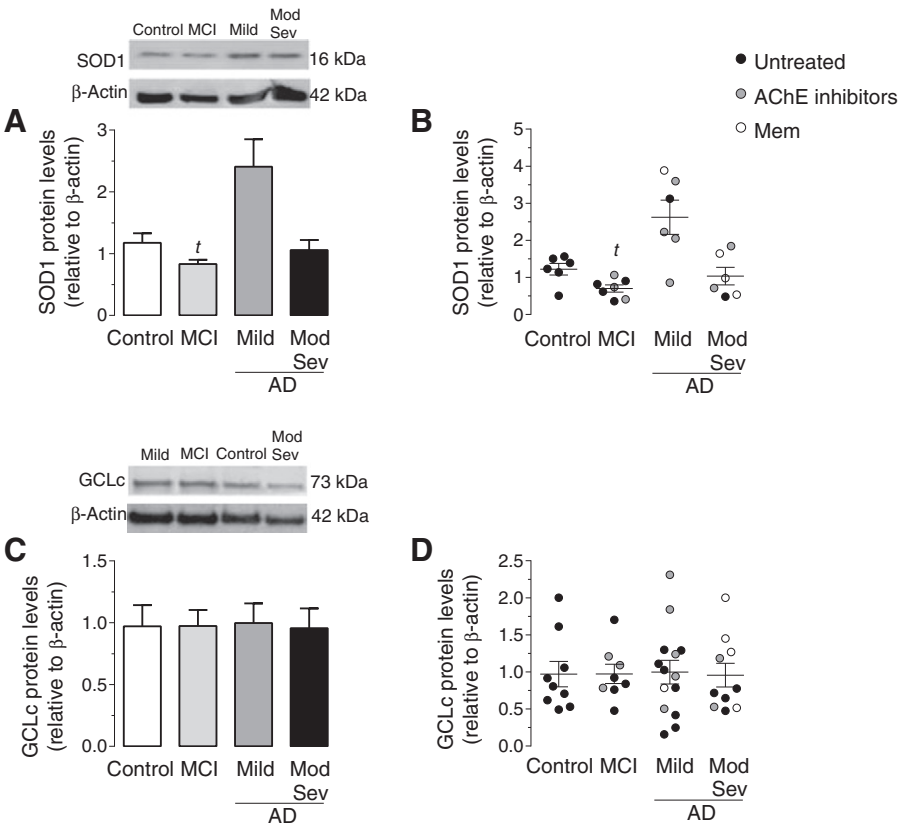

Fig. 6. Levels of SOD1 and GCLc in PBMCs of controls, MCI and AD individuals. Levels of SOD1 (A, B) and GCLc (C, D) were analyzed by Western blotting. In B, D patients medicated with Mem, AChE or no medication (untreated) are represented with white, gray or black circles, respectively. Results are expressed in arbitrary units relative to β-actin as the mean ± SEM of 5–14 individuals per group. Statistical analysis: $^{t}p < 0.05$ significantly different when compared with control individuals (Student's *t*-test).

Accordingly to the "amyloid cascade hypothesis", Aβ (particularly in the oligomeric form) triggers several pathological mechanisms leading to synaptic and neuronal dysfunction, which appear to underlie cognitive deficits and dementia [7]. This hypothesis is supported, among other evidences, by the discovery of an APP mutation that decreases Aβ levels *in vitro* and protects against AD-associated cognitive decline [39]. Previously, we demonstrated that NMDAR-mediated $Ca^{2+}{}_i$ dyshomeostasis is induced by Aβ oligomeric forms in cultured neurons [17,25,26,30,67]. Furthermore, this peptide was shown to be an ER stressor that activates the UPR and deregulates ER Ca^{2+} homeostasis, increasing cytosolic Ca^{2+} levels, finally leading to apoptosis [17,29,37,67]. Interestingly, previous studies revealed increased cytosolic Ca^{2+} levels in AD lymphocytes, when compared to control individuals [1]. In the present work, we demonstrated that basal $Ca^{2+}{}_i$ levels were significantly higher in the initial stage of cognitive deficits (mild AD patients); interestingly, MCI subjects and mild AD patients were previously reported to exhibit synaptic modifications [71]. Furthermore, in PBMCs from MCI individuals the increase in cytosolic Ca^{2+} correlated with decreased ER Ca^{2+} levels, indicating a deregulation in ER Ca^{2+} buffering capacity, an early event implicated in ER stress response [42]. In the presence of oxidant toxic stimuli (glutamate or H_2O_2), PBMCs from MCI individuals showed a reduced $Ca^{2+}{}_i$ response, indicating increased susceptibility to Ca^{2+} deregulation following a stress insult. These results show that during the initial stages of the disease, PBMCs exhibit significant alterations in regulation of $Ca^{2+}{}_i$ homeostasis by the ER. Interestingly, we did not observe alterations in Ca^{2+} levels in PBMCs isolated from moderate–severe AD patients, suggesting that alternative mechanisms may occur in peripheral cells at later disease stages.

Changes in ER Ca^{2+} content in PBMCs isolated from MCI subjects and in the early stages of the disease were followed by enhanced levels of GRP78 and XBP-1, valuable markers of ER stress previously found to be up-regulated in AD brains [84,85]. Our results suggest that, in an early stage of AD, GRP78 detaches from the stress sensor IRE1α allowing its dimerization and XBP1 splicing [58], which may then up-regulate chaperones to cope with misfolded proteins. Prolonged ER stress can trigger apoptotic cell death, which may occur through activation of the transcription factor GADD153/CHOP [63,70]. However, we did not find evidences for the involvement of GADD153/CHOP in peripheral cell damage or in brain cortical samples from 3xTg-AD mice. In these mice, results also suggest an age- and gender-dependent induction of ER stress which occurs later in males, as suggested by increased GRP78 and XBP-1 levels at 12 months of age, and earlier in females, as demonstrated by the increase in GRP78 levels at 3 months of age.

Previous studies showed that lymphocytes from MCI subjects and AD patients exhibited increased basal ROS levels, when compared with lymphocytes obtained from age-matched control subjects [43,53]. Mórocz and colleagues (2002) also found oxidized purines in nuclear DNA isolated from AD lymphocytes and diminished repair capacity of H_2O_2-induced oxidized purines [54]. Our results are in accordance with these observations, since PBMCs from MCI individuals exhibited an increase in ROS production under basal conditions. Importantly, this increase was accompanied by enhanced Nrf2 (ROS-related transcription factor) activation and decreased levels of SOD1 protein levels, although Nrf2 is known to regulate SOD1 transcription, among several other targets;, suggesting an impairment of defensive oxidative stress pathways activation. Furthermore, thapsigargin induced a significant rise in ROS levels in MCI PBMCs suggesting that ER might also be an important source of ROS under conditions of ER Ca^{2+} dyshomeostasis and decreased SOD1. Cumulating evidences suggest that ER stress induction upon depletion of ER Ca^{2+} and generation of ROS are closely linked events [46]. Indeed, Ca^{2+} released from ER leads to the generation of ROS [30] and depletion of reduced glutathione (GSH), which in turn activate the mitochondrial-mediated apoptotic cell death pathway in cultured cortical neurons [29]. Results obtained in human PBMCs suggest

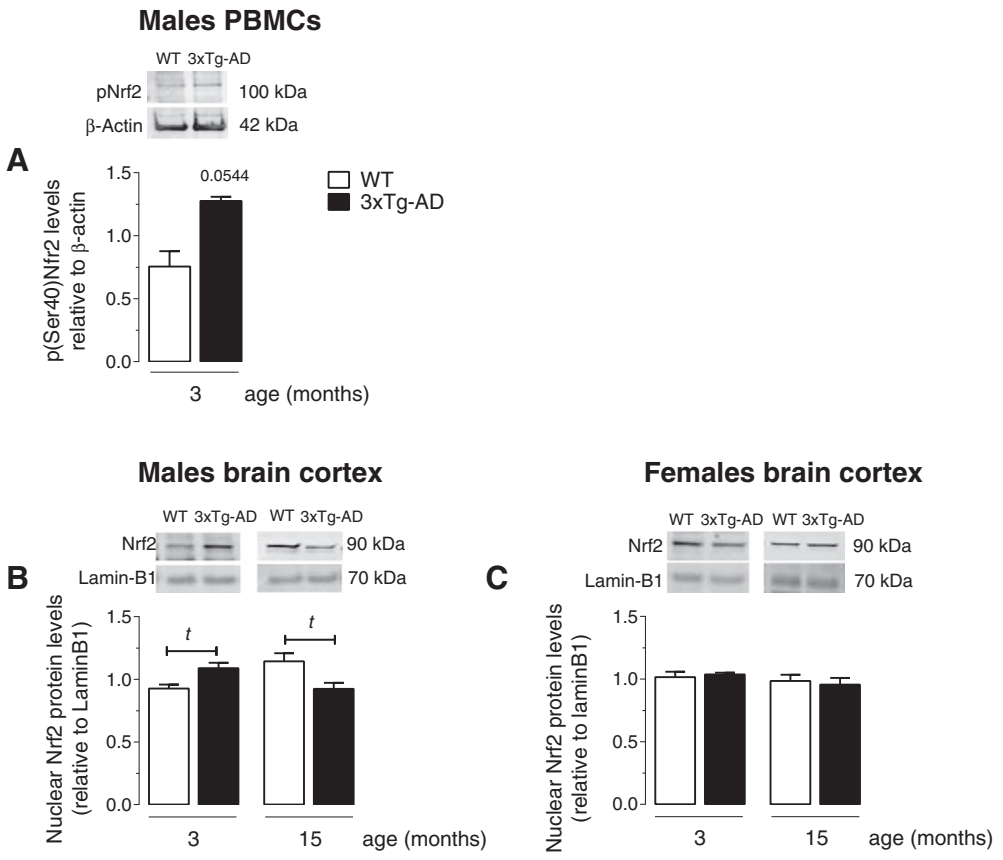

Fig. 7. Levels of phosphorylated Nrf2 and Nrf2 in young and old 3xTg-AD *versus* WT mice. Levels of p(Ser40)Nrf2 in PBMCs from 3 month-old WT and 3xTg-AD mice were evaluated by Western blotting (A). Nuclear levels of Nrf2 (B, C), both in 3 and 15 month-old males (B) and females (C) were analyzed by Western blotting. Results are expressed in arbitrary units relative to β-actin (A) or lamin B1 (B, C) as the mean ± SEM of 2 (A) or 4 (B, C) animals per group. Statistical significance: $^{t}p < 0.05$ *versus* control (Student's *t*-test).

that during MCI, oxidative stress is related, at least in part, to deregulated ER Ca^{2+} homeostasis and correlates with decreased antioxidant defenses resulting from a probable impairment in Nrf2 pathway.

In vivo oxidative damage was shown to directly correlate with the presence of Aβ deposits [75]. Similarly to our findings in human PBMCs, we obtained evidences for deregulation of defensive oxidative pathways in both PBMCs and brain of 3xTg-AD pre-symptomatic mice, at 3 months of age, presenting intracellular Aβ accumulation [56]. Young 3xTg-AD mice males exhibited increased p(Ser40)Nrf2 levels in PBMCs similarly to those observed in MCI PBMCs, and increased nuclear Nrf2 levels in brain cortex, which occurred concomitantly with diminished SOD1 and HO-1 mRNA levels, largely suggesting an impairment in the Nrf2 transcriptional activity. Our previous studies showed evidences for early oxidative stress in 3–5 month-old 3xTg-AD females total brain homogenates, namely decreased levels of GSH and vitamin E, increased activity of glutathione peroxidase and superoxide dismutase and lipid peroxidation products [68]. However, in the cortex of 3xTg-AD young females we did not observe alterations in SOD1, GCLc or nuclear Nrf2 levels. We previously demonstrated age-dependent differences between genders in 3xTg-AD mice regarding GluN2B subunit of NMDARs and Src activation [56]. In humans, differences between genders in AD patients have been reported and the incidence of the disease was described to be higher in post-menopausal women than in age-matched men [9,32], but this is still questionable.

Our results are in accordance with the literature and differences observed between males and females at the same age suggest gender-

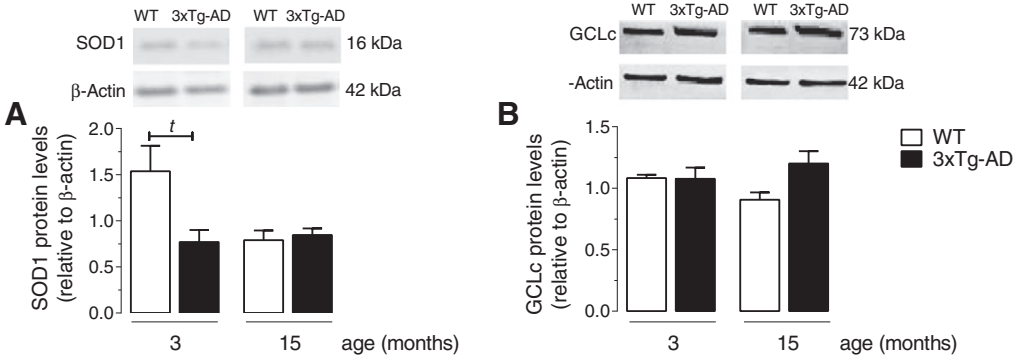

Fig. 8. Levels of SOD1 and GCLc in young and old 3xTg-AD *versus* WT male mice. Levels of SOD1 (A) and GCLc (B) levels in 3xTg-AD *versus* WT ale mice with 3 and 15 months of age were analyzed by Western blotting. Results are expressed in arbitrary units relative to β-actin as the mean ± SEM of 6 animals per group. Statistical significance: $^{t}p < 0.05$ *versus* control (Student's *t*-test).

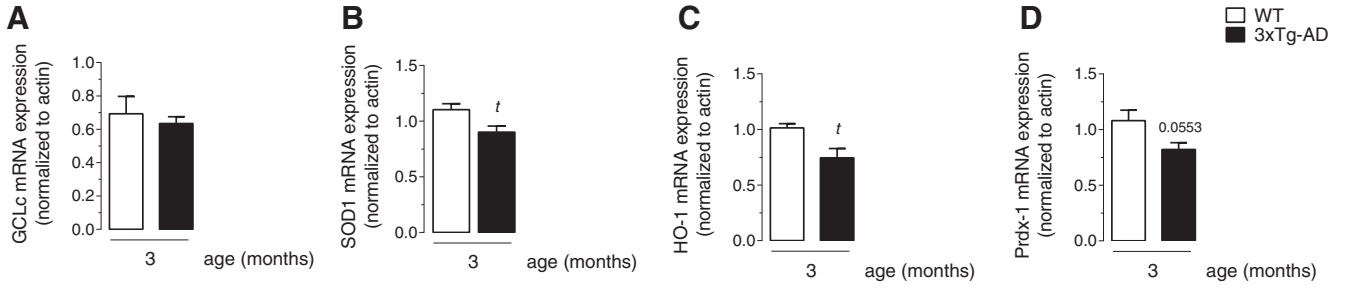

Fig. 9. mRNA levels of Nrf2 targets in young 3xTg-AD *versus* WT males. mRNA was extracted from 3 month-old mice male cortex and quantitative real time PCR was performed for analysis of GCLc (A), SOD1 (B), HO-1 (C) and Prdx-1 (D) mRNA. mRNA levels were normalized to actin mRNA levels. Data are the mean ± SEM of 5–6 animals per group. Statistical significance: $^{t}p < 0.05$ (Student's *t*-test).

dependent mechanisms regulating antioxidant gene expression, which may be mediated by differential activity of sex steroid hormones [12]. A late (15 month-old) decrease in nuclear Nrf2 in the cerebral cortex of 3xTg-AD males, compared to WT mice, might be concordant with the decrease in nuclear Nrf2 levels in aged human AD brains [66] suggesting that Nrf2-mediated transcription is not induced at late AD stage.

In the present study increased Nrf2 activation determined by increased p(Ser40)Nrf2 and enhanced nuclear Nrf2 levels was not associated with increased expression of Nrf2 targets. For instance, GCLc levels were consistently unaffected in AD models, namely human PBMCs and 3xTg-AD mice cerebral cortex, suggesting that early features of oxidative stress and ER stress in AD are unrelated with modified GSH synthesis. Data also support a decrease in expression of other Nrf2 targets acting as antioxidants and/or detoxifying proteins, namely SOD1, HO-1 and Prdx-1, revealing a possible impairment in Nrf2 activity as a transcription factor.

Importantly, our results support that changes detected in peripheral cells may reflect mechanistic alterations that occur in the brain, as depicted by the increase in p(Ser40)Nrf2 in PBMCs from prodromal MCI individuals and pre-symptomatic/young 3xTg-AD mice, along with increased nuclear Nrf2 in the cerebral cortex of 3 month-old 3xTg-AD mice males. We previously demonstrated similarities between human PBMCs and 3xTg-AD brain regarding BACE1 mRNA [48]. Moreover, a recent report demonstrated that in 3xTg-AD mice, the behavioral deficits develop simultaneously with systemic autoimmune/inflammatory disease, which in turn occurs before AD-like neuropathology, thus supporting a causal link between changes in peripheral cells and aberrant behavior [47]. Similarly to that was observed in the 3xTg-AD mice model, we can hypothesize that human PBMCs may reflect mechanistic alterations occurring in the brain and be used to identify new markers of disease progression.

In human PBMCs we did not observe significant differences between moderate plus severe AD patients and control individuals. Characterization of our population shows that groups are similar in terms of gender, age, ApoE genotype and educational level. AD patients could be distinguished from MCIs by the presence of lower levels of $A\beta_{1-42}$ and higher t-tau and p-tau levels in the CSF. Interestingly, correlation analysis showed R^2 values of 0.803 or 0.947, respectively, for correlations between CSF Aβ levels and pNrf2 or ROS levels in human PBMCs, indicating that decreased CSF Aβ levels positively correlate with diminished oxidative stress markers in human PBMCs. In fact, patients with lower levels of CSF Aβ (late-stage AD patients) presented lower levels of ROS and pNrf2 in PBMCs. Therefore, we hypothesize that unaltered oxidative stress parameters observed in PBMCs from moderate plus severe AD patients may be due to the fact that they are highly medicated, not only with AD-associated compounds, but also with psychopharmaceuticals, anti-cholesterol and anti-coagulation drugs, which may interfere with the measured parameters. In fact, ROS production by peripheral cells was shown to be inhibited by antipsychotic drugs such as risperidone [13] or anti-cholesterol drugs, namely statins [81,82]. ER stress was also shown to be affected by antipsychotic compounds [78]. Importantly, our study failed to show significant effects of Mem or AChE inhibitors, the most commonly prescribed molecules in AD pathology, in PBMCs, which, however, cannot exclude their beneficial effects in the central nervous system [61].

5. Conclusions

In conclusion, results obtained in this study provide evidence for an early cell dysfunction possibly arising from impairment in Nrf2 transcriptional activity and oxidative stress, along with ER stress-related Ca^{2+} dyshomeostasis, as found in PBMCs from human subjects at different stages of cognitive impairment and from 3xTg-AD mice regarding pNrf2. Moreover, data obtained using brain cortex from 3xTg-AD mice appear to correlate with the results obtained in both 3xTg-AD mice and human PBMCs, suggesting a concordance between the evidences of oxidative and ER stress markers observed in AD mice brain and the blood peripheral model of AD. These findings support that alterations occurring in PBMCs during pre-clinical and initial stages of AD might reflect brain modifications, possibly linked to synaptic dysfunction and neuronal loss. Therefore, studies conducted in PBMCs might be useful to identify possible targets for earlier detection and therapeutic intervention in AD.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.03.015.

Author's disclosure statement

None of the authors have any actual or potential financial conflict of interest related to this study.

Transparency document

The Transparencydocument associated with this article can be found, in the online version.

Acknowledgements

The authors thank the patients and families who generously contributed to this study. This work was supported by project QREN Diamarker "Desenvolvimento e Operacionalização da Investigação de Translação (DOIT), Lundbeck Foundation, and "*Fundação para a Ciência e a Tecnologia (FCT)*, Portugal, grant PEst-C/SAU/LA0001/2013-2014 and by the "*Programa Operacional Temático Factores de Competitividade*" (COMPETE), supported by the European community fund FEDER, and by FCT fellowships SFRH/BD/43430/2008, SFRH/BPD/99219/2013, SFRH/BD/28403/2006 and SFRH/BD/47573/2008.

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