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Original Contribution

Reductive stress in young healthy individuals at risk of Alzheimer disease

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ABSTRACT

Oxidative stress is a hallmark of Alzheimer disease (AD) but this has not been studied in young healthy persons at risk of the disease. Carrying an Apo ε4 allele is the major genetic risk factor for AD. We have observed that lymphocytes from young, healthy persons carrying at least one Apo ε4 allele suffer from reductive rather than oxidative stress, i.e., lower oxidized glutathione and P-p38 levels and higher expression of enzymes involved in antioxidant defense, such as glutamylcysteinyl ligase and glutathione peroxidase. In contrast, in the full-blown disease, the situation is reversed and oxidative stress occurs, probably because of the exhaustion of the antioxidant mechanisms just mentioned. These results provide insights into the early events of the progression of the disease that may allow us to find biomarkers of AD at its very early stages.

Introduction

Oxidative stress is a hallmark of Alzheimer disease (AD) [1–3]. However, the majority of studies dealing with this problem have measured oxidative stress markers in full-blown AD. It is very difficult, probably impossible, to foresee if a person will develop the disease later in life. But the events leading to AD start years, or even decades, before the onset of clinical symptoms of the disease [4]. Carrying an ApoE 4/4 genotype is one of the clearest and best established genetic risk factors for AD. ApoE 3/4-carrying persons also have an intermediate increased risk of developing the disease and those carrying ApoE 3/3 have lower risk [5,6]. We have shown in the past that proteins that are the result of an adaptation to oxidative damage (such as RCAN1) are increased in ApoE 4-carrying individuals compared with those carrying ApoE 3/3 [7]. On the other hand recent evidence has shown, using human embryonic kidney cells, that reductive stress may later on lead to oxidative stress in vitro [8]. p38 is a MAP kinase that is activated by oxidative stress and is involved in the pathophysiology of many neurodegenerative diseases [9], including AD [10]. It has a pivotal role in AD pathophysiology because of its activation in inflammation [11] and oxidative stress [12] and its role as a tau kinase of importance in the pathophysiology of AD [13–15].

The aim of this work was to determine markers of oxidative stress in healthy individuals who are descendants of AD patients and to relate them to their ApoE 4 genotype. We report that two markers of oxidative stress, p38 phosphorylation (P-p38) and glutathione oxidation, are increased in AD patients but, paradoxically, are decreased in healthy individuals at risk of developing the disease. The most relevant fact reported here is that healthy individuals at risk of developing AD suffer reductive stress years before the development of the disease. This stress may be due to a hyperactivation of antioxidant defenses, which are exhausted later in life. When this occurs oxidative stress takes place and the person may develop AD. Thus targeting reductive stress may be a strategy to delay, or even prevent, the onset of AD.

Material and methods

Healthy subjects

Fifty-four young healthy subjects were recruited, 33 carrying at least one Apo ε4 allele ($n = 17$ ε3/ε4 and $n = 16$ ε4/ε4), descendants of AD patients, and 21 carrying the ApoE 3/3 genotype (controls) without antecedents of familial AD. The exclusion criteria were situations that caused an increase in

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oxidative stress, such as neoplastic diseases, diabetes mellitus, uncontrolled hypertension, chronic renal failure, alcoholism, menopause, chronic inflammation, autoimmune diseases, chronic infection, or treatment with any antioxidant drug or refusal to sign the informed consent. The age range was 20–55 years.

We collected personal and demographic data, employment status and educational level, medical history, and treatment with drugs. We investigated stress and depression and subjective memory complaints. We also performed a neuropsychological evaluation in which we assessed memory using the Rey memory test and the Golden Stroop test. All subjects signed an informed consent. We took 10 ml of blood sample from the antecubital vein of each person.

AD patients

We recruited 58 patients diagnosed with probable AD at a mild to moderate stage diagnosed following the recommendations of NINCDS-ADRA. The inclusion criteria were not having concomitant diseases that could alter the oxidative status and not consuming antioxidants. We also recruited 25 age-matched subjects as controls with the same inclusion criteria except that of dementia diagnosis. All patients and controls signed an informed consent. We took a 10-ml blood sample from the antecubital vein of each person.

All protocols were approved by the ethics committee of the Hospital Clinico Universitario, Valencia, and all procedures were performed according to the Declaration of Helsinki for the ethical principles for medical research involving human subjects.

Analysis of the isoforms of ApoE

The analysis of the ApoE genotype was performed on lymphocytes by PCR. The gels were developed by staining with silver nitrate, using the method described by Beidler et al. [16].

Isolation of lymphocytes

We used Vacutainer CPT tubes (Becton–Dickinson, Rutherford, NJ, USA) to separate plasma, white cells, and erythrocytes. We removed the white ring containing mononuclear cells after centrifugation. Mononuclear cells were washed twice in phosphate-buffered saline and finally in fetal bovine serum to eliminate platelets. After incubation of cells on petri plates in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum for 3 h, the lymphocytes remained in the supernatant, whereas the rest of the cells adhered to the plates.

Determination of glutathione values

Oxidized glutathione (GSSG) was measured following an HPLC method developed in our laboratory and previously described in [17].

Western blotting analysis

Protein extracts from lymphocytes were mixed with an equal volume of sodium dodecyl sulfate (SDS) buffer (0.125 M Tris–HCl, pH 6.8, 2% SDS, 0.5% (v/v) 2-mercaptoethanol, 1% bromophenol blue, and 19% glycerol) and then boiled for 5 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, which were incubated overnight at 4 °C with appropriate primary antibodies: anti-p38 and anti-P-p38 (Cell Signaling; EMD Millipore Corp., Billerica, MA, USA). The protein levels of β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were measured as a loading control. Thereafter, membranes were incubated with a secondary antibody for 1 h at room temperature. Specific proteins were visualized by using the enhanced chemiluminescence procedure as specified by the manufacturer (Amersham, GE Healthcare Europe GmbH, Barcelona, Spain) and quantified by densitometry using a Bio-Rad scanning densitometer (Bio-Rad, Hercules, CA, USA). The protein of interest was normalized to the β-actin expression for each densitometry. We have included only a representative Western blot in each figure.

PCR

The RNA was isolated from lymphocytes with the PARIS Protein and RNA isolation kit (Ambion Austin, TX, USA) according to the manufacturer's instructions. For the reverse transcription (RT) reaction, 1 μg of purified RNA was transcribed using random hexamers with the cDNA Archive High Capacity kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription conditions were an initial incubation at 25 °C for 10 min, followed by cDNA synthesis reaction at 37 °C for 120 min and a final inactivation step of 5 min at 95 °C.

The measurement of mRNA levels was determined by quantitative PCR with the ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems). The specific primers used were obtained from Qiagen (Applied Biosystems). The PCR conditions were 10 min at 95 °C to activate the enzyme, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured in all samples with the aim of normalizing the expression of each gene, RNA quality, and efficiency of RT. The primers were from TaqMan Gene Expression Assays GCGC, Hs00155249_m1; GCLM, Hs00157694_m1; and GPX1, Hs00829989_gH. Each sample was tested in triplicate and the expression was calculated according to the method $2^{-\Delta\Delta Ct}$.

Statistical analysis

Results are expressed as means ± SD. Statistical analysis was performed by the least significant difference test, which consists of two steps: first an analysis of variance was performed. The null hypothesis was accepted for all numbers of those sets in which F was nonsignificant, at the level of $p>0.05$. Second, the sets of data in which F was significant were examined by the modified t test using $p\leq 0.05$ as the critical limit. We used the Student t test to compare two means in parametric samples and the Mann–Whitney test for no parametric samples. When we compared more than two means, we used the ANOVA for parametric samples and the Kruskal–Wallis test for nonparametric samples.

Results

p38 phosphorylation and glutathione oxidation in AD patients and in healthy individuals carrying ApoE 3/3, 3/4, or 4/4 genotype

Lymphocytes from AD patients exhibit an increase in P-p38 of approximately fivefold compared with controls (Fig. 1A). In contrast, healthy individuals who carry the ApoE 4/4 or 3/4 genotype have lower levels of P-p38 than controls (Fig. 1B).

We also measured glutathione oxidation in whole blood from AD patients and found that they have higher GSSG levels than age-matched nondemented individuals (Fig. 2A). As with P-p38, we found that descendants of AD patients who were still healthy but who carried the ApoE 3/4 or 4/4 genotype have a lower GSSG level than those carrying the ApoE 3/3 genotype (Fig. 2B).

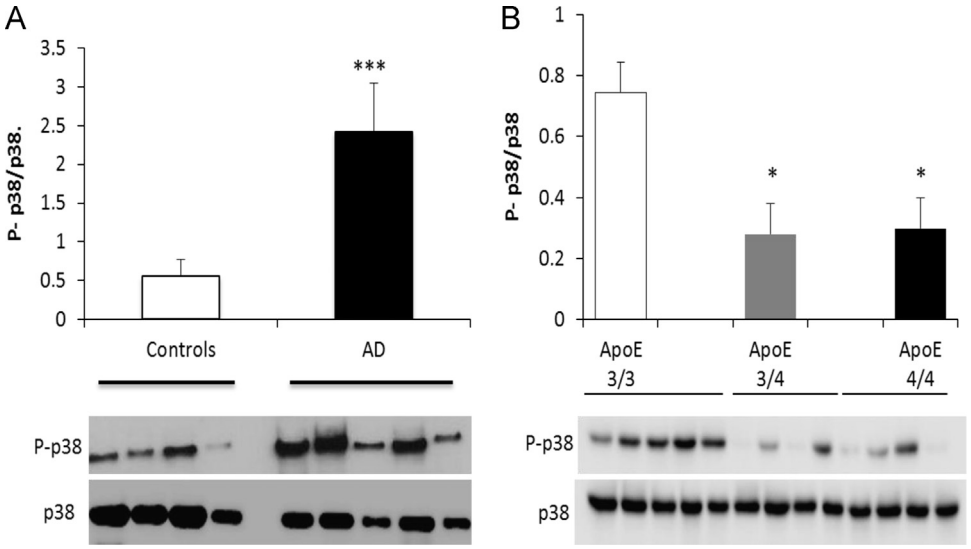

Fig. 1. Phospho-p38 levels in lymphocytes (A) of Alzheimer patients and age-matched nondemented controls and (B) of healthy young individuals carrying the ApoE 3/3, 3/4, or 4/4 genotype. P-p38 levels are higher in AD patients than in controls (indicating oxidative stress) but lower in healthy persons at risk of developing the disease, i.e., carrying the ApoE 3/4 or 4/4 genotype (indicating reductive stress). Protein levels were normalized to β-actin expression in each densitometry assay. Representative Western blots are shown. Values are means ± SD for the number of patients indicated under Material and methods. $^{***}p < 0.001$ vs controls (A) and $^{*}p < 0.05$ vs ApoE 3/3 (B).

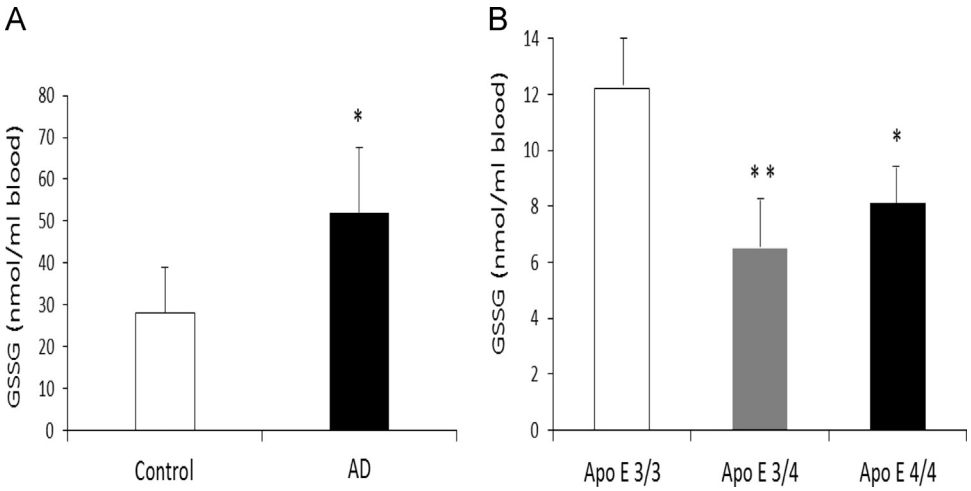

Fig. 2. GSSG levels in whole blood (A) of Alzheimer patients and age-matched nondemented controls and (B) of healthy young individuals carrying the ApoE 3/3, 3/4, or 4/4 genotype. GSSG levels were higher in AD patients than in controls (indicating oxidative stress) but lower in healthy persons at risk of developing the disease, i.e., carrying the ApoE 3/4 or 4/4 genotype (indicating reductive stress). Values are means ± SD for the number of patients indicated under Material and methods. $^{*}p < 0.05$ vs controls (A) and $^{*}p < 0.05$, $^{**}p < 0.01$ vs ApoE 3/3 (B).

We used our GSSG/GSH value to calculate the $NADP^+$/NADPH ratio. The detailed procedures were as follows: knowing that the equilibrium constant for the glutathione reductase reaction is $1.98 \times 10^{-2}\ M^{-1}$ [18], we used this constant to calculate the $NADP^+$/NADPH ratio. Values for this ratio in healthy persons, persons who are at risk of Alzheimer disease, and Alzheimer patients are reported in Table 1.

Thus we observed that reductive stress occurs in healthy individuals carrying the ApoE 4 compared with those carrying the ApoE 3/3 genotype. This turns to oxidative stress in patients suffering from full-blown AD.

Glutathione metabolism in AD

Glutamylcysteinyl ligase (GCL) is the major regulatory enzyme in the whole glutathione cycle [19]. It has been shown that overexpression of either the GCL catalytic subunit (GCLC) or the

Table 1
$NADP^+$/NADPH ratio in Alzheimer patients and in age-matched nondemented controls and healthy young individuals carrying the ApoE 3/3, 3/4, or 4/4 genotype.

Subjects	$NADP^+$/NADPH ratio
Old healthy subjects (controls)	0.98×10^{-3}
AD patients	1.6×10^{-3}
Young subjects ApoE 3/3	0.04×10^{-3}
Young subjects ApoE 3/4	0.02×10^{-3}
Young subjects ApoE 4/4	0.02×10^{-3}

We used our GSSG/GSH values to calculate the NADP/NADPH ratio knowing that the equilibrium constant for the glutathione reductase reaction is $1.98 \times 10^{-2}\ M^{-1}$ (see Ref. [18]).

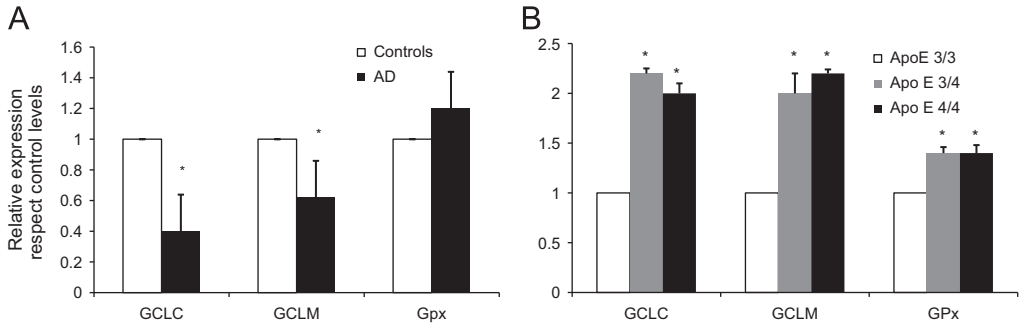

Fig. 3. Glutamylcysteinyl ligase (catalytic and regulatory subunits: GCLC and GCLM) and glutathione peroxidase (Gpx) levels measured by PCR in lymphocytes (A) of Alzheimer patients and age-matched nondemented controls and (B) of healthy young individuals carrying the ApoE 3/3, 3/4, or 4/4 genotype. The results are normalized to GAPDH gene expression level. Glutathione-related enzyme levels are lower in AD patients than in controls (A) but higher in healthy persons at risk of developing the disease, i.e., carrying the ApoE 3/4 or 4/4 genotype. Expression is presented relative to control levels, which were set at 1. Values are means ± SD for the number of patients indicated under Material and methods. $^{*}p < 0.05$ vs controls (A) or vs ApoE 3/3 (B).

GCL modifier subunit (GCLM) results in a reduced glutathione redox ratio [8]. Thus we measured GCLC and GCLM expression in lymphocytes from young, healthy individuals carrying the ApoE 4/4 or 3/4 genotype and from AD patients. Lymphocytes from AD patients (Fig. 3A) have a significantly lower expression of GCL than those from nondemented age-matched controls. In contrast, young healthy people at risk of developing the disease have an increased expression of this enzyme (see Fig. 3B). Glutathione peroxidase, another important enzyme catalyzing the reduction of GSSG, was higher in individuals at risk of developing the disease (see Fig. 3B) but was the same in AD patients and in normal age-matched controls. These results explain the paradoxical effect of having reductive stress in healthy persons at risk of developing the disease but oxidative stress in full-blown AD.

Discussion

The concept of oxidative stress as originally coined by Helmut Sies [20] has received plenty of attention in the scientific literature. Oxidative stress occurs in individuals suffering from the disease as well as in experimental models of AD. Healthy individuals at risk of developing AD because they are descendants of patients carrying the ApoE 4/4 genotype have not been studied in the context of oxidative stress. One of the major facts reported in this paper is that these young healthy individuals at risk of AD suffer from reductive (and not oxidative) stress. Reductive stress was observed recently in cultured cells and it triggers mitochondrial dysfunction and cytotoxicity [8]. The antioxidant response becomes exhausted probably because of the continuous formation of high levels of reactive oxygen species associated with the interaction between mitochondria and Aβ peptide. We acknowledge that this is not the only mechanism by with Aβ causes free radicals. But it is a “metabolic” one involving mitochondria. The persistent formation of radicals, we postulate, overruns the capacity of the cells to react and the cell passes from a status of reductive stress to one of oxidative stress. We have observed that, in fact, individuals enrolled in this study show an increased expression of stress-related proteins such as RCAN and calcineurin [7].

We used lymphocytes from patients because, for obvious ethical reasons, we cannot obtain brain samples. Previous research has shown that changes in lymphocytes reflect changes that are observed in brain [7,21,22]. Thus lymphocytes may be the best cells to search for biomarkers that reflect alterations in brain associated with Alzheimer disease.

Patients suffering from AD have an increased expression of P-p38 in their lymphocytes as well as an increased level of oxidized glutathione in their blood. Oxidative damage occurs in lymphocytes from patients with AD. Calabrese et al. [23] have shown that oxidative and nitrosative stress results in impaired protein metabolism.

In clear contrast, healthy individuals at risk of developing the disease have lower levels of P-p38 in their mononuclear cells and lower levels of oxidized glutathione in their blood than individuals at low risk.

Our results suggest an explanation for this paradoxical effect. We show that young healthy individuals at risk of developing AD overexpress antioxidant enzymes (Fig. 3). For instance, glutamylcysteinyl ligase, one of the most heavily regulated enzymes involved in antioxidant defense, is lower in AD patients than in controls, but is higher in healthy individuals at risk than in controls. Thus, it seems that persons at risk of developing AD have an increased generation of radicals [7], which leads to a hormetic overresponse in terms of antioxidant enzymes. When this response is exhausted, antioxidant enzymes become low. The continuous production of radicals, mainly due to the presence of Aβ, as we have previously observed [24], leads to a massive oxidative stress in cells that have lost the capacity to respond to it and then cell damage occurs and the clinical symptoms of the disease appear. This explains the apparently paradoxical fact that healthy individuals who are likely to develop AD suffer reductive stress that at some point disappears and then an “oxidative stress burst” occurs, contributing to the development of the full-blown disease. These young healthy people carrying the ApoE 4 genotype report more subjective complaints [7] and slightly impaired cognitive function [25] and have lower cerebrospinal fluid Aβ levels [26] than those carrying ApoE 3/3.

The hypothesis we are proposing fits within the general concept of the hormetic response. This has been previously postulated in the context of, for instance, physical exercise. In exercise, oxidative stress occurs especially because of the activation of NADPH oxidase and xanthine oxidase [27]. This stress leads to such an overactivation of antioxidant enzymes that exercise can be considered an antioxidant [28]. The difference in oxidative stress associated with AD is that in the case of the disease the radical production caused by Aβ could be of such magnitude that even if at first the cell responds with a hormetic antioxidant defense, this is lost after a so-far undefined period of time, thus leading to the oxidative stress seen in the full-blown disease.

Proteomic studies in brain from patients at early stages of AD show elevated levels of antioxidant proteins [29]. The lower markers of oxidative stress that we have seen in persons at risk had previously been observed in animal models. Shea et al.[38] observed that there are increased antioxidant levels in transgenic mice lacking ApoE compared to normal mice of identical genetic

background. Early work by Smith, Perry, and their colleagues [30] showed that there was a reductive compensation to oxidative stress in AD, but its occurrence in healthy young persons at risk was not studied. Another paradoxical effect, in this case in humans, is that the brain of young individuals carrying ApoE 4 have a significantly higher in vivo glucose consumption (as determined by positron emission tomography) than those carrying the ApoE 3 genotype [31,32]. Moreover, using functional (f)MRI, it was shown that in cognitively normal ApoE 4 carriers, the magnitude of brain activation in the parietal and prefrontal regions during memory tasks is higher than in controls and that the extent of brain activation correlates with subsequent memory decline in these subjects [33]. This hyperactivation at least temporarily coincides with the periods of reductive stress that we describe here.

Clifford et al. [4] have proposed a bilogistic model of AD and thus favor the idea of compensation. The results we report here and results from other authors from both fMRI and biochemical studies [34–36] support the idea that functional compensation is a major event in the course of AD.

Conclusions

The major facts reported in this paper are that persons at risk of AD suffer from reductive stress (indicators of oxidative stress being lower in healthy individuals at risk than in those with low risk of developing the disease) and that when the hormetic hyperresponse of antioxidant defenses collapses oxidative stress occurs and compensatory mechanisms such as the activation of p38 operate, leading to a hyperphosphorylation of tau and to the development of the dementia. Our results support the biphasic hypothesis of the pathogenesis of AD [37].

Acknowledgments

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