Free Radical Biology and Medicine 63 (2013) 274–279

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Reductive stress in young healthy individuals at risk of Alzheimer disease

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ARTICLE INFO

Article history:
Received 4 February 2013
Received in revised form
12 April 2013
Accepted 1 May 2013
Available online 7 May 2013

Keywords:
Apo E4
Antioxidants
Glutathione
Oxidative stress
Free radicals

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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http://dx.doi.org/10.1016/j.freeradbiomed.2013.05.003

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).