Oxidative imbalance in patients with mild cognitive impairment and Alzheimer’s disease

Ilaria Guidi a,1, Daniela Galimberti a,6,∗, Silvia Lonati b, Cristina Novembrino c, Fabrizia Bamonti b, Marco Tiriticco b, Chiara Fenoglio a, Eliana Venturelli a, Pierluigi Baron a, Nereo Bresolin a, Elio Scarpini a

a Department of Neurological Sciences, “Dino Ferrari” Center, University of Milan, IRCCS Ospedale Maggiore Policlinico, Via F. Sforza 35, 20122 Milan, Italy
b Department of Medical Sciences, University of Milan, IRCCS Ospedale Maggiore Policlinico, Via F. Sforza 35, 20122 Milan, Italy
c Department of Surgical Sciences, University of Milan, IRCCS Ospedale Maggiore Policlinico, Via F. Sforza 35, 20122 Milan, Italy

Received 23 October 2004; accepted 11 January 2005

Abstract

Increasing evidence supports a role of oxidative imbalance, characterized by impaired antioxidant enzymatic activity and increased reactive oxygen species (ROS) production, in mild cognitive impairment (MCI) and Alzheimer’s disease (AD) pathogenesis. Hyperhomocysteinemia, another risk factor for AD, also contributes to oxidative damage. Plasma total homocysteine (tHcy) and ROS levels, and total antioxidant capacity (TAC) were determined in 71 AD, 36 MCI and 28 vascular dementia (VaD) patients as well as in 44 age-matched controls. tHcy levels were significantly increased in patients with AD and VaD and a trend towards an increase in multiple domain MCI was observed. TAC was significantly decreased in AD as well as MCI, but not in VaD patients. In AD patients, a negative correlation was found between TAC and disease duration. ROS levels did not differ among groups, but were correlated with age.

In conclusion, a pattern characterized by increased tHcy levels and decreased TAC is present in AD as well as MCI patients. While increased tHcy levels were also found in VaD, TAC modifications occur specifically in AD. ROS levels appear to be correlated with age rather than with a specific dementing disorder, thus leading to the hypothesis that oxidative imbalance observed in AD could be due to a decreased TAC.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Mild cognitive impairment; Alzheimer’s disease; Total plasma homocysteine; Total antioxidant capacity; Reactive oxygen species

1. Introduction

Oxidative stress is supposed to play a relevant role in the pathogenesis of several neurodegenerative diseases, including Alzheimer’s disease (AD), which is the most common form of dementia in the elderly. Amyloid beta [1–42] deposition into the brain is considered a crucial step in AD development, as it originates a cascade of neuroinflammatory events leading to irreversible neuronal damage [1]. In particular, Aβ and other lesion-associated proteins are a major source of reactive oxygen species (ROS) and other toxic radicals [36]. Increasing evidence supports a role of oxidative stress and impaired energy metabolism in the pathogenesis of the disease: an increase in DNA, lipid and protein oxidation metabolites has been observed in blood as well as post-mortem brain samples from AD patients compared with healthy subjects [15]. Free radicals are produced by mitochondria, as a side product, during the reduction of molecular oxygen. The production of radicals is thought to be higher in cerebral tissue, particularly vulnerable to free radical damage because of its low content of antioxidants, high content of polyunsaturated fatty acids in neuronal membranes and high oxygen requirements for its metabolic process [6]. Further observation indicates reduced cerebral metabolism in AD [6] as well as reduced activities of specific mitochondrial enzyme complexes, such as cytochrome oxidase [8,11,16,20].
The alterations in these key enzymes can favour the aberrant production of ROS. Intracellular oxidative balance is tightly regulated and, therefore, an upregulation of antioxidant compensatory mechanisms would be expected in AD. The induction of Cu/Zn superoxide dismutase, catalase, glutathione peroxidase (GSH-Px), glutathione reductase (GSSG-R), peroxiredoxins and a number of heat shock proteins [2] suggests that vulnerable neuronal cells mobilize antioxidant defence in the face of increased oxidative stress [36]. On the other hand, the total antioxidant capacity (TAC, including glutathione, ascorbic acid, uric acid and bilirubin) was shown to be reduced by 24% in plasma samples from AD patients [28].

A link between oxidative stress and hyperhomocysteinemia, which is a known risk factor for the development of AD [32], has been hypothesized, as homocysteine (Hcy) influences DNA repair, promoting the accumulation of DNA damage caused by oxidative stress [13]. Recent in vitro studies demonstrate that Hcy increases levels of thiobarbituric acid reactive substances, which represent an index of peroxidation, and decreases levels of total-trapping antioxidant enzymes. Individuals with MCI, and subsequently without a simultaneous activation of new molecules of antioxidant enzymes [30] . In order to explain these results it has been suggested that the increased free radical production in MCI might lead to a rapid consumption of plasma antioxidants and subsequent oxidative stress are early events in AD evolution, and are probably secondary to other mechanisms specific to AD but not present in other neurodegenerative diseases [26]. On the basis of these studies, suggesting that oxidative imbalance may help to understand whether MCI is a prodromal stage of AD and whether a common pathogenesis between AD and MCI occurs, ROS, tHcy, and TAC were evaluated in samples from patients with AD, MCI and VaD, compared with age-matched healthy subjects. The correlation with age, gender, mini mental state examination (MMSE) score, age at onset, duration of the disease and with the presence of the Apol: e4 allele, a major genetic risk factor for sporadic AD [7], was also analyzed in the same groups of patients in order to verify the relationship of these factors with the most relevant clinical variables.

2. Materials and methods

2.1. Subjects

Between April 2002 and December 2003, the following subjects were consecutively recruited at the Alzheimer Unit of Ospedale Maggiore-IRCCS (Milan): 36 individuals with MCI, 71 with AD (38 with probable and 33 with possible AD), 28 with VaD. Seventy out of the 71 AD patients were LOAD. All patients underwent a standard battery of examinations, including medical history, physical and neurological examination, screening laboratory tests (including Hcy, folate and vitamin B12 determination), neurocognitive evaluation (to assess memory, language and constructional praxis), brain magnetic resonance imaging (MRI) or computed tomography (CT) and, if indicated, positron emission computed tomography (PET). Dementia severity was assessed by the clinical dementia rating (CDR) and the MMSE. Disease duration was defined as the time in years between the first symptoms (by history) and the clinical diagnosis. MCI diagnosis was made in accordance with Petersen et al.’s criteria (1999) [24]. According to recently proposed clinical criteria [23], 7 MCI patients presented only memory impairment (amnestic-MCI), 25 had a multiple domain impairment (MD-MCI), and 4 patients showed impairment in a single cognitive domain other than memory (SD-MCI). AD patients were diagnosed by exclusion according to NINCDS-ADRDA criteria [17] and VaD diagnoses met the NINDS-AIREN criteria. The control group consisted of 44 subjects matched for ethnic background and age, examined at the Department of Neurological Sciences of the Ospedale Maggiore-IRCCS (Milan). These subjects underwent the standard battery of examinations, and cognitive
impairment was excluded. All these control subjects did not develop dementia after 6 months’ follow up. An informed consent to participate in this study was given by all individuals or their caregivers. Both patients and controls were genotyped for ApoE status. Individuals with folate or Vitamin B12 low levels, malnutrition, major organ failure, smoking habit or vitamin supplementation have not been included. All the patients’ and controls’ characteristics are summarized in Table 1.

2.2. ApoE genotyping
High-molecular weight DNA was isolated from whole blood using a Flexigene Kit (Qiagen, Hilden, Germany) as described by the manufacturer. ApoE genotype was determined by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) assay. Briefly, genomic DNA was amplified using specific primers and then digested with HhaI. Fragments have been visualized on an agarose gel, as previously described [9].

2.3. Total Hcy determination
Plasma tHcy levels were measured using a commercial kit (IMx tHcy, Abbott Laboratories, Abbott Park, IL) on IMx analyzer (Diag cron). This method is based on HClO capacity to oxidize the physiologic antioxidant reef, including uric acid, glutathione, thiol groups, vitamins, glutathione-peroxidase, superoxide dismutase and catalase, as described by Campise et al. [5].

2.4. ROS determination
Serum ROS concentrations were measured by a spectrophotometric method using a commercial kit (d-ROMs test, Diacron, Grosseto, Italy) on FREE analyzer (Diacron), as previously described [5].

2.5. TAC determination
Serum TAC concentrations were measured by a spectrophotometric method using a commercial kit (OXY-Adsorbent test, Diacron, Grosseto, Italy) on FREE analyzer (Diacron). This method is based on HClO capacity to oxidize the physiologic antioxidant reef, including uric acid, glutathione, thiol groups, vitamins, glutathione-peroxidase, superoxide dismutase and catalase, as described by Campise et al. [5].

2.6. Statistical analysis
ApoE allelic and genotypic frequencies were obtained by direct counting. Hardy Weinberg equilibrium was tested by using a $\chi^2$ goodness of fit test. Fisher’s exact test was used to test for differences in allele distribution between the groups. Total Hcy, ROS and TAC values are expressed as mean ± S.E.M. Non-parametric Wilcoxon rank sum test incorporating the Bonferroni correction for multiple testing was used for comparisons among groups.

Table 2
<table>
<thead>
<tr>
<th>tHcy (µM)</th>
<th>Controls (n = 44)</th>
<th>MCI (n = 36)</th>
<th>AD (n = 71)</th>
<th>VaD (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>12.89 ± 0.62</td>
<td>14.80 ± 0.73</td>
<td>19.10 ± 0.85</td>
<td>21.05 ± 1.43</td>
</tr>
<tr>
<td>Males</td>
<td>13.81 ± 0.83</td>
<td>15.90 ± 1.26</td>
<td>19.53 ± 1.63</td>
<td>18.29 ± 1.41</td>
</tr>
<tr>
<td>Females</td>
<td>12.59 ± 0.79</td>
<td>14.31 ± 0.89</td>
<td>18.90 ± 0.99</td>
<td>24.24 ± 2.38</td>
</tr>
<tr>
<td>ROS (U. Carr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>351.44 ± 9.83</td>
<td>367.78 ± 10.41</td>
<td>353.39 ± 9.20</td>
<td>355.39 ± 11.69</td>
</tr>
<tr>
<td>Males</td>
<td>321.14 ± 12.53</td>
<td>365.27 ± 24.54</td>
<td>311.43 ± 12.05</td>
<td>273.47 ± 14.73</td>
</tr>
<tr>
<td>Females</td>
<td>361.53 ± 12.01</td>
<td>369.00 ± 10.83</td>
<td>373.50 ± 11.20</td>
<td>372.38 ± 14.76</td>
</tr>
<tr>
<td>TAC (µmol HClO/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>402.80 ± 11.49</td>
<td>370.00 ± 9.75</td>
<td>368.38 ± 5.70</td>
<td>402.03 ± 11.69</td>
</tr>
<tr>
<td>Males</td>
<td>419.54 ± 13.37</td>
<td>365.45 ± 17.51</td>
<td>371.22 ± 9.62</td>
<td>405.33 ± 14.70</td>
</tr>
<tr>
<td>Females</td>
<td>397.21 ± 10.67</td>
<td>372.00 ± 11.96</td>
<td>367.02 ± 7.14</td>
<td>398.23 ± 19.21</td>
</tr>
</tbody>
</table>

$^a$ P < 0.001, AD and VaD vs. controls.
$^b$ P < 0.001, females vs. males.
$^c$ P < 0.005, AD vs. either controls or VaD.
Spearman test was used for correlation with age and clinical data. The odds ratio (OR) was calculated along with its 95% CI.

3. Results

Total Hcy, ROS and TAC mean values ± S.E.M. in both patients and controls are reported in Table 2. Total Hcy levels were significantly increased in AD patients compared with controls (19.10 ± 0.85 μM versus 12.89 ± 0.62 μM, P < 0.001, Fig. 1A), with no difference between probable and possible AD patients (18.94 ± 1.07 and 19.29 ± 1.36 μM, respectively). Total Hcy concentrations were significantly higher in VaD patients (21.05 ± 1.43 μM) than in controls as well (P < 0.001, Fig. 1A). Notably, tHcy levels slightly increased were found in MCI patients (14.80 ± 0.73 μM) compared with controls (Fig. 1A). When dividing MCI patients according to the different impaired function, a trend towards an increase in tHcy levels was observed only in patients with MD-MCI, who can progress not only to AD but also to other kind of dementias [21,22], compared both with amnestic-MCI and SD-MCI patients (15.60 ± 0.98 μM versus 13.33 ± 1.14 and 12.37 ± 0.84 μM, respectively, Table 3, Fig. 1B). Moreover, tHcy highest mean levels were found in 10 patients with MD-MCI who showed a vascular component at imaging (17.35 ± 2.01 μM). On the basis of our patients’ data, Hcy levels higher than 14 μM (cut-off for increased AD risk [32]) increase the risk of developing either AD (OR: 5.69, 95% CI: 2.50–12.94) or VaD (OR: 16.11, 95% CI: 4.18–62.15).

As regards ROS levels, no significant difference was shown between patients and controls (Table 2, Fig. 2A), but significantly higher levels were found in females than in males (368.90 ± 6.24 versus 313.97 ± 8.56 U. Carr, P < 0.001, Fig. 2B). This tendency was observed in all groups studied, with significant differences in AD and VaD patients (Table 2).

TAC was significantly lower in AD patients than in either healthy subjects or VaD patients (368.38 ± 5.70 μmol HClO/ml versus 402.80 ± 11.49 or 402.03 ± 11.69 μmol HClO/ml, respectively, P < 0.005, Fig. 3), and TAC was almost the same in both probable and possible AD patients (371.92 ± 7.84 and 364.30 ± 8.40 μmol HClO/ml). Interestingly, TAC mean values in MCI patients were lower (370.00 ± 9.75 μmol HClO/ml, Fig. 3) than in controls and VaD patients. No difference was observed among MCI subtypes (Table 3). No correlation between ROS and TAC levels in each subject was observed (ρ = −0.01, P = 0.19). Any correlation between these variables in different groups was excluded by calculating the ratio between ROS and TAC levels.
Table 3
tHcy, ROS and TAC mean values ± S.E.M. in MCI patients

<table>
<thead>
<tr>
<th></th>
<th>Amnestic (n = 7)</th>
<th>Multiple domain (n = 25)</th>
<th>Single domain (language, n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tHcy (µM)</td>
<td>12.37 ± 0.84</td>
<td>15.60 ± 0.98</td>
<td>13.33 ± 1.14</td>
</tr>
<tr>
<td>ROS (U. Carr)</td>
<td>346.75 ± 14.87</td>
<td>364.24 ± 14.46</td>
<td>392.43 ± 13.13</td>
</tr>
<tr>
<td>TAC (µmol HClO/ml)</td>
<td>364.75 ± 22.99</td>
<td>371.36 ± 15.03</td>
<td>368.14 ± 19.45</td>
</tr>
</tbody>
</table>

Fig. 3. TAC mean values ± S.E.M. in MCI (n = 36), AD (n = 71) and VaD (n = 28) patients compared with control subjects (n = 44). *: P < 0.005 for AD vs. either controls or VaD patients.

Fig. 4. TAC values lower than 350 µmol HClO/ml (cut-off [5]) do not seem to increase the risk of developing AD.

A significantly positive correlation between tHcy levels and age was found in controls (ρ = 0.45, P = 0.002, Fig. 5), while tHcy levels were markedly higher in AD and VaD patients than in controls, independent of age.

Considering AD patients, a significantly positive correlation between ROS levels and age was found (ρ = 0.38, P = 0.001, Fig. 6A), while TAC did not correlate with age (data not shown). Besides, a tendency towards a slight negative correlation between TAC and the duration of the disease was found (ρ = −0.11, Fig. 6B). Total Hcy, ROS and TAC values did neither correlate with MMSE score or age at onset, nor any evidence of correlation was found among these parameters. Similarly, no correlation was found between ApoE status and the evaluated analytes. However, as expected, the frequency of the ε4 allele was significantly higher in AD patients than in controls (38.0% versus 11.3%, P < 0.003, Table 1), whereas in MCI and in VaD patients it was higher than controls (27.8 and 25.0%, respectively), but not to a significant extent (Table 1). The presence of a single copy of this allele increases the risk of developing AD (OR: 4.79, 95% CI: 1.68–13.64).

4. Discussion

According to the results shown, an alteration of some biochemical factors involved in oxidative stress occurs in AD patients. In fact, an increase in tHcy levels and a decrease...
in TAC were found in AD patients compared with controls, while no differences in ROS levels were found between AD patients and healthy subjects. However, higher tHcy levels were found not only in AD but also in VaD patients, to an even higher extent than in AD patients, while a decrease in TAC occurred specifically in AD patients. Both tHcy and TAC modifications seemed to be early events in the pathogenesis of AD, as a trend towards an alteration of these parameters was also observed in MCI, which is considered a transitional state between the cognition of normal ageing and mild dementia [23].

Our data on tHcy levels in AD patients are in accordance with previous findings [32], whereas the increase in tHcy levels observed in our MCI patients is in contrast to previous observations [27]. However, in the above mentioned study, the control group showed tHcy levels higher than the proposed cut-off value (14 μM), and this could cause a lack of significance when comparing controls with MCI patients. Indeed, the increase of tHcy in our MCI group is quite slight compared with tHcy levels in AD. Considering that 70 out of the 71 patients were LOAD and they were older than MCI patients, it is reasonable a co-occurrence of other pathological condition which may influence tHcy levels in this group, as previously suggested [12].

On the basis of recently proposed clinical criteria [22,23], MCI is a heterogeneous pathological condition including different clinical subtypes: amnestic-, MD- and SD-MCI. In this regard, our MD-MCI patients, who can theoretically progress towards many different forms of dementia, including VaD, had higher levels of tHcy than amnestic-MCI patients, who are likely to convert to AD [18]. Moreover, within each subtype, there are a variety of causes, with a prevalent degenerative or vascular component [22]. Among our MD-MCI patients with a vascular component at the imaging had even higher tHcy levels than MCI patients with only a degenerative component, again supporting the hypothesis that tHcy might be related mainly to vascular elements. However, our MCI groups are too small to reach statistically significant conclusions. Notably, as lower folate and Vitamin B12 concentrations may lead to an increase in tHcy levels in a normal population [29], only subjects with Vitamins B12 and folate levels within the relevant reference interval were included in this study. Although a correlation between tHcy and age has been found in controls, tHcy levels were markedly higher in AD and VaD patients than in healthy subjects, and no correlation was found either with age at time of sampling or at onset.

In contrast to tHcy, there was a decrease of TAC mean values in AD, but not in VaD, supporting the hypothesis that oxidative status may be specifically altered in AD but not in other neurodegenerative diseases [26], such as VaD. Decreased TAC was found in MCI as well, independently of the different subtypes. However, a longitudinal analysis of each MCI patient would be needed to clarify whether TAC impairment is associated with conversion to AD. Our data on TAC are in accordance with previous results [30], demonstrating that plasma antioxidants are similarly depleted in both MCI and AD patients, although the antioxidant panel in our study was determined using a different method. Moreover, these findings further strengthen previous observations indicating that oxidative damage is likely to be an early event during AD pathogenesis [19].

The decreased TAC mean values observed in AD and MCI compared with controls are within the reference levels indicated by the supplier (>350 μmol HClO/ml), and OR calculated with this cut-off suggests that decreased TAC does not seem to act as a risk factor for either AD or MCI. Nevertheless, it should be taken into account that we are at present faced with a lack of information about TAC normal reference levels in elderly. On the basis of our results, there is no correlation between TAC and aging, but further studies on a larger population are needed to verify if a cut-off value for increased risk could be identified in elderly people. In AD patients, TAC was inversely correlated with the duration of the disease, suggesting a possible role during progression, even though no correlation was found with the degree of cognitive impairment quantified with the MMSE.

ROS levels appear to be correlated with age rather than with a specific dementing disorder. This consideration leads to the hypothesis that oxidative imbalance observed in AD is mainly due to a decreased TAC rather than to an increased production of ROS. This hypothesis previously suggested also by other authors [26,30] is strengthened by the absence of correlation between ROS and TAC levels as well as by different ratios between these two variables in studied groups (Fig. 4). The observed trend towards a negative correlation between TAC levels and the duration of the disease further supports the role of oxidative imbalance during AD progression. Indeed, as previous findings indicate an increase in oxidative species in AD pathogenesis [2,6,15], it cannot be excluded that the observed decreased TAC levels could be the result of antioxidants being used up to maintain ROS at normal level.

As expected, the presence of the ApoE ε4 allele increases the risk of developing AD, while it is not a susceptibility factor for VaD. In this regard, there is conflicting evidence, as some studies demonstrated an increased ε4 frequency in VaD, similar to that found in AD [35], whereas recent findings failed to confirm any association of this allele with VaD [10,34].

In conclusion, oxidative imbalance seems to play a role in the pathogenesis of AD, for a specific pattern, characterized by higher tHcy and lower TAC levels, was observed in AD patients. Increased tHcy levels were found in VaD as well, while decreased TAC was specific to AD only. These modifications are present in MCI but not to a significant extent. ROS levels did not differ among the groups analyzed, implying that TAC is likely to be the crucial factor leading to oxidative imbalance. However, these preliminary data need further confirmation. A longitudinal analysis of MCI patients will be the next step of the research.
Acknowledgements

This work was supported by grants from Associazione “Amici del Centro Dino Ferrari”, CARIPLO and Monzino Foundations, IRCCS Ospedale Maggiore Milano and Centre of Excellence for Neurodegenerative Diseases of the University of Milan. The authors are very grateful to Mrs. Mary Coduri for linguistic consultation.

References


