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Original Contribution

INCREASED LIPOPROTEIN OXIDATION IN ALZHEIMER'S DISEASE

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Abstract—Oxidation has been proposed to be an important factor in the pathogenesis of Alzheimer's disease (AD) and amyloid β is considered to induce oxidation. In biological fluids, including cerebrospinal fluid (CSF), amyloid β is found complexed to lipoproteins. On the basis of these observations, we investigated the potential role of lipoprotein oxidation in the pathology of AD. Lipoprotein oxidizability was measured in vitro in CSF and plasma from 29 AD patients and found to be significantly increased in comparison to 29 nondemented controls. The levels of the hydrophilic antioxidant ascorbate were significantly lower in CSF and plasma from AD patients. In plasma, α-carotene was significantly lower in AD patients compared to controls while α-tocopherol levels were indistinguishable between patients and controls. In CSF, a nonsignificant trend to lower α-tocopherol levels among AD patients was found. Polyunsaturated fatty acids, the lipid substrate for oxidation, were significantly lower in the CSF of AD patients. Our findings suggest that (i) lipoprotein oxidation may be important in the development of AD and (ii) the in vitro measurement of lipid peroxidation in CSF might become a useful additional marker for diagnosis of AD. © 2000 Elsevier Science Inc.

Keywords—Alzheimer's disease, Antioxidants, Cerebrospinal fluid, Free radicals, Lipid peroxidation, Lipoproteins, Plasma

INTRODUCTION

Alzheimer's disease (AD) is a demential disorder with increasing prevalence in the elderly population in the Western world. Senile plaques and neurofibrillary tangles are salient features in AD brains at autopsy and the histopathological hallmarks of clinical dementia. Apart from rare cases of early onset AD with causative mutations in the amyloid precursor protein (APP) or presenilin (*PS 1* and *PS2*) genes [1], the etiology of AD is multifactorial [2]. In the framework of such a concept, several authors have proposed a pivotal role for oxidation in the pathogenesis of AD in recent years [3–7]. The central nervous system is especially vulnerable to oxidative stress as a result of the brain's high oxygen consumption, abundant lipid content, and relative paucity of antioxidant compounds compared with other tissues [8,9]. Evidence for increased oxidative damage in AD includes studies showing that brain tissue from AD patients has higher levels of oxidized proteins [3], advanced glycation end products [10,11], and 4-hydroxynonenal-derived adducts [12,13] than tissue from nondemented elderly controls. Lipid peroxidation is increased in the brain in AD [14] and the transition metal ions Cu(II) and Fe(III), capable of producing reactive oxygen species, have been shown to be elevated in AD brain tissue [15,16]. In addition, APP can reduce Cu(II) to Cu(I), a highly reactive form [17].

The oxidation hypothesis is supported by the initial large clinical trial that proposed a beneficial effect of α-tocopherol and selegiline by slowing the progression of the disease [18]. Alpha-tocopherol is also known to be effective against lipid peroxidation and to reduce the neurotoxicity of amyloid β (Aβ), a major component of senile plaques [19]. The exact mechanisms responsible for increased oxidation in AD brain remain unclear and there is not yet enough evidence to decide whether oxidation is a primary phenomenon inducing neurodegeneration or is secondary to cell death and loss of neurons.

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Aβ has been implicated as an oxidant involved in the pathogenesis of AD [5,6,20]. Two facts make Aβ potentially important for lipid peroxidation: soluble Aβ is found in biological fluids like cerebrospinal fluid (CSF), which is in direct contact with the brain [21], and in both CSF [22] and plasma [23], Aβ is complexed to lipoproteins.

Lipoproteins in the density range of plasma high-density lipoproteins (HDL) have been found in CSF [24,25]. They contain polyunsaturated fatty acids (PUFA), the major substrate for lipid peroxidation, as well as lipophilic antioxidants such as tocopherols. Plasma lipoproteins have been shown to be highly susceptible to oxidative modifications [26], a mechanism playing a crucial role in the pathogenesis of atherosclerosis. Changes in the chemical composition of CSF lipoproteins have been recently reported in AD [27]. We recently found that lipoproteins of human CSF are easily oxidized in vitro [28]. The increased oxidation damage in the brain of AD patients led us to believe that lipoprotein oxidation could be important in AD as it is in atherosclerosis. To assess this hypothesis, we analyzed plasma and CSF samples from 29 AD patients and 29 nondemented controls for their lipid content, the amount of lipophilic and hydrophilic antioxidants, and the oxidizability of the samples in vitro. We found that CSF lipoproteins in AD patients were more susceptible to oxidation than those from nondemented controls. The increased lipoprotein oxidation in CSF can provide a useful additional marker in the clinical diagnosis of AD and in particular it might allow a verification of the effects of antioxidant therapy.

MATERIALS AND METHODS

Subjects

AD patients ($n = 29$) and control subjects ($n = 29$) were recruited in the psychiatric clinic and the neurological clinic of Hamburg University Hospital. The AD patients were all seen in the outpatient "memory clinic" and diagnosed as "clinically probable" according to the NINCDS-ADRDA and DSM-IV criteria for primary degenerative dementia, Alzheimer type [29]. All AD patients were in an early stage of the disease, mobile, in a good general nutritional state, and did not take antioxidant supplements. The control subjects attended the neurological clinic and underwent lumbar puncture for diagnostic purpose. Patients with degenerative disorders were excluded, as were all patients with clinically evident cognitive impairment. Informed consent according to the declaration of Helsinki was obtained before lumbar puncture and the study was approved by the Ethical Committee, Hamburg.

Sample collection and preservation

From each patient, 1 ml of CSF (obtained as a surplus of diagnostic lumbar puncture) and 10 ml of ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood were sampled at the same visit and immediately placed on ice. Blood was centrifuged at 4°C for 10 min at 2500 rpm to obtain plasma and the cellular buffy coat for DNA preparation. CSF and plasma were freshly frozen under argon or nitrogen at −80°C, not later than 30 min after puncture. The buffy coat was stored at −20°C. Samples were not stored longer than 3 months. The samples were thawed at room temperature immediately before analysis.

CSF and plasma oxidation kinetics

Oxidation of CSF and plasma was monitored as a change in the sample absorbance at 234 nm. This parameter has been shown to reflect the level of lipid hydroperoxides in isolated low-density lipoprotein (LDL) oxidized under in vitro conditions [30]. Lipid hydroxides are other products of LDL oxidation that have conjugated diene structure and specifically absorb at 234 nm. However, they comprise only a small percentage of hydroperoxides formed during lipoprotein oxidation [31]. When lipoproteins are oxidized in diluted plasma or CSF, changes in the absorbance at 234 nm correlate with other indices of lipid peroxidation, such as consumption of PUFAs and accumulation of cholesterol linoleate hydroperoxide [28,32]. Furthermore, when oxidized plasma was treated with sodium borohydride, to eliminate hydroperoxides, and then extracted with hexane, no increase in the absorbance of the extract at 234 nm was found in comparison with unoxidized samples (data not shown). These data justify the use of absorbance at 234 nm as a specific measure for the accumulation of lipid hydroperoxides in the lipoproteins.

To register oxidation kinetics, CSF was diluted 10-fold with phosphate-buffered saline (PBS), containing 0.6 M NaCl, pH 7.4, treated with Chelex 100 ion-exchange resin (Bio-Rad, Munich, Germany) for 1 h to remove transition metal ions. The samples were oxidized at 37°C either in the absence (autoxidation condition) or in the presence of the exogenous oxidant 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH; Polysciences, Inc., Warrington, PA, USA) at 100 μM. The absorbance was continuously registered spectrophotometrically at 5 min intervals over 50 h at 37°C in quartz cuvettes tightly sealed with Nescofilm to prevent evaporation.

Plasma was diluted 150-fold with PBS and incubated at 37°C for 20 h in the absence of exogenous oxidants (autoxidation condition) or in the presence of AAPH (330 μM) [33]. The absorbance was measured at 234 nm

as described for CSF and the formation of conjugated dienes was quantified by the mean oxidation rate during the initial linear phase.

CSF and plasma lipids

CSF and plasma fatty acids and CSF cholesterol were measured by capillary gas chromatography with flame ionization detection [34]. One hundred microliters of CSF were mixed with 2 ml chloroform/methanol (2:1 vol/vol), and 100 μl of heptadecanoic acid (200 mg/l) and 25 μl 5α-cholestane (100 mg/l) were added as internal standards; 25 μl butylated hydroxytoluene (BHT, 0.2 M) was added as antioxidant. The chloroform extract was evaporated under nitrogen, the dried lipids were dissolved in 250 μl toluene, and fatty acids were derivatized with 500 μl of 0.5 M anhydrous sodium methoxide for 15 min at 50°C. The mixture was neutralized with 1 ml 2.5% acetic acid and extracted with 250 μl hexane. The supernatant was evaporated under nitrogen and 100 μl dimethylformamide were added. Cholesterol was silylated by incubation with N,O-bis(trimethylsilyl)trifluoroacetamide for 30 min at room temperature. After a final evaporation, the pellet was dissolved in 20 μl toluene, 2 μl of which were injected into a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett Packard, Palo Alto, CA, USA). CSF saturated fatty acids (SFA) were calculated as the sum of palmitic, stearic, and arachidic acids; monounsaturated fatty acid (MUFA) was defined as oleic acid; and PUFA was defined as the sum of linoleic and arachidonic acids.

Plasma fatty acids were measured by capillary gas chromatography with flame ionization detection as described elsewhere [34]. Plasma cholesterol and triglycerides were quantified by commercially available enzymatic kits (Boehringer Mannheim, Mannheim, Germany).

Lipophilic antioxidants

Alpha-tocopherol, α- and β-carotene, ubiquinol-10, and ubiquinone-10 were measured as the main lipophilic antioxidants in plasma. For CSF, only the levels of α-tocopherol and β-carotene were determined. The antioxidant content was quantified by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection as described elsewhere [35], except that the system was calibrated using an external standard method.

Hydrophilic antioxidants

Ascorbate, the major hydrophilic antioxidant in CSF, was measured by reversed-phase HPLC with ultraviolet (UV) detection at 267 nm [36]. One hundred microliters CSF were diluted 1:1 with 10% meta-phosphoric acid and centrifuged for 3 min at 13,000 rpm and 4°C. One hundred microliters of the supernatant were injected into the HPLC system using a solution of 0.1 M Na_2HPO_4, 2.5 mM EDTA, and 2.0 mM tetrahexyl ammonium chloride, pH 3.0, as a mobile phase, running at 1.0 ml/min. Plasma ascorbate was measured photometrically as described elsewhere [37].

Apolipoprotein E genotyping

The apolipoprotein (Apo) E genotype was determined using the restriction isotyping method as described elsewhere [38].

Statistical analysis

Between-group differences in continuous variables were analyzed by Student's *t*-test for independent groups. Differences in dichotomous variables were analyzed by Fisher's exact test. Pearson's moment-product correlation coefficients were calculated to evaluate relationships between variables. Multiple regression was performed on oxidation parameters to elucidate the extent to which the oxidizability was specifically influenced by the presence of the disease, rather than by other independent factors. All results are expressed as means $\pm$ standard deviations. The quality of the assays was controlled by measuring the assay variability, which was not higher than 8% for all the parameters measured [28,33,35].

RESULTS

Characterization of patients

Clinical data of 29 AD patient and 29 controls are given in Table 1. The AD patients had a mean Mini Mental Status Examination score of 19 $\pm$ 5. Due to the significantly higher mean age of the AD patients (72 vs. 55 years), we performed a subgroup analysis including only individuals $\geq$ 60 years to eliminate a possible age-related influence on the data. Twenty-six patients remained in the AD group and 14 in the age-matched control subgroup. All data were analyzed for the total group and the individual subgroups. The mean age of onset was 68 years in the total AD group and 70 years in the AD subgroup, and the mean time since diagnosis of the disease was 3.6 years in both groups.

As expected, the frequency of the ε4 allele of Apo E was significantly higher in the AD patients, .36 vs. .07 in controls. This frequency is in good accordance with epidemiological data [39]. The between-group comparison revealed a significantly higher rate of cur-

Table 1. Study Population

	AD patients		Control subjects	
	All (n = 29)	≥60 (n = 26)	All (n = 29)	≥60 (n = 14)
Age	71.7 ± 10.1*	73.9 ± 7.6	55.1 ± 18.8	70.3 ± 8.3
Sex (M/F)	14/15	11/15	16/13	8/6
Smoking (y/n)	2/27*	2/24	9/20	3/11
CHD (y/n)	2/27	2/24	4/25	4/10
Hypertension (y/n)	9/20	9/17	7/22	5/9
Diabetes (y/n)	1/28	1/25	2/27	2/12
Plasma cholesterol (mg/dl)	216.6 ± 43.7	218.5 ± 41.4	206.5 ± 45.6	208.6 ± 46.9
Plasma triglycerides (mg/dl)	133.0 ± 52.9	126.8 ± 45.2	157.4 ± 70.0	160.7 ± 59.4
Apo E ε4 allele frequency	0.36*	0.33†	0.07	0.07

* $p < .01$, † $p < .05$ vs. corresponding control group.

rent smokers among the controls. No other parameter of potential interest in the context of oxidative stress, such as presence of coronary heart disease (CHD), hypertension and diabetes or plasma lipids showed a significant between-group difference (Table 1).

Lipoprotein oxidation in CSF

Given the difficulties in measuring oxidative stress in vivo, we decided to assess lipid peroxidation in biological fluids, in which lipids are present as lipoproteins. We previously developed a method to record lipoprotein oxidation in human plasma via photometric measurement of the kinetic of conjugated diene formation [33], a protocol that was subsequently adapted for the characterization of lipoprotein oxidation in CSF [28].

A typical time course of the absorbance at 234 nm exhibited three consecutive phases: the lag phase, during which the oxidation rate was close to zero; the propagation phase, representing rapid accumulation of lipid peroxides; and the plateau phase with an oxidation rate close to zero again (Fig. 1A). The absorption of oxidizing CSF was found to parallel the time course of phosphatidylcholine hydroperoxides accumulation measured by HPLC with UV detection (Fig. 1B) and was correlated with the consumption of antioxidants in the sample [28].

Typical oxidation kinetics from three AD patients and three controls are shown in Fig. 2. AD patients exhibited an increased oxidation rate in the lag phase and a clearly shorter lag phase duration, indicating a more rapid oxidation of CSF lipoprotein in AD patients compared with controls. To compare the kinetics for all subjects, the mean oxidation rate during the initial phase and the lag

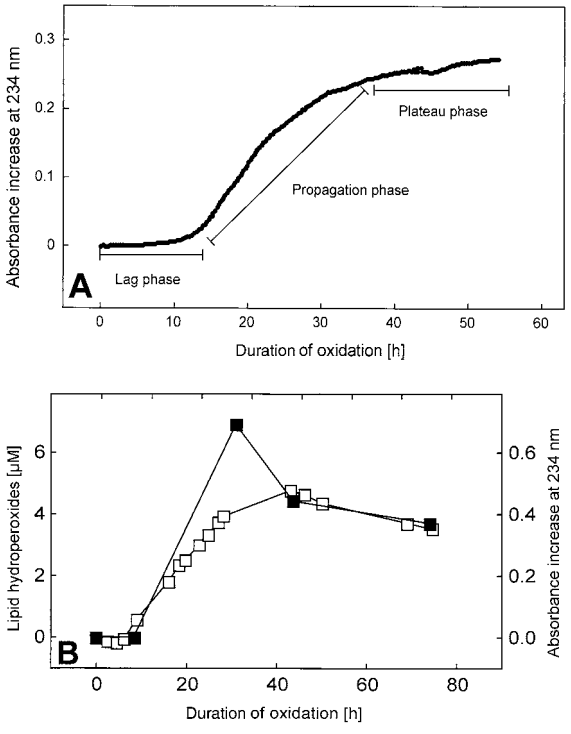

Fig. 1. (A) Typical absorbance increase of CSF at 234 nm during its autoxidation. Time points were taken every 5 min. CSF was diluted 10-fold and incubated at 37°C in the sealed cuvettes. (B) Lag, propagation, and plateau phases of the oxidation. As in (A), but the accumulation of lipid hydroperoxides was measured by absorption increase at 234 nm (□) and HPLC (■); fewer time points were sampled, as indicated by the symbols.

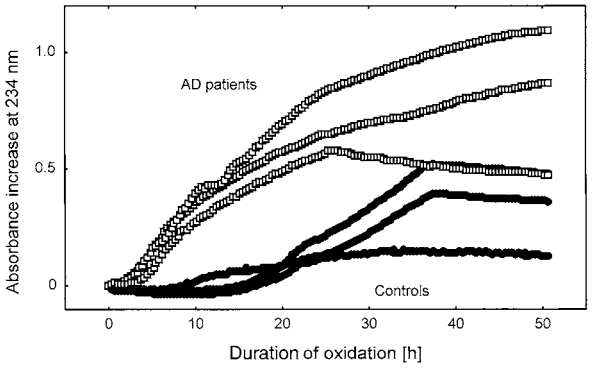

Fig. 2. Typical absorption increase at 234 nm of CSF obtained from three representative AD patients (□) and three representative control subjects (●). CSF was diluted 10-fold and incubated at 37°C in the absence of exogenous oxidants to determine autoxidation.

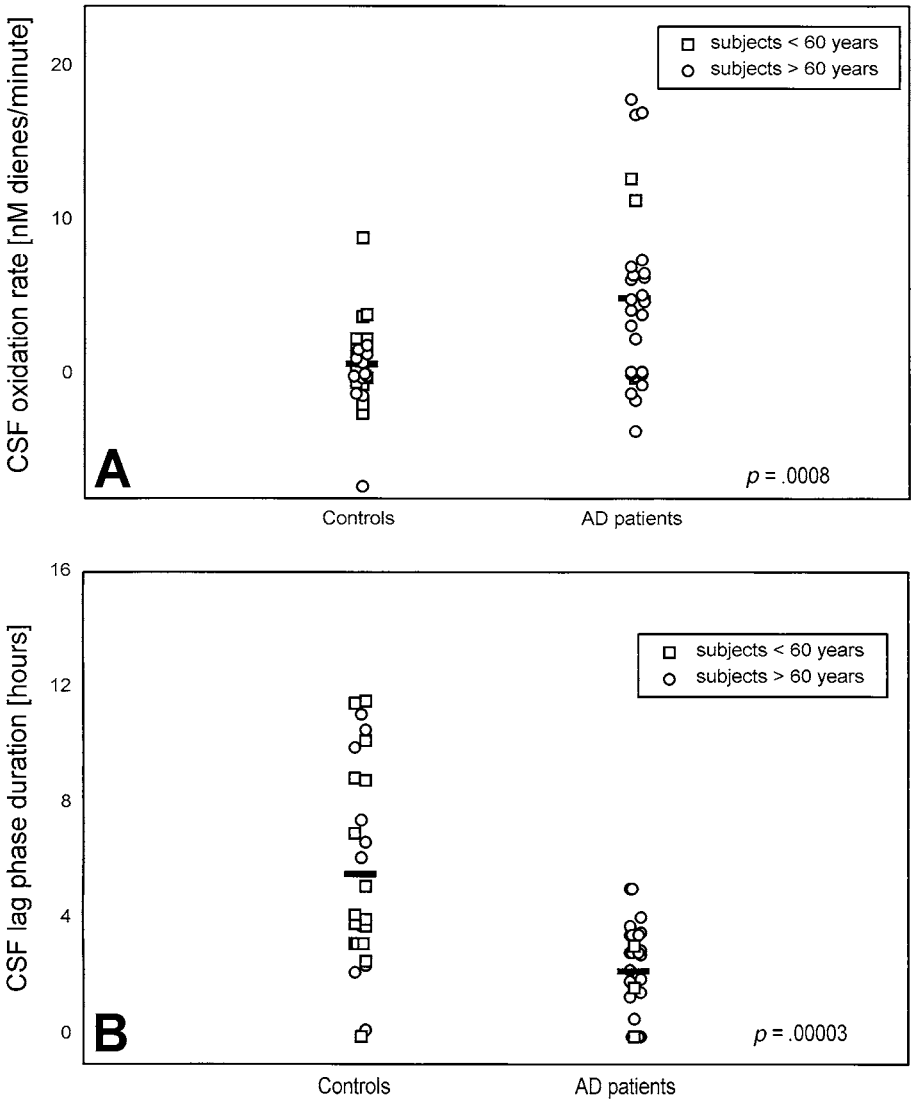

Fig. 3. (A) Rate and (B) lag phase of autoxidation of CSF obtained from AD patients and control subjects. CSF was diluted 10-fold with PBS and incubated at 37°C in the absence of exogenous oxidants. Bars correspond to the mean values calculated for each group. Each open circle (○) and open square (□) corresponds to one subject older and one subject younger than 60 years, respectively. Significance of the difference between the groups is shown as a p value.

phase duration were calculated for each curve. When both parameters were compared between all AD patients and all controls, the oxidizability of CSF from patients with AD was found to be significantly higher. The mean oxidation rate during the initial phase expressed as nanomoles of dienes liter per minute (nM/min), was significantly higher ($p = .0008$; Fig. 3A) and the duration of the lag phase was significantly shorter ($p = .00003$; Fig. 3B) in the AD group. The age of the patients had no influence on the sample oxidizability, so that the difference remained similarly significant in the age-matched subgroups. The differences were similarly pronounced when AAPH, a chemical initiator of the oxidation, was added to the samples (data not shown).

There was no significant difference between the CSF samples of patients with AD and controls in the levels of total protein, cholesterol, total fatty acids (TFA), and MUFA (Table 2). These data indicate that differences observed in other parameters cannot be due to differences in CSF volume. The relative level of PUFA in CSF (expressed as a percentage of TFA) was significantly lower in the patients with AD ($p = .001$), whereas SFA were found to be relatively increased ($p = .02$; Table 2). There was a negative correlation between the relative PUFA content and both the autoxidation rate and the oxidation rate with AAPH ($r = -0.29$, $p = .03$; $r = -0.43$, $p = .001$, respectively).

To complement these methods, the levels of hydro-

Table 2. Protein, Lipids, and Antioxidants in CSF of AD Patients and Control Subjects

	AD patients		Control subjects	
	All (n = 29)	≥60 (n = 26)	All (n = 29)	≥60 (n = 14)
Total protein (mg/l)	439 ± 131	438 ± 130	438 ± 167	447 ± 123
Cholesterol (mg/l)	6.8 ± 2.5	6.9 ± 2.5	7.5 ± 2.6	7.8 ± 2.9
TFA (μM)	39.7 ± 34.5	41.4 ± 36.2	28.3 ± 21.5	31.6 ± 30.9
PUFA (%)[a]	11.0 ± 4.9*	10.8 ± 5.1[‡]	15.0 ± 2.7	15.6 ± 2.8
MUFA (%)[a]	35.4 ± 13.0	34.3 ± 13.4	37.9 ± 7.1	35.6 ± 8.7
SFA (%)[a]	53.6 ± 16.4[†]	54.9 ± 16.8	46.1 ± 3.2	46.8 ± 3.6
Ascorbate (μM)	166.7 ± 40.9[†]	163.8 ± 40.6[†]	193.4 ± 27.7	199.5 ± 28.1
α-Tocopherol (nM)	45.5 ± 34.6	46.1 ± 34.9	56.7 ± 28.4	64.3 ± 31.6
β-Carotene (nM)	2.1 ± 0.9	2.1 ± 0.9	1.9 ± 1.4	1.7 ± 1.0

[a] Weight percentage of TFA. * $p < .001$; [†] $p < .01$; [‡] $p < .05$ vs. corresponding control group.

philic and lipophilic antioxidants were measured in the same samples (Table 2). Ascorbate, the major hydrophilic antioxidant in CSF [40], was significantly lower in the both the total group and the age-matched AD subgroup compared to controls ($p = .006$ and .007, respectively). Additionally, as expected, ascorbate levels were negatively correlated with CSF autoxidation rate ($r = -0.32$, $p = .02$). Alpha-tocopherol, the major lipophilic antioxidant in lipoproteins, tended to decrease in AD patients' CSF but this difference did not reach statistical significance. The difference in β-carotene values was negligible (Table 2).

Lipoprotein oxidation in plasma

The plasma oxidation kinetics observed in the present study were in accordance with previously reported data in patients without AD [33]. When the mean initial oxidation rates were compared, the rates in AD plasma samples were significantly higher. This was the case for both autoxidation and oxidation by AAPH, which increased the oxidation rates in both groups in parallel (Fig. 4).

When plasma lipids of patients with AD and controls were compared, neither triglycerides and total cholesterol (Table 1), nor total fatty acids, saturated, monounsaturated, and polyunsaturated fatty acids (data not shown) differed between groups.

The levels of antioxidants in plasma were in good accordance with the results of the oxidation kinetics (Table 3). In the total AD group, the hydrophilic antioxidant ascorbate was significantly decreased ($p = .02$), but was only slightly lower in the age-matched AD subgroup

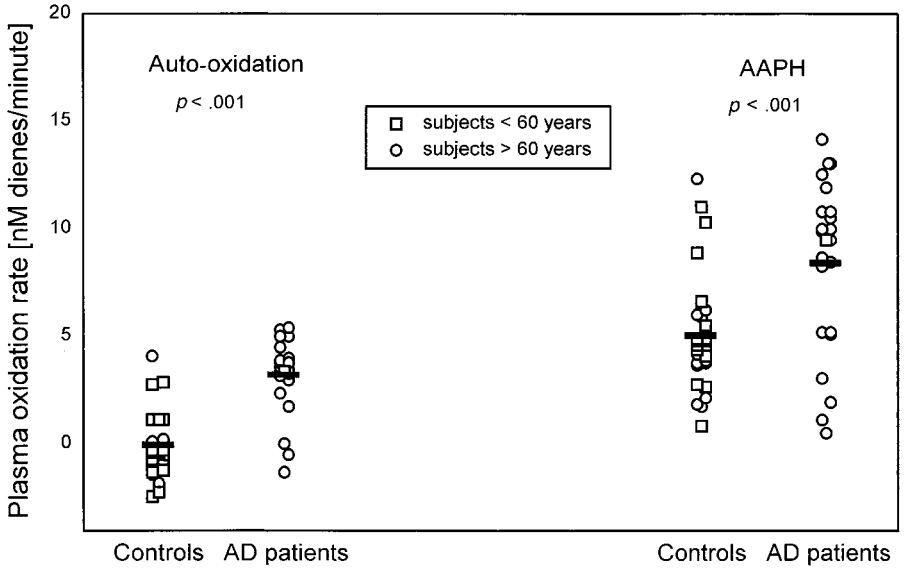

Fig. 4. Initial oxidation rate of plasma obtained from AD patients and control subjects. Plasma was diluted 150-fold with PBS and incubated at 37°C in the absence of exogenous oxidants (autoxidation) and in the presence of AAPH (330 μM). Bars correspond with the mean values calculated for each group. Each open circle (○) and open square (□) corresponds to one subject older and one subject younger than 60 years, respectively. Significance of the difference between the groups is shown as a p value.

Table 3. Antioxidants in Plasma of AD Patients and Control Subjects

	AD patients		Control subjects	
	All (n = 29)	≥60 (n = 26)	All (n = 29)	≥60 (n = 14)
Ascorbate (μM)	35.0 ± 18.6†	35.8 ± 18.8	48.8 ± 18.5	41.9 ± 18.6
α-Tocopherol (μM)	22.3 ± 10.8	22.9 ± 10.7	24.6 ± 7.7	23.3 ± 6.8
α-Carotene (μM)	0.06 ± 0.07*	0.07 ± 0.07*	0.18 ± 0.06	0.19 ± 0.08
β-Carotene (μM)	0.54 ± 0.51	0.57 ± 0.51	0.56 ± 0.34	0.45 ± 0.33

* $p < .001$; † $p < .05$ vs. corresponding control group.

($p = .40$). The only lipophilic antioxidant that showed significant difference between the groups was α-carotene ($p = .00001$). There were no differences in the levels of α-tocopherol, β-carotene (Table 3), ubiquinol-10, and ubiquinone-10 (data not shown). These results were consistent with the age-matched analysis (Table 3) and the lipid-normalized values expressed as picomoles per milligram (pmol/mg) total cholesterol and triglycerides (data not shown). The latter can be explained by the comparable lipid levels in both groups. The plasma autoxidation rate was negatively correlated with the content of ascorbate ($r = -0.29$, $p = .05$) and with the lipophilic antioxidants α-tocopherol and α-carotene ($r = -0.37$, -0.73; $p = .01$, $< .001$, respectively), as was plasma oxidation rate by AAPH with the levels of ascorbate and α-carotene ($r = -0.30$ and -0.51; $p = .04$ and $< .001$, respectively).

Correlations and results of multiple regression

To elucidate a potential relationship between the extent of oxidizability of CSF and plasma, we examined the correlation between CSF and plasma oxidizability parameters. The autoxidation rate in CSF correlated positively with plasma oxidation rates under both oxidizing conditions (autoxidation: $r = 0.56$, $p < .001$ and oxidation by AAPH: $r = 0.49$, $p < .001$). CSF lag phase duration also showed a negative correlation with plasma autoxidation rate ($r = 0.45$, $p = .001$).

To detect a potential influence of factors other than AD on the oxidation kinetics, multiple regression was performed on oxidation parameters as dependent variables, using the categoric variables AD, sex, Apo E ε4 allele, current smoking, increased alcohol intake, presence of CHD and hypertension, and continuous variable age as independent variables. The presence of AD significantly influenced every oxidation parameter in CSF and plasma. From the other parameters only sex entered the final regression equation once. The relatively small number of patients did not, however, allow a correlation between the severity of disease and oxidation parameters.

DISCUSSION

Our study revealed a highly significant increase in lipoprotein oxidizability in vitro for both CSF and plasma samples from AD patients compared with controls. In parallel, a decrease in antioxidant levels in both biological fluids was demonstrated. These data support the concept of oxidation as an important factor in the pathogenesis of AD and might provide a useful additional tool in the diagnosis of the disease.

Oxidation of plasma lipoproteins has been studied quite extensively [26,41] and data obtained in plasma samples of patients with CHD and hyperlipidemia versus controls demonstrated differences between these groups [33]. Human CSF contains lipoproteins with properties similar to those of HDL from blood plasma [24,25,42]. CSF lipoproteins have, however, not yet been fully characterized, and no data on their oxidation are available. We have recently shown that lipoproteins of human CSF are oxidatively modified during CSF incubation at 37°C [28]. Our present data show reproducible differences between AD patients and controls in the time course of CSF oxidation in vitro. In addition, CSF PUFA, the main substrate for lipid peroxidation, was found relatively reduced in AD, which was in accordance with data published by others [27]. While the increased formation of conjugated dienes can also be shown in plasma, the relative reduction of PUFA cannot be demonstrated in plasma samples. This observation can be due to the much higher amount of fatty acids in plasma.

It has been previously shown that the oxidizability of plasma LDL is determined by three major factors: (i) levels of antioxidants; (ii) levels of substrate for oxidation, such as PUFA or other lipids; and (iii) levels of preformed oxidation products or other substances able to accelerate LDL oxidation [43,44]. Increased oxidizability of plasma and CSF lipoproteins in AD might therefore be theoretically related to (i) lower levels of antioxidants, (ii) higher levels of substrate for oxidation, and (iii) presence of (yet unidentified) oxidants. We did find lower levels of antioxidants in CSF and plasma from AD patients. However, the CSF level of oxidation substrate (PUFA) was significantly lower in AD. This finding

could be due to elevated oxidation of CSF lipids in AD in vivo, i.e., to higher "preoxidation" of CSF samples used to measure the oxidizability. This phenomenon might be associated, in turn, with elevated levels of oxidation products, or other substances able to accelerate in vitro oxidation, in CSF from AD patients. The finding that CSF oxidizability was increased in AD despite lower PUFA levels, might therefore be explained by increased levels of these, yet unidentified, oxidants.

We have previously reported that diluted CSF can be oxidized without adding exogenous oxidants, i.e., autocatalytically [28]. The autoxidation of CSF was completely inhibited by EDTA, indicating that it was catalyzed by transition metal ions, such as Cu(II) and/or Fe(III). These ions are present in native CSF as redox-inactive complexes with the metal-binding proteins ceruloplasmin and transferrin [45] but can be released, when intact protein structure is disturbed under some pathologic conditions, which may occur in vivo [46,47]. Prolonged incubation in vitro at 37°C is likely to disturb the structure of metal-binding proteins, enabling release of Cu(II) and Fe(III) in a catalytically active form. Cu(II) and Fe(III) [15,16] as well as their carriers ceruloplasmin, transferrin, and p97 [45,48-50] have all been shown to be elevated in AD. These data implicate transition metal ions as potential oxidants for CSF lipoproteins in AD [51,52].

Reduction of catalytically active protein-bound transition metal ions must take place to initiate oxidation, and reductants, such as ascorbate or tocopherol, are able to mediate reduction [34,53]. However, it has been shown that this particular pro-oxidative activity of ascorbate does not results in its total pro-oxidative action on lipoprotein oxidation; the well-known radical-scavenging action of ascorbate appears to prevail and ascorbate remains an antioxidant even in the presence of redox-active transition metal ions [54,55]. The lower levels of ascorbate we measured in the CSF of AD patients are therefore in accordance with higher CSF oxidizability.

The high oxygen consumption in the central nervous system implies a potentially increased production of oxygen radicals and there is a elevated requirement for antioxidative molecules. However, ascorbate alone is three to five times higher in CSF, while all lipophilic molecules are around 100–500 times less concentrated in CSF as compared to plasma. Therefore, ascorbate might be of special relevance in antioxidative protection of lipoproteins in CSF. Antioxidant levels could be expected to be decreased in the CSF of AD patients as a consequence of high levels of oxidative stress in vivo. Our finding that ascorbate is significantly decreased and lipophilic antioxidants are slightly decreased among AD patients therefore strongly suggests that oxidation is a current feature of AD pathology in vivo. This finding is in accordance with recent studies showing that AD brain tissue has higher amounts of oxidatively modified biomolecules, as well as higher basal levels of lipid peroxidation, than tissue from control subjects [3,10–14].

Alternatively, low levels of antioxidants in AD may result from insufficient dietary supply. However, this is unlikely to be the case in our study, since a subset of 10 AD patients showed normal plasma levels of vitamin B_{12} and normal body mass index values (data not shown). In addition, we recruited AD patients who were in the early stage of disease, as indicated by the short time since its diagnosis. This suggests the patients with AD had a normal dietary status.

Because APP, Aβ, and senile plaques, the main markers for AD, have been shown to cause increased oxidative stress [5,6,17,56], oxidation seems to be secondary to the formation of Aβ and senile plaques, thus promoting the course of the disease rather than being causal. Oxidation can induce protein dysfunction and cell death, and neuronal cell death contributes to further oxidation. Therefore, oxidation might be both a cause and consequence of the degenerative processes in AD brains.

The correlation found between plasma oxidation rates and antioxidant levels underlines the value of the in vitro measurement of oxidation as a marker for oxidative stress in vivo. The multiple regression analysis showed that the increased oxidation levels were specifically correlated with the presence of the disease rather than with other factors. A comparison of CSF oxidation parameters between AD patients and patients with other degenerative neurological diseases, such as Parkinson's disease and amyotrophic lateral sclerosis, is currently being performed.

In conclusion, it should be noted that AD, a multifactorial disease, is neither genetically fully elucidated, nor are all the factors influencing its pathogenesis known. The present study indicates that increased lipoprotein oxidation in CSF may be an additional important factor in the progression of the disease. The recently published trial on the antioxidative treatment of patients with AD, proposing a positive effect on the course of the disease, supports this hypothesis [18]. In vitro measurements of lipoprotein oxidation in CSF might be useful as an additional tool for the clinical diagnosis of AD. They can also be of particular interest as biochemical markers for the evaluation of antioxidant treatment of AD patients.

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ABBREVIATIONS

AD—Alzheimer's disease
Aβ—amyloid β
APP—amyloid precursor protein
AAPH—2,2′-azobis-(2-amidinopropane) hydrochloride
BHT—butylated hydroxytoluene
CSF—cerebrospinal fluid
CHD—coronary heart disease
EDTA—ethylenediaminetetraacetic acid
HDL—high-density lipoproteins
HPLC—high-performance liquid chromatography
LDL—low-density lipoprotein
MUFA—monounsaturated fatty acids
PBS—phosphate-buffered saline
PUFA—polyunsaturated fatty acids
SFA—saturated fatty acids
TFA—total fatty acids