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Time-course of Oxidation of Lipids in Human Cerebrospinal Fluid *In Vitro*

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Oxidative mechanisms play an important role in the pathogenesis of Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. To assess whether the oxidation of brain lipoproteins plays a role in the development of these pathologies, we investigated whether the lipoproteins of human cerebrospinal fluid (CSF) are susceptible to oxidative modification *in vitro*. We studied oxidation time-course for up to 100 h of human CSF in the absence (autooxidation) or presence of exogenous oxidants. Autooxidation of diluted CSF was found to result in a slow accumulation of lipid peroxidation products. The time-course of lipid hydroperoxide accumulation revealed three consecutive phases, lag-phase, propagation phase and plateau phase. Qualitatively similar time-course has been typically found in human plasma and plasma lipoproteins. Autooxidation of CSF was accelerated by adding exogenous oxidants, delayed by adding antioxidants and completely inhibited by adding a chelator of transition metal ions. Autooxidation of CSF also resulted in the consumption of endogenous ascorbate, α-tocopherol, urate and linoleic and arachidonic acids. Taking into account that (i) lipid peroxidation products measured in our study are known to be derived from fatty acids, and (ii) lipophilic antioxidants and fatty acids present in CSF are likely to be located in CSF lipoproteins, we conclude that lipoproteins of human CSF are modified *in vitro* during its autooxidation. This autooxidation appears to be catalyzed by transition metal ions, such as Cu(II) and Fe(III), which are present in native CSF. These data suggest that the oxidation of CSF lipoproteins might occur *in vivo* and play a role in the pathogenesis of neurodegenerative diseases.

Keywords: Cerebrospinal fluid, oxidation, antioxidants, lipoproteins, neurodegenerative diseases

Abbreviations: Aβ, β-amyloid peptide; AAPH, 2,2′-azobis-(2-amidinopropane)hydrochloride; AD, Alzheimer's disease; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid; 9-HODE, 9-hydroxyoctadecadienoic acid; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; PD, Parkinson's disease; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substances

INTRODUCTION

There is good evidence that increased oxidative stress plays an important role in the development of Alzheimer's disease (AD),[1,2] Parkinson's

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disease (PD)[3] and other neurodegenerative pathologies. The levels of various oxidation products have been shown to be higher in the brains of AD[4,5] and PD[6,7] patients compared to controls.[8] However, the mechanisms of oxidative degeneration in AD and PD remain largely unknown.

β-Amyloid peptide (Aβ), a major component of senile plaques, has been implicated as an oxidant involved in the pathogenesis of AD.[2] It has been suggested that Aβ, a 40–43 amino acid peptide, can spontaneously produce free radicals on incubation in an aqueous milieu.[9] Aβ radicals might react with various biomolecules, such as lipids and proteins resulting in their dysfunction. This mechanism of free radical oxidation has been suggested to be responsible for the toxicity of Aβ towards neurons.[10] Neurons have been accordingly proposed as the major target for oxidative damage in AD. Other oxidants potentially involved in the development of AD include transition metal ions, such as Cu(II) and Fe(III), which seem to be increased in this pathology.[11,12] These transition metals are also thought to play a role in other neurodegenerative diseases, such as PD and multiple sclerosis.[13,14]

Aβ is synthesized by neuronal cells and secreted into the interstitial fluid[15] and is therefore present at low concentrations in cerebrospinal fluid (CSF) and blood plasma.[16] Besides Aβ, human CSF contains Cu(II) and Fe(III) which are carried by the proteins ceruloplasmin and transferrin in a catalytic-inactive form.[17] Importantly, human CSF also contains lipoproteins whose physical properties are similar to those of high-density lipoproteins (HDL) from blood plasma.[18,19] It is well established that plasma lipoproteins are highly vulnerable to oxidative modification by different oxidants *in vitro*.[20,21] Oxidation of plasma lipoproteins is known to be important for the development of atherosclerosis.[22] Since lipoproteins are present in CSF together with several oxidants, we think it is likely that oxidation of CSF lipoproteins may play a role in the pathogenesis of neurodegenerative diseases. To assess this hypothesis, we investigated whether the lipoproteins of human CSF are susceptible to oxidative modification *in vitro*. We found that lipoproteins of human CSF were modified *in vitro* during both, CSF autooxidation and oxidation by exogenous oxidants. CSF autooxidation was catalyzed by transition metal ions, such as Cu(II) and Fe(III), and was completely inhibited by endogenous ascorbate, the major CSF antioxidant.

MATERIALS AND METHODS

Materials

Chelex 100 resin (50–100 mesh) was obtained from Bio-Rad (München, Germany). 9-Hydroxyoctadecadienoic acid (9-HODE) was from Cascade Biochem (Reading, UK). Anhydrous sodium methoxide was from Alltech (Unterhaching, Germany). Egg yolk phosphatidylcholine, bovine brain phosphatidylserine and bovine brain sphingomyelin were obtained from Serva (Heidelberg, Germany). Soybean lipoxygenase and Aβ peptide 1–40 were from Sigma (Deisenhofen, Germany). All other chemicals and solvents were obtained from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

The reagents and phosphate-buffered saline (PBS) used for CSF oxidation were made up in Chelex-treated, double-distilled deionized water to effectively remove transition metal ions. The absence of catalytically active transition metal ions in PBS and water was verified by the ascorbate autooxidation method.[23]

Isolation of CSF

CSF was obtained by lumbar puncture from eight adult subjects aged 26–76 years being examined for possible neurological disorders. Five of the subjects had no neurological disorder and three suffered from pseudotumor cerebri (CSF overproduction). No significant difference in oxidation time-course between the subjects without

neurological disorders and those with CSF overproduction was found. CSF was put on ice immediately after collection and frozen at −80°C under argon within 1 h. The samples were stored under these conditions for no longer than 3 months. Freshly isolated CSF was used in some experiments to assess a potential influence of freezing/thawing on the oxidation time-course. CSF centrifuged at 4°C for 10 min at 3000 rpm to remove potentially present cells, was also studied to assess their role for CSF oxidation.

Characterization of CSF Chemical Composition

α-Tocopherol, ascorbate and urate were measured as major antioxidants of human CSF.[24] α-Tocopherol was quantified by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection as described elsewhere.[25,26] δ-Tocopherol was used as an internal standard. Ascorbate and urate were measured by reversed-phase HPLC with UV detection at 280 nm.[27] An aliquot of the sample corresponding to 100 µl undiluted CSF was diluted 1 : 1 (v/v) with 10% meta-phosphoric acid and centrifuged at 13 000 rpm for 3 min at 4°C. The supernatant was subjected to reversed-phase HPLC using an aqueous solution containing 0.1 M Na_2HPO_4, 2.5 mM Na_2EDTA and 2.0 mM tetrahexylammonium chloride, pH 3.0, at 1.0 ml/min as a mobile phase.

CSF fatty acids and cholesterol were measured by capillary gas chromatography with flame ionization detection.[28] CSF samples corresponding to 100 µl undiluted CSF were mixed with heptadecanoic acid and 5α-cholestane which were used as internal standards for fatty acids and cholesterol, respectively. t-Butyl hydroxytoluene was added as an antioxidant and the mixture was extracted with 9 volumes of chloroform/methanol (2 : 1 v/v). The chloroform phase was collected, evaporated under nitrogen and fatty acids in the residue were methylated by anhydrous sodium methoxide for 15 min at 50°C. To measure cholesterol, the reaction product was silylated by incubation with N,O-bis-(trimethylsilyl)trifluoro acetamide with 1% trimethylchlorosilane for 1 h at 80°C under argon. CSF polyunsaturated fatty acids (PUFAs) were calculated as a sum of linoleic and arachidonic acids. Individual fatty acids and cholesterol were identified by gas chromatography-mass spectrometry using a Hewlett Packard MS 5972 mass selective detector (Hewlett Packard, Palo Alto, USA).

Copper and iron were measured as potential oxidants present in CSF. Total copper was quantitated as its complex with bathocuproinedisulfonic acid[28] after reduction with ascorbate. Five hundred µl CSF was mixed with 100 µl of ascorbate solution (100 mM) and 400 µl bathocuproinedisulfonic acid (100 µM in PBS), and sample absorbance was measured at 480 nm. Total iron was measured photometrically using a commercially available kit (Sigma, Deisenhofen, Germany).

Oxidation of CSF

Frozen CSF samples were thawed at room temperature immediately before oxidation and pooled when necessary. The samples (undiluted or diluted 10-fold with PBS, pH 7.4) were oxidized at 37°C under air either in the absence (autooxidation) or in the presence of exogenous oxidants or antioxidants. Cu(II), 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH), lipoxygenase or Aβ peptide 1–40 were used as oxidants; ethylenediaminetetraacetic acid (EDTA), α-tocopherol and ascorbate were used as antioxidants. Aβ 1–40 was dissolved in Chelex-treated, double-distilled deionized water, stored at −80°C for no longer than 2 months and thawed immediately before use. Absence of catalytically active transition metal ions in Aβ solutions was verified by the ascorbate autooxidation method.[23]

To continuously register the time-course of oxidation, the oxidation was performed in a thermostated spectrophotometrical cuvette and an accumulation of lipid peroxidation products having conjugated diene structure was measured

(see below). The oxidation was also performed in a water bath to discontinuously measure antioxidants, fatty acids and oxidation products. Aliquots of the samples were removed after incubation time specified and oxidation was stopped by freezing at −80°C until analysis.

Characterization of the Time-course of CSF Oxidation

Lipid peroxidation products (mostly hydroperoxides[29]) with a conjugated diene structure and thiobarbituric acid-reactive substances (TBARS) were measured as CSF oxidation products. Accumulation of conjugated dienes was registered as an absorbance increase of CSF samples at 234 nm. This parameter is known to reflect the level of conjugated dienes in plasma low-density lipoprotein (LDL)[29] and has been shown to correlate with other indices of lipid peroxidation and to be indicative of lipoprotein oxidation in human plasma.[30–32] The absorbance was measured over a period of up to 100 h. Individual lipid hydroperoxides with a conjugated diene structure were analyzed using reversed-phase HPLC with UV detection at 234 nm.[33,34] An aliquot of the sample corresponding to 100 µl undiluted CSF or an aliquot of lipid standard or plasma lipoproteins was extracted with 5 volumes of hexane and 1 volume of methanol. Hexane extract was evaporated under nitrogen, dissolved in methanol/tert-butanol (1 : 1 v/v) and subjected to reversed-phase HPLC using methanol/tert-butanol (1 : 1 v/v) at 1.0 ml/min as a mobile phase. The system was calibrated using oxidized stearoylarachidonoyl phosphatidylcholine. The concentration of lipid hydroperoxides in oxidized stearoylarachidonoyl phosphatidylcholine was measured spectrophotometrically using 9-HODE as a standard. The recovery of phospholipids by the extraction method used was characterized using human plasma HDL. Known amount of HDL was extracted as described above and phospholipids were measured before and after extraction using a commercially available kit (Boehringer Mannheim, Mannheim, Germany). The recovery was 91.0 ± 4.2% ($n = 3$).

TBARS were measured after reaction of the diluted CSF samples (200 µl) with thiobarbituric acid using HPLC with fluorescent detection at 515 nm excitation/553 nm emission.[35] CSF oxidation was also characterized by a consumption of antioxidants, fatty acids and cholesterol which were measured as described above.

Oxidation of Plasma Lipoproteins and Standard Lipids

Plasma LDL and HDL were isolated using density gradient ultracentrifugation of heparin plasma for 20 h at 4°C in the presence of 1.5 mM EDTA.[36] Plasma was obtained from a local blood bank. LDL (80 mg total cholesterol/dl) and HDL (50 mg total cholesterol/dl) were oxidized by AAPH (10 mM) at 37°C for 18 h. Egg yolk phosphatidylcholine, bovine brain phosphatidylethanolamine, bovine brain phosphatidylserine, bovine brain sphingomyelin, bovine heart cardiolipin and synthetic cholesteryl linoleate, cholesteryl arachidonate, stearoyllinoleyl phosphatidylcholine, palmitoylarachidonoyl phosphatidylcholine (PAPC) and stearoylarachidonoyl phosphatidylcholine (200 mg/dl of each) were oxidized as standard lipids. The oxidation was performed at 37°C in the absence of exogenous oxidants for 64 h or in the presence of AAPH (10 mM) for 18 h. Total cholesterol in plasma lipoproteins was measured using commercially available kit (Boehringer Mannheim, Mannheim, Germany). All data are shown as mean ± SEM, unless otherwise indicated.

RESULTS

To characterize the oxidizability of CSF lipoproteins, undiluted and 10-fold diluted CSF was incubated at 37°C for up to 100 h in the absence of exogenous oxidants. Whereas undiluted CSF was well protected against autooxidation, slow

accumulation of lipid peroxidation products was observed in the diluted samples (Figure 1). Thus, the autooxidation was accelerated by CSF dilution. This effect was not related to freezing and thawing the samples, since similar time-courses were recorded in fresh CSF (Figure 1A) and in CSF frozen at −80°C and thawed (Figure 1B).

Conjugated dienes (mostly lipid hydroperoxides[29]) were detected in CSF extracted with methanol and hexane after a separation using reversed-phase HPLC by their specific absorbance at 234 nm.[29] A peak at 3.2 min was newly found in oxidized CSF in comparison to unoxidized samples (Figure 1C). At the same position, a peak was also seen in oxidized HDL (Figure 1C) and LDL (data not shown). When different unsaturated lipids were oxidized *in vitro*, a peak at 3.1 min was only found in oxidized PAPC (Figure 1C), stearoylarachidonoyl phosphatidylcholine and stearoyllinoleyl phosphatidylcholine (data not shown), suggesting that phosphatidylcholine hydroperoxides were the major hydroperoxide species formed during CSF autooxidation.

The time-course of CSF absorbance at 234 nm was found to parallel the time-course of lipid hydroperoxides measured by HPLC (Figure 2). Absorbance increase at 234 nm was not associated with an increased turbidity of the samples, since no appreciable increase in absorbance at 400 and 600 nm was measured (data not shown). This justified using the absorbance increase at 234 nm as a measure of oxidation of CSF lipids.

Centrifugation of CSF at 3000 rpm to remove contaminating cells had no influence on the time-course of absorbance at 234 nm (Figure 3A), demonstrating that oxidized lipids were not derived from CSF cells. The time-course of both the absorbance increase at 234 nm and lipid hydroperoxide accumulation in the diluted CSF revealed three consecutive phases, the lag-phase, propagation phase and plateau phase. The oxidation rate was close to zero in the lag-phase, maximal in the propagation phase and again close to zero in the plateau phase. The lag-phase lasted 2–20 h and the propagation phase 5–15 h,

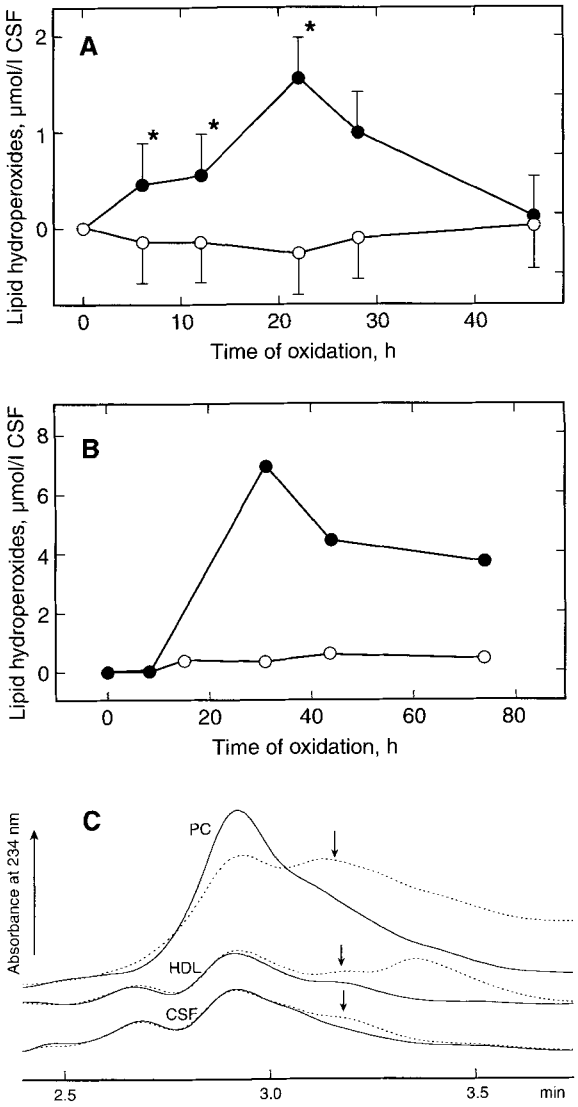

FIGURE 1 Influence of dilution on the time-course of CSF oxidation. Accumulation of lipid hydroperoxides in fresh (A) and frozen at −80°C/thawed (B) CSF incubated at 37°C in the absence of exogenous oxidants is shown. Time-courses measured for undiluted (○) and 10-fold diluted with PBS (●) CSF are given. Data are the mean ± SEM for three different CSF samples (A) or means for two other samples (B). Lipid hydroperoxides were extracted from CSF by methanol and hexane and measured by HPLC with UV-detection at 234 nm; $^*p < 0.05$ vs. undiluted CSF. (C) Chromatograms of unoxidized (solid lines) and oxidized (dotted lines) CSF, HDL and PAPC measured by HPLC with UV-detection at 234 nm. CSF was diluted 10-fold with PBS and incubated for 43 h at 37°C. PAPC (200 mg/dl) and HDL (30 mg total cholesterol/dl) were oxidized at 37°C in the presence of AAPH (10 mM) for 18 h. Before separation, the samples were extracted by methanol and hexane (see Materials and Methods). A peak newly found in oxidized samples is shown with arrows.

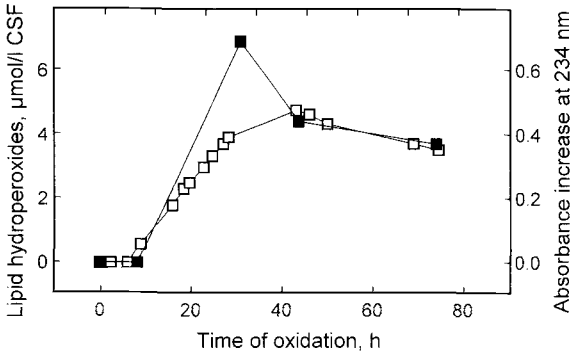

FIGURE 2 Accumulation of lipid hydroperoxides measured by HPLC (■) and absorbance increase at 234 nm (□) in 10-fold diluted CSF incubated at 37°C in the absence of exogenous oxidants. Data shown are the mean of three measurements and are representative of two separate experiments performed with two different CSF samples.

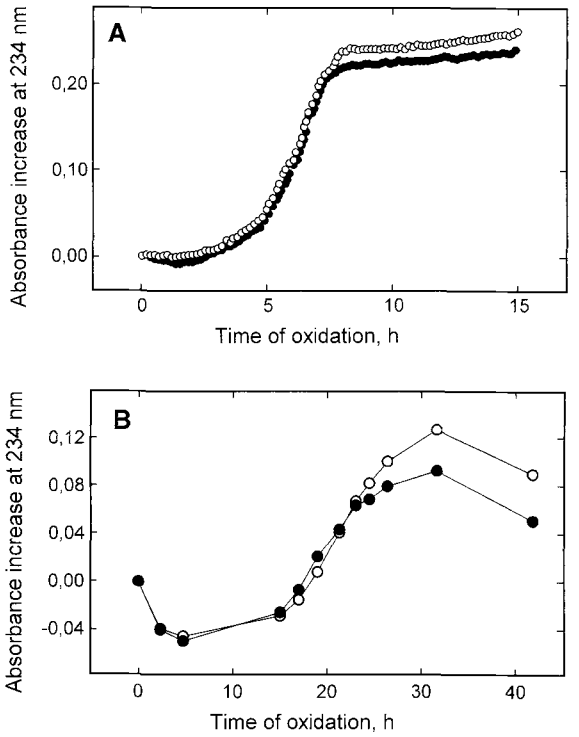

FIGURE 3 Influence of CSF centrifugation (A) and freezing/thawing (B) on the absorbance increase at 234 nm in 10-fold diluted CSF incubated at 37°C in the absence of exogenous oxidants. (A) CSF was diluted and oxidized before (○) and after (●) 10 min centrifugation at 3000 rpm to remove CSF cells. (B) CSF was diluted and oxidized before (○) and after (●) freezing at −80°C and thawing. Data shown are the mean of two (A) or three (B) separate experiments.

demonstrating substantial variability in the oxidizability between different individuals. Qualitatively similar time-course has been typically found in human blood plasma[30–32] and plasma lipoproteins[29] where three similar phases are also observed. The triphasic time-course of CSF absorbance at 234 nm was not influenced by freezing the samples at −80°C and thawing (Figure 3B). Time-course of oxidation measured in parallel in one particular sample was reproducible, seen as similar characteristics of oxidation phases, e.g. the duration of the lag-phase (16.4 ± 1.7 h, $n = 8$) and propagation phase (10.6 ± 1.2 h, $n = 8$).

Accumulation of lipid hydroperoxides in the diluted CSF was accelerated by adding exogenous oxidants, such as lipoxygenase and AAPH, to the CSF prior to oxidation (Figure 4A). Adding EDTA, a chelator of transition metal ions, completely inhibited autooxidation. The oxidants significantly and dose-dependently increased CSF oxidation rate in the propagation phase and slightly shortened this phase in comparison to the samples unsupplemented with oxidants (Figure 4B). Acceleration of lipid hydroperoxide accumulation was also seen after adding Aβ 1–40 (1 µM) to CSF prior to oxidation (Figure 5). The CSF oxidation rate in the propagation phase was increased (13.1 ± 2.7 vs. 15.9 ± 5.1 nM dienes/min, $n = 3$, in the absence and presence of Aβ 1–40, respectively), and the lag-phase was shortened (3.2 ± 1.1 vs. 1.1 ± 1.0 h, $n = 3$). Adding Cu(II) to a final concentration of 10 or 50 µM caused a slow decrease in the absorbance at 234 nm (data not shown).

Accumulation of conjugated dienes in 10-fold diluted CSF was delayed by adding the antioxidants ascorbate and α-tocopherol. When the CSF was oxidized in the presence of 50 nM α-tocopherol, the oxidation lag-phase was prolonged from 9.1 ± 4.2 to 10.4 ± 2.5 h (mean ± SD, $n = 3$). When a CSF sample from another patient was oxidized in the presence of 500 µM ascorbate, the oxidation lag-phase was prolonged from 2.5 ± 1.3 to 3.7 ± 0.2 h (mean ± SD, $n = 3$).

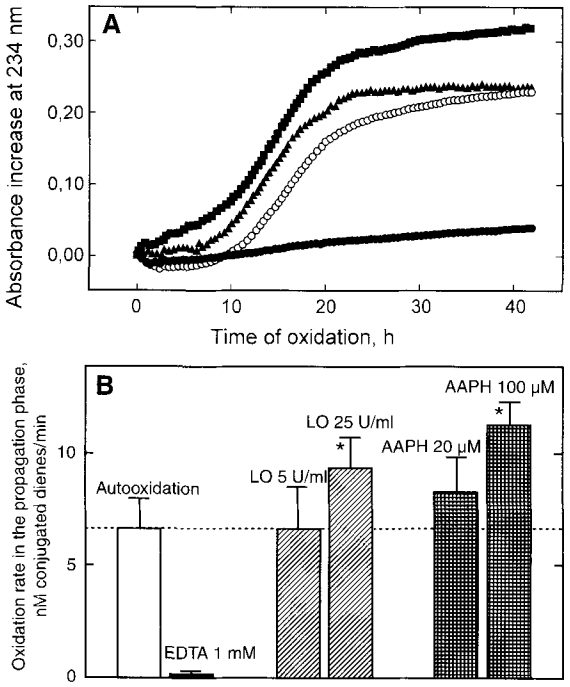

FIGURE 4 (A) Absorbance increase at 234 nm in 10-fold diluted CSF incubated at 37°C in the absence of exogenous oxidants (autooxidation; ○) and in the presence of AAPH (100 µM; ■), lipoxygenase (25 U/ml; ▲) or EDTA (1 mM; ●). (B) Oxidation rate in the propagation phase in 10-fold diluted CSF incubated at 37°C in the absence of exogenous oxidants (autooxidation) and in the presence of AAPH, lipoxygenase or EDTA. Data shown are representative of five separate experiments performed with two different CSF samples (A) and mean ± SEM of four experiments (B). Oxidation rate averaged over the whole incubation time is shown for CSF incubated with EDTA. LO, lipoxygenase; $^*p < 0.05$ vs. autooxidized CSF.

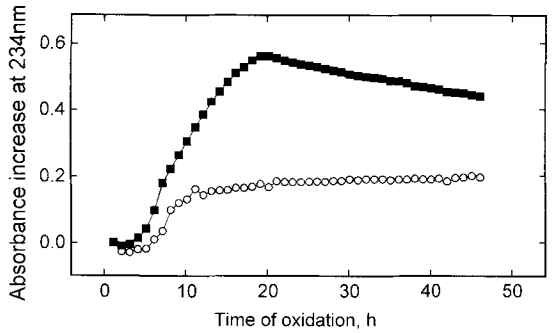

FIGURE 5 Absorbance increase at 234 nm in 10-fold diluted CSF incubated at 37°C in the absence of exogenous oxidants (autooxidation; ○) and in the presence of full Aβ peptide 1–40 (1 µM; ■). Data shown are representative of four separate experiments performed with three different CSF samples.

Autooxidation of diluted CSF at 37°C was also seen as an accumulation of TBARS, which increased by 58.5 ± 11.5 nM after 100 h incubation ($n = 3$). TBARS time-course revealed a lag-phase of about 75 h and was accelerated by 50 µM Cu(II) (increase by 95.6 ± 17.4 nM after 100 h) and inhibited by 1 mM EDTA (increase by 0.3 ± 19.7 nM after 100 h). Linoleic and arachidonic acids, the major PUFAs in CSF,[37] were consumed during CSF autooxidation. PUFA content of CSF decreased from 3.28 ± 1.04 to 1.57 ± 0.58 µM after 40 h autooxidation ($n = 3$), corresponding to their consumption rate of 1.3% of total CSF PUFAs per hour. In contrast, no decrease in CSF PUFAs was measured in the presence of EDTA. Consumption of CSF cholesterol revealed similar time-course (data not shown).

CSF oxidation resulted in changes in the concentration of α-tocopherol, the major lipophilic antioxidant in CSF.[38] After 75 h incubation at 37°C, 71.0 ± 5.1% ($n = 3$) of the initial concentration of 5.1 nM was consumed in autooxidizing CSF. There was a lag-phase of about 25 h, during which no α-tocopherol consumption was observed (Figure 6). Adding 50 µM Cu(II) slightly accelerated α-tocopherol consumption (73.7 ± 4.1% consumed after 75 h, $n = 3$) and adding

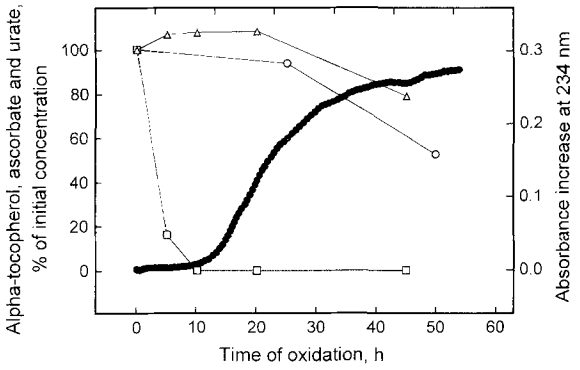

FIGURE 6 Time-course of the absorbance at 234 nm (●), α-tocopherol (○), ascorbate (□) and urate (△) simultaneously measured in 10-fold diluted CSF autooxidized at 37°C. Initial concentration of ascorbate, urate and α-tocopherol in whole CSF were 138 µM, 36 µM and 5.1 nM, respectively. Data shown are representative of three separate experiments.

1 mM EDTA delayed it (24.8 ± 26.9% consumed after 75 h, $n = 3$).

Ascorbate and urate, the most important hydrophilic antioxidants in human CSF,[24] were also consumed during CSF autooxidation. All CSF ascorbate was oxidized within the first 10 h of incubation, whereas urate consumption was only seen after 45 h (Figure 6). Adding EDTA delayed ascorbate consumption (86.0 ± 0.75% consumed after 10 h, $n = 3$).

When time-courses of the different antioxidants and oxidation products measured in the same sample were compared, ascorbate was found to be the antioxidant first consumed during the oxidation, followed by α-tocopherol and urate (Figure 6). The point at which ascorbate was totally consumed approximately coincided with the end of the lag-phases of conjugated diene accumulation and α-tocopherol consumption. As long as ascorbate was present, no appreciable accumulation of oxidation products and consumption of other antioxidants was detected in the samples. After complete loss of ascorbate, accumulation of oxidation products began, despite the presence of α-tocopherol.

CSF samples used in our study contained transition metal ions. In accordance with previously reported data,[11,12] CSF levels of total copper and iron were 0.64 ± 0.43 and 0.57 ± 0.27 μM ($n = 5$), respectively.

DISCUSSION

Human CSF was found to be oxidized during its incubation at 37°C either in the absence or in the presence of exogenous oxidants. CSF oxidation was seen as a consumption of hydrophilic and lipophilic antioxidants and accumulation of oxidation products. Human CSF contains lipoproteins whose properties are similar to those of HDL from blood plasma.[18,19] These lipoproteins carry most, if not all, CSF lipids and are expected to carry other lipophilic compounds, such as lipophilic antioxidants and fatty acids. Our study shows that lipoproteins of human CSF are oxidatively modified during CSF oxidation. This was concluded from the following observations: (i) phosphatidylcholine hydroperoxides were detected in oxidized CSF using reversed-phase HPLC; (ii) the concentration of hydroperoxides and TBARS increased during incubation; (iii) both lipid hydroperoxides and TBARS are known to be derived from PUFAs;[20] (iv) α-tocopherol, the major lipophilic antioxidant, and linoleic and arachidonic acids, the major PUFAs in CSF, were consumed during the incubation; (v) neither the time-course of oxidation was influenced by CSF cells nor were the oxidation products derived from the cells. These results indicate that lipoproteins of human CSF are prone to oxidative modification *in vitro*.

CSF oxidation did not require exogenous oxidants but occured autocatalytically. The autooxidation 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[17] but can be released, when intact protein structure is disturbed, e.g.[39,40] 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 free form.[41]

Another potential source of transition metals is PBS which normally contains traces of them. To remove transition metals, PBS was made up in double-distilled, deionized water treated with Chelex 100 ion-exchange resin. Absence of transition metals in PBS was then verified by the ascorbate autooxidation method.[23] The detection limit of this method is about 1 nM Cu(II), which is much more active than Fe(III) in catalyzing lipoprotein oxidation.[20] CSF samples used in our study contained about 0.5 μM copper. This implies that in our incubations (CSF diluted 10-fold), the level of endogenous copper was much higher than exogenous (50 vs. ≤ 1 nM), implying that endogenous, rather than exogenous, metal ions catalyzed CSF oxidation in our study.

Besides transition metals, other oxidants were able to oxidize CSF in our study. Adding exogenous oxidants accelerated oxidation of CSF lipoproteins. For lipoxygenase, AAPH and Cu(II), this was in agreement with their ability to accelerate oxidation of plasma lipoproteins.[20,21] The prooxidant activity of Aβ 1–40 at high (micromolar) concentrations can be explained by its reported ability to produce free radicals.[9] Accelerated oxidation was seen as a more rapid increase in CSF absorbance at 234 nm (in the presence of AAPH, lipoxygenase or Aβ 1–40) or more rapid consumption of antioxidants and fatty acids and accumulation of TBARS (in the presence of Cu(II)). The decrease in the absorbance at 234 nm found after addition of Cu(II) was likely to be due to the precipitation of CSF lipoproteins at such high Cu(II) concentrations.

Following initiation, the time-course of CSF oxidation (measured as an accumulation of lipid hydroperoxides) revealed three consecutive phases, the lag-phase, propagation phase and plateau phase, independently of which oxidant was used. No major changes in the CSF levels of lipophilic compounds, such as α-tocopherol, PUFAs, lipid hydroperoxides and TBARS, were found during the lag-phase. The end of the lag-phase approximately coincided with the time-point of total ascorbate consumption. This suggests that endogenous ascorbate was able to fully protect CSF lipoproteins against oxidation. This effect was in accordance with the ability of ascorbate to completely inhibit oxidation of plasma lipoproteins and to be the most efficient antioxidant in human plasma.[42] After ascorbate consumption, oxidation of CSF lipoproteins began, as indicated, with an increase in lipid hydroperoxides and a decrease in α-tocopherol. The propagation phase of the oxidation was reached despite the presence of α-tocopherol which was clearly unable to protect CSF lipids after ascorbate was consumed. The presence of urate, another hydrophilic antioxidant known to delay late stages of lipoprotein oxidation,[43,44] was also unable to inhibit CSF oxidation in the absence of ascorbate.

Such oxidative time-course measured by us in human CSF can be explained using the α-tocopherol-mediated model of lipoprotein oxidation developed for the oxidation of isolated plasma lipoproteins by Stocker *et al.*[21,43,45] According to this model, α-tocopheroxyl radical, a primary product of α-tocopherol oxidation, can propagate oxidation of isolated lipoproteins under mild oxidative conditions. This prooxidant activity of α-tocopherol evolves into antioxidant activity, when oxidative conditions become stronger. The presence of co-antioxidants,[46] such as ascorbate, capable of reducing α-tocopheroxyl radical back to tocopherol, represents another factor determining α-tocopherol activity towards oxidation. We have recently shown that in the presence of high concentrations of co-antioxidants, α-tocopherol behaves as an antioxidant independently of the oxidative conditions.[47] This antioxidant activity may, however, evolve into prooxidant, when the co-antioxidants are exhausted under conditions of mild oxidation. According to this model, oxidation of CSF lipoproteins is completely inhibited in the presence of ascorbate, which efficiently eliminates α-tocopheroxyl radicals formed from the lipoprotein particles. As a result, concentration of ascorbate decreases and that of α-tocopherol remains unchanged. Under these conditions α-tocopherol functions as an antioxidant, which is in accordance with the prolongation of the lag-phase after CSF supplementation with α-tocopherol. After ascorbate is consumed, CSF lipoproteins become oxidized via an α-tocopheroxyl radical-mediated mechanism. The order of the antioxidant consumption found (ascorbate followed by α-tocopherol and urate) was in accordance with this model. Long (up to 20 h) lag-phase and low CSF concentrations of transition metals suggest that in our study CSF was oxidized under mild conditions, which strongly supports the mechanism proposed.

This mechanism implies that ascorbate is the most important antioxidant in human CSF. The central role of ascorbate for antioxidative

protection of CSF lipoproteins can explain why its CSF concentration is maintained at such a high level (3–5-fold higher than its plasma concentration).[48] Since autooxidation of undiluted CSF occured very slowly, ascorbate seems to provide sufficient protection of CSF *in vivo*. Antioxidant function of ascorbate is also consistent with the observation that CSF autooxidation was delayed by adding this antioxidant. Dilution of CSF might weaken an interaction between water-soluble ascorbate and lipoprotein located α-tocopherol and thereby enable CSF oxidation, as it has already been described for plasma.[47]

As long as the physiological relevance of CSF dilution is unclear, our system remains rather artificial. However, our data suggest that oxidation of CSF lipoproteins might gradually occur *in vivo*, if ascorbate is consumed under the action of some oxidants. This might happen in a local microenvironment between neurons and neuroglia when high amounts of oxidants are secreted, e.g. by activated microglia. Physiological oxidants able to drive this process include, e.g. transition metal ions, Aβ[2] and lipoxygenase, which is expressed in brain.[49] Once formed, oxidized lipoproteins might initiate various pathological changes in the brain, since oxidized plasma lipoproteins are known to be toxic for different cells and can initiate pathological processes.[22] Lipid transport within the brain, the major function of CSF lipoproteins, might also be impaired. Since Aβ, a major marker for AD, can generate free radicals,[9] oxidation seems to be secondary to its formation, thus promoting the course of the disease rather than being causal. Moreover, oxidation can be induced by neuronal cell death and thereby might be a consequence of the degenerative processes in the brain.

It is not known how important the oxidation of CSF lipoproteins may be for the development of neurodegenerative diseases. In a recent study we found that the *in vitro* oxidizability of CSF lipoproteins was considerably higher in patients with AD, PD and multiple sclerosis than in age-matched controls (Schippling *et al.*, submitted). Thus oxidized lipoproteins may be more easily formed in the brain of patients with neurodegenerative diseases in comparison with healthy subjects, suggesting that the oxidation of CSF lipoproteins might play a role in the pathogenesis of neurodegenerative diseases.

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