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Resistance of Human Cerebrospinal Fluid to *in vitro* Oxidation is Directly Related to its Amyloid-β Content

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Amyloid-β (Aβ) peptide, a major constituent of senile plaques and a hallmark of Alzheimer's disease (AD), is normally secreted by neurons and can be found in low concentrations in cerebrospinal fluid (CSF) and plasma where it is associated with lipoproteins. However, the physiological role of Aβ secretion remains unknown. We measured the resistance to *in vitro* oxidation of CSF obtained from 20 control subjects and 30 patients with AD, and correlated it with CSF levels of antioxidants, lipids and Aβ. We found that the oxidative resistance, expressed as a duration of the oxidation lag-phase, was directly related to CSF levels of $A\beta_{1-40}$, $A\beta_{1-42}$ and ascorbate and inversely to levels of fatty acids. These data suggest that, besides ascorbate, Aβ is another major physiological antioxidant for CSF lipoproteins.

Keywords: Amyloid-β; Cerebrospinal fluid; Antioxidants; Ascorbate; Lipid peroxidation; Alzheimer's disease

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β peptide; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline

INTRODUCTION

Amyloid-β peptide (Aβ) is a major constituent of senile plaques in the brain of patients with Alzheimer's disease (AD).[1] The ability to spontaneously aggregate and, at high concentrations, to induce oxidation of biomolecules are the best known pathological properties of Aβ.[2] In brain, Aβ is normally produced and secreted by neurons as a part of the processing of amyloid-precursor protein.[3,4] Aβ can be found in low concentrations (about 0.1–1 nM) in cerebrospinal fluid (CSF) and plasma of both AD patients and healthy subjects.[5] Being highly lipophilic, Aβ is associated with lipoprotein particles in both CSF[6] and plasma.[7,8] *In vitro* neurons and some other cells are able to secrete Aβ[9] which is also lipoprotein associated.[10] However, the physiological function of Aβ production and secretion remains unknown.

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It has been shown that Aβ at physiological levels can interact with extracellular matrix and promote neurite outgrowth.[11] Aβ has neurotrophic effects and it is able to promote neuronal adhesion and can stimulate protein kinase C.[12,13] Aβ fragment 25–35 can inhibit lipid peroxidation at low concentrations, as a result of its interaction with membrane lipid bilayer.[14] In addition, Aβ efficiently binds transition metal ions, which are potent initiators of oxidation.[15–17]

Lipid peroxidation is a process involved in the pathogenesis of various diseases. Oxidation of plasma lipoproteins in the arterial wall is firmly established to be essential for the development of atherosclerosis.[18] There are several lines of evidence indicating that increased oxidative stress is important for the development of AD.[19–22] Lipoproteins of human CSF contain oxidizable lipids[23,24] which may similarly represent a substrate for pathological oxidation.

Therefore, we recently proposed that oxidation of CSF lipoproteins might be important in the pathophysiology of AD.[25,26] We found that CSF lipoproteins are susceptible to oxidative modification *in vitro*[27] and that their oxidizability is significantly higher in AD patients in comparison with control subjects.[25,26,28] These data have been recently confirmed by others.[29] In addition, we showed that at concentrations measured in biological fluids (0.1–1.0 nM), both $A\beta_{1-40}$ and $A\beta_{1-42}$ strongly inhibit oxidation of CSF and plasma lipoproteins.[30] Together with a decreased CSF concentration of Aβ frequently measured in AD,[31] these findings suggest that Aβ may act as a physiological antioxidant for CSF and plasma lipoproteins.

To further assess this suggestion, we investigated which factors determine the resistance to *in vitro* oxidation of human CSF. We measured the oxidative resistance of CSF obtained from 20 control subjects and 30 patients with AD, and correlated it with CSF levels of antioxidants, lipids and Aβ.

MATERIALS AND METHODS

Chemicals

Both $A\beta_{1-40}$ and $A\beta_{1-42}$ were purchased from Biosource International (USA). Aβ was dissolved in hexafluoropropanol, transferred into dimethyl sulfoxide and stored at −80°C. All other chemicals and solvents were either from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Subjects

AD patients ($n = 30$) and control subjects ($n = 20$) were recruited in the psychiatric clinic and the neurological clinic of Hamburg University Hospital. The AD patients (age 71.9 ± 9.7 years) 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.[32] All AD patients were in an early stage of the disease, mobile, in a good general nutritional state and took no antioxidant supplements. The control subjects (age 58.6 ± 17.3 years) 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 was obtained before lumbar puncture and the study was approved by the Ethical Committee, Hamburg.

Sample Collection and Preservation

From each subject 1 ml CSF, obtained as a surplus of diagnostic lumbar puncture, was sampled, immediately placed on ice and frozen under argon or nitrogen at −80°C, not later than 30 min after the puncture. Samples were not stored longer than 3 months and thawed at room temperature directly before analysis.

Resistance To *In Vitro* Oxidation

Oxidation of CSF was monitored as a change in the sample absorbance at 234 nm. This parameter reflects the level of lipid hydroperoxides in isolated lipoproteins oxidized under *in vitro* conditions.[33] When lipoproteins are oxidized in diluted CSF, change in the absorbance at 234 nm correlates with other indices of lipid peroxidation, such as consumption of PUFAs and accumulation of cholesterol linoleate hydroperoxide measured by HPLC.[27,34] These data justify using absorbance at 234 nm as a specific measure for the accumulation of lipid hydroperoxides in CSF 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. Absence of transition metals in PBS was then verified by the ascorbate auto-oxidation method.[35] The samples were oxidized at 37°C either in the absence (auto-oxidation) or in the presence of the exogenous oxidant 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH; Polysciences, Warrington, 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 avoid evaporation. In a separate experiment, either $A\beta_{1-40}$ or $A\beta_{1-42}$ was added to the samples directly before oxidation to the final concentration of 0.1 nM.

Analytical Measurements

α-Tocopherol and ascorbate were measured as main antioxidants in CSF. α-Tocopherol was quantified by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection[36] as described elsewhere.[27,28] Ascorbate was measured by reversed-phase HPLC with UV detection at 267 nm[37] as previously described.[28]

CSF fatty acids and cholesterol were measured by capillary gas chromatography with flame ionization detection.[27,28,38]

$A\beta_{1-40}$ and $A\beta_{1-42}$ were quantified using a commercially available ELISA obtained from Biosource International (USA).

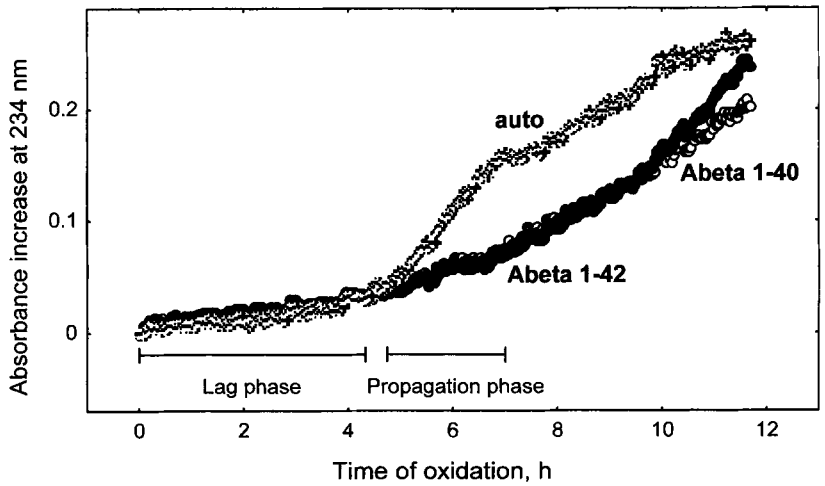
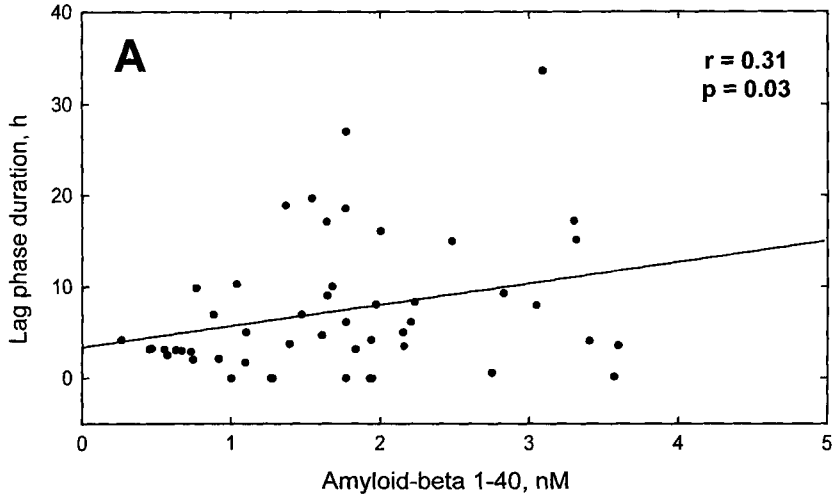
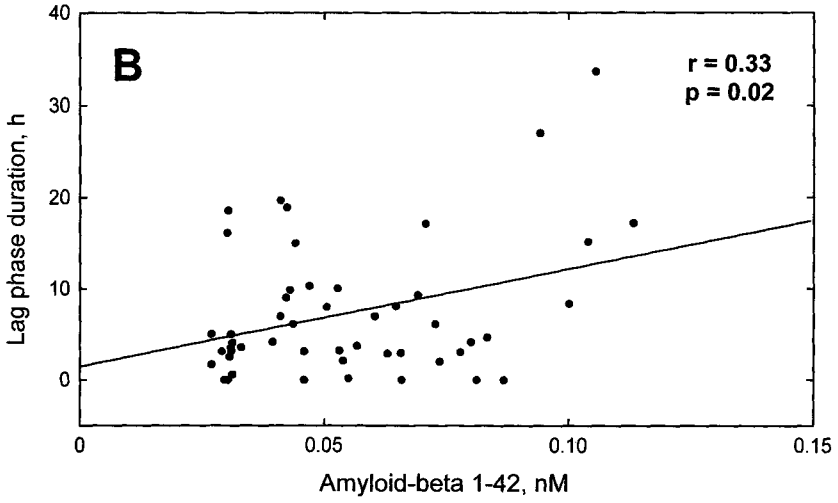
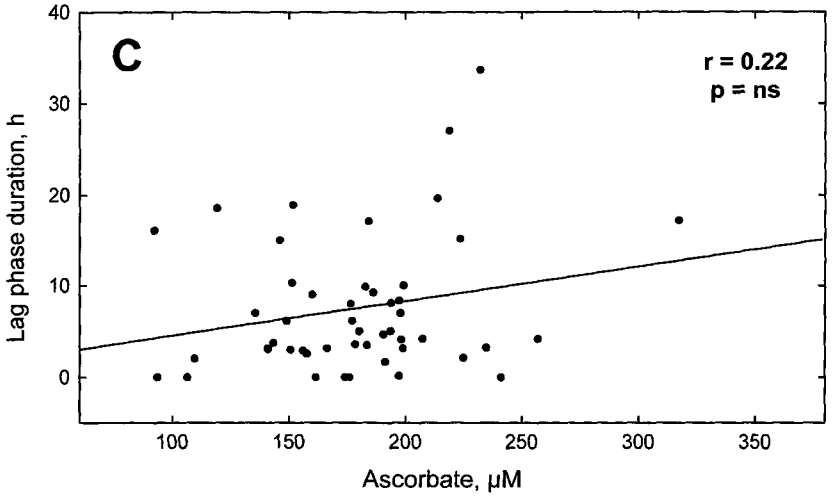


FIGURE 1 Typical auto-oxidation kinetics of human CSF in the presence of nanomolar concentrations of exogenously added $A\beta_{1-40}$ and $A\beta_{1-42}$. The lag and propagation phases are schematically shown for a curve measured without added Aβ. CSF was diluted 10-fold with PBS and incubated at 37°C either without or with 0.1 nM $A\beta_{1-40}$ or $A\beta_{1-42}$. Absorbance increase at 234 nm was recorded as a measure of oxidation of CSF lipoproteins. Data shown are representative of five separate experiments performed with two different CSF preparations.







Statistical Analysis

Pearson's moment-product correlation coefficients were calculated to evaluate relationships between variables. Between-group differences were analyzed by Student's *t*-test for independent groups. All results are expressed as means±standard deviations. The quality of the assays was controlled by calculating the assay variability which was not higher than 8% for all the parameters measured.[28,36,39]

RESULTS

As we had previously reported,[26-28] the auto-oxidation kinetics of diluted CSF were characterized by three consecutive phases: the lag-phase, during which the oxidation rate was low; the propagation phase, presenting a fast accumulation of lipid peroxides; the plateau phase with a lower oxidation rate again (Fig. 1). Addition of AAPH to a final concentration of 100 μM slightly accelerated CSF auto-oxidation and did not qualitatively change its kinetics.[27] The oxidative resistance of CSF was therefore described as a duration of the oxidation lag-phase.[33] The lag and propagation phases were approximated using two straight lines produced using linear regression analysis of the both phases as described before for the calculation of the lag-phase of lipoprotein oxidation.[33,40,41] The abscissa at the intersection was used as a lag-phase duration. The non-linear regression analysis of the whole oxidation curve (its non-linear approximation using two linear functions) revealed very close values for the lag-phase duration ($r = 0.93$ for the correlation between the two methods obtained in a subset of 10 oxidation curves, data not shown).

To investigate which factors determine oxidative resistance CSF, we measured the lag-phase of CSF auto-oxidation and oxidation by the exogenous oxidant AAPH in the samples obtained from 20 control subjects and 30 patients with AD, and correlated it with CSF levels of antioxidants, lipids and Aβ. We found that the duration of the lag-phase of auto-oxidation positively correlated with the CSF levels of both $A\beta_{1-40}$ (Fig. 2A) and $A\beta_{1-42}$ (Fig. 2B). The correlations were of a similar significance. The duration of the lag-phase of oxidation by AAPH positively correlated with the CSF level of $A\beta_{1-42}$, whereas a correlation with the level of $A\beta_{1-40}$ did not reach significance (Table I). Interestingly, the significance of the correlations was considerably improved, when $A\beta_{1-40}$ was calculated per unit of fatty acids.

Of the two major CSF antioxidants, ascorbate and α-tocopherol measured in our study, only the former revealed a significant positive correlation with the duration of the lag-phase of oxidation by AAPH ($r = 0.33$, $p = 0.02$). Similar correlation calculated for the lag-phase of auto-oxidation did not reach significance (Fig. 2C). Any significant correlation was not found between CSF oxidative resistance and its level of α-tocopherol (data not shown).

Of CSF lipids, total fatty acids revealed a significant negative correlation with the lag-phase ($r = -0.31$, $p = 0.03$, and $r = -0.36$, $p = 0.01$, for auto-oxidation and oxidation by AAPH, respectively). All major classes of fatty acids (saturated, mono- and polyunsaturated) showed negative correlations of a comparable significance (data not shown). In contrast, no significant correlation was found between CSF oxidative resistance and its level of cholesterol (data not shown).

FIGURE 2 Correlations between the duration of the lag-phase of CSF auto-oxidation and CSF levels of $A\beta_{1-40}$ (A), $A\beta_{1-42}$ (B) and ascorbate (C). CSF was diluted 10-fold with PBS and incubated at 37°C. Absorbance increase at 234 nm was recorded as a measure of oxidation of CSF.

TABLE I Correlation coefficients between the lag-phase of CSF oxidation and its Aβ levels (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

	Units for Aβ	Auto-oxidation	Oxidation by AAPH
$A\beta_{1-40}$	nM	0.31*	0.23
	nmol/mg cholesterol	0.37**	0.29*
	nmol/mg fatty acids	0.51***	0.47**
$A\beta_{1-42}$	nM	0.33*	0.40**
	nmol/mg cholesterol	0.31*	0.37**
	nmol/mg fatty acids	0.48**	0.56***

When CSF parameters were compared between the two subgroups of our study population, AD patients and control subjects, the levels of ascorbate and $A\beta_{1-42}$ were found to be significantly lower in the patient group (Table II). This was accompanied by a slightly shorter oxidation lag-phase in this group. We have previously reported that the lag-phase was significantly shorter in 29 AD patients compared to 29 control subjects.[28] Lower significance of this difference reported here can be related to the lower number of subjects included in our current study.

In a separate experiment, we studied whether kinetics of CSF oxidation can be influenced by Aβ at concentrations close to those measured *in vivo* (0.1–1.0 nM). We found that adding either $A\beta_{1-40}$ or $A\beta_{1-42}$ to a 10-fold diluted CSF at a final concentration of 0.1 nM considerably delayed CSF auto-oxidation (Fig. 1). This was in accordance with our recent data.[30]

DISCUSSION

The major finding of this study was that the oxidative resistance of CSF, expressed as a duration of the oxidation lag-phase, was directly related to CSF levels of $A\beta_{1-40}$, $A\beta_{1-42}$ and ascorbate, and inversely to levels of fatty acids.

Oxidative resistance of lipoprotein-containing biological fluids, such as plasma and CSF, is determined by a subtle balance of anti- and pro-oxidative factors.[27,42,43] Major antioxidative factors are hydrophilic and lipophilic antioxidants. Pro-oxidative factors embrace potential oxidants, such as transition metal ions, products of oxidation which are able to accelerate it, such as lipid hydroperoxides, and substrate for oxidation, such as oxidizable lipids. Our data thus imply that $A\beta_{1-40}$, $A\beta_{1-42}$ and ascorbate are the most important antioxidants, which determine oxidative resistance of CSF *in vitro*, whereas fatty acids represent a most important pro-oxidative factor.

TABLE II CSF parameters of the study population (***$p < 0.001$, *$p < 0.05$ vs. control subjects)

	Control subjects ($n = 20$)	AD patients ($n = 30$)
Ascorbate (μM)	205 ± 42	161 ± 32***
α-Tocopherol (nM)	35 ± 19	45 ± 21
Cholesterol (mg/l)	6.1 ± 3.3	4.9 ± 3.3
Total fatty acids (mg/l)	19.9 ± 15.2	13.8 ± 10.1
$A\beta_{1-40}$ (nM)	1.59 ± 1.03	1.79 ± 0.80
$A\beta_{1-42}$ (nM)	0.065 ± 0.027	0.048 ± 0.019*
Duration of lag-phase of auto-oxidation (h)	8.21 ± 9.87	6.79 ± 5.22
Duration of lag-phase of oxidation by AAPH (h)	7.81±8.96	5.66 ±4.53

Ascorbate is well known as a major hydrophilic antioxidant in human CSF.[28,44] It efficiently inhibits oxidation of lipoproteins in both plasma[45] and CSF.[27] Ascorbate is the antioxidant first consumed during the oxidation of plasma[45] and CSF[27] and builds, together with ubiquinol-10, a first line of their antioxidative defense. We used an absorbance increase at 234 nm as a measure of CSF oxidation. This parameter reflects accumulation of lipid hydroperoxides and hydroxides having a conjugated diene structure, i.e. oxidation of CSF lipoproteins.[27] We showed that the end of the lag-phase of the oxidation of CSF lipoproteins corresponds to a complete loss of ascorbate which, as long as present, is able to completely protect the lipoproteins.[27] This protection is due to recycling of lipoprotein-located α-tocopheroxyl radicals as well as direct scavenging of other free radicals by ascorbate.[46] The positive correlation between the lag-phase duration and CSF level of ascorbate can therefore be explained by its radical-eliminating activity.

In contrast to ascorbate, Aβ peptides have been barely studied as antioxidants. Aβ fragment 25–35 has been reported to inhibit lipid peroxidation at low concentrations, as a result of its interaction with membrane lipid bilayer.[14] We have recently found that, at the concentrations measured in biological fluids (0.1–10 nM), $A\beta_{1-40}$ and $A\beta_{1-42}$ efficiently inhibit auto-oxidation of lipoproteins in CSF and plasma.[30] *In vitro* auto-oxidation of CSF is induced by transition metal ions which are present in native CSF as redox-inactive complexes with metal-binding proteins.[27] The antioxidative properties of Aβ have been therefore ascribed to its metal-chelating activity.

Aβ possesses properties typical of a metal-chelating antioxidant. It inhibits metal-induced oxidation at concentrations slightly higher than that of metals, but do not influence metal-independent oxidation induced by free radicals.[30] Strong metal binding by Aβ has been recently reported for Cu(II) and Fe(III).[16] The histidine residue at position 13 and the tyrosine at position 10 are likely to form a metal-binding site for $A\beta_{1-40}$ and $A\beta_{1-42}$.[16,47] Chelation of metals at this site may be responsible for the correlations we observed. Since $A\beta_{1-42}$ has been reported to have a stronger chelating activity than $A\beta_{1-40}$,[16] this can explain the stronger correlations between CSF oxidative resistance and concentration of $A\beta_{1-42}$, than $A\beta_{1-40}$.

In addition to its ability to bind metal ions, Aβ also efficiently binds to lipids.[48,49] This makes Aβ a lipophilic metal chelator with a metal-reducing activity, a novel combination of redox properties. The physiological metal chelators known to date are mainly hydrophilic proteins with a metal-oxidizing activity.[50] Lipophilic properties of Aβ may allow chelation of metal ions which have avoided binding by hydrophilic chelators and reached lipoproteins.

It should be noted that, unlike many other metal chelators, Aβ has been reported to possess pro-oxidative properties. At high (micromolar) concentrations, it can reduce transition metal ions, such as Cu(II) and Fe(III), to the high-active state and increase generation of reactive oxygen species.[51] The only methionine residue of Aβ, at position 35, may be responsible for the metal reduction which might form a basis for a pro-oxidative activity of Aβ at high concentrations. We hypothesize that, to behave as a pro-oxidant, Aβ must form aggregates, where the atoms of transition metal not only interact with the Aβ metal binding site (formed by histidine and tyrosine residues) but are also available for the reduction by the Met 35. However, this effect had no relevance under our experimental conditions, where Aβ concentration was in a low-nanomolar range (Table II). In a separate experiment, we confirmed the antioxidative activity of both $A\beta_{1-40}$ and $A\beta_{1-42}$ at these concentrations (Fig. 1).

Increased oxidative stress is related to the development of AD[19] and Aβ is considered as an oxidant in this process.[2] However, to accelerate oxidation, Aβ must be present in

concentrations greatly exceeding those measured in biological fluids (i.e. micromolar vs. nanomolar).[2,6,7,52] It seems likely that the pro-oxidant activity of Aβ requires its aggregation by transition metal ions[16] and is related to their reduction by Aβ with a subsequent production of hydrogen peroxide,[51] a major intermediate of Aβ toxicity,[53] in accordance with the mechanism proposed above. In contrast, at low-nanomolar concentrations (i.e. those circulating in CSF and plasma), Aβ is non-toxic, has beneficial effects on neurite outgrowth, neuronal adhesion and cell signaling.[11,12] These effects may be related to antioxidative properties of the peptide. This is in line with the fact that Aβ is normally secreted by neurons and some other cells.[9,10]

The physiologic relevance of the antioxidative properties of Aβ is determined by its physiologic concentration and by the level of oxidative stress *in vivo*. Both in the cell medium[10] and CSF,[6] Aβ is co-localized with lipoproteins similar to plasma high density lipoprotein. The level of Aβ in CSF (0.1–1.0 nM)[5,54–56] corresponds to about one molecule of Aβ per 100 lipoprotein particles. We found that exogenous Aβ at 0.1 nM, i.e. at about one molecule of Aβ per 100 lipoprotein particles, significantly delayed auto-oxidation of 10-fold diluted CSF. This implies that the endogenous level is enough to considerably delay auto-oxidation. Mild oxidation of lipoproteins *in vitro* (such as auto-oxidation) should better reflect their hypothetical *in vivo* oxidation, than strong oxidation by high amounts of oxidants (see Ref. [46] for discussion). Taken together, these data suggest that Aβ may well function as a physiological antioxidant for CSF lipoproteins under normal physiological conditions (i.e. in the absence of amyloid plaques). In contrast, aggregated Aβ in the plaques may behave as a pro-oxidant through the reduction of transition metals which are known to be present in plaques.[57]

The antioxidative properties of Aβ may have wide implications for the biochemistry of AD. Aβ can function as a lipoprotein-bound metal chelator, which binds transition metal ions in inactive form and prevents them from catalyzing lipoprotein oxidation. Production of Aβ by neurons might increase under conditions of increased oxidative stress, i.e. increased requirement of antioxidants, representing a regulatory response, similar to increased synthesis of antioxidant enzymes documented in AD.[58] This has been reported for *in vitro* conditions of cell culture, where increased Aβ production by cells subjected to oxidative stress is observed.[59–61] Transition metal ions are highly enriched in amyloid plaques.[57] Accumulation of such Aβ–metal complexes can thus be considered as a final stage of this protective pathway and should inversely correlate with oxidative damage, and has been recently found.[62,63] However, whereas low levels of produced Aβ might beneficially influence oxidation and thereby decrease oxidative stress, abnormally high Aβ production might further increase it, providing a feedback loop mechanism to further worsen the situation. Highly increased Aβ production is a key feature of transgenic mice which express genes found in the early-onset form of AD.[64] Massive accumulation of Aβ in the brain of AD patients might be accordingly considered as a hyper-response to increased oxidative stress in aging.

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