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Journal
Brain Research, 839(2)

ISSN
1385-299X

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Publication Date
1999-08-01

DOI
10.1016/s0006-8993(99)01694-7

ELSEVIER

Brain Research 839 (1999) 221–226

BRAIN RESEARCH

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Research report

The stabilization of ferrous iron by a toxic β-amyloid fragment and by an aluminum salt

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Accepted 1 June 1999

Abstract

Aluminum is a recognized neurotoxin in dialysis encephalopathy and may also be implicated in the etiology of neurodegenerative disease, particularly Alzheimer's disease. Alzheimer's disease is suspected to be associated with oxidative stress, possibly due to the pro-oxidant properties of β-amyloid present in the senile plaques. The underlying mechanism by which this occurs is not well understood although interactions between amyloid and iron have been proposed. The presence of low molecular weight iron compounds can stimulate free radical production in the brain. This study provides a possible explanation whereby both aluminum and β-amyloid can potentiate free radical formation by stabilizing iron in its more damaging ferrous (Fe^{2+}) form which can promote the Fenton reaction. The velocity, at which Fe^{2+} is spontaneously oxidized to Fe^{3+} at 37°C in 20 mM Bis–Tris buffer at pH 5.8, was significantly slowed in the presence of aluminum salts. A parallel effect of prolongation of stability of soluble ferrous ion, was found in the presence of β-amyloid fragment (25–35). Ascorbic acid, known to potentiate the pro-oxidant properties of iron, was also capable of markedly stabilizing ferrous ions.

Keywords: Aluminum; Iron; Ferrous ion; β-Amyloid; Ascorbic acid; Free radical; Alzheimer's disease; Bathophenanthroline

1. Introduction

Iron is an essential element needed by all mammalian cells. It plays a key role in the function of many proteins such as hemoglobin, and the cytochromes of the mitochondrial respiratory chain, in microsomal electron transfer chains and in many enzymes including several hydroylases and catalases [40]. Iron within the brain is mostly bound to different iron-sequestering molecules [2]. Two proteins, ferritin and transferrin, are the main proteins which store and transport iron in the brain. Iron complexes formed with these proteins are incapable of catalyzing free radical reactions [5,41]. Binding to these proteins can prevent iron from forming insoluble precipitates and can also reduce the exposure of cells to oxidative species [38]. Normally, the amount of unbound low molecular weight iron in the brain is very low [25].

Aluminum is not a transition metal and hence cannot itself initiate any oxidation/reduction reactions. However, the ability of aluminum to potentiate the iron-catalyzed free radical production and to initiate lipid peroxidation has been repeatedly described [6,29,41]. The mechanisms underlying this interaction are unclear. Some studies have proposed that aluminum is capable of competing with iron for the binding sites on ferritin and transferrin, thus leading to more accumulation of free iron in the brain [12,15]. Others have suggested that aluminum can substitute the Fe^{3+} in the Fe^{2+}–O_2–Fe^{3+} complex to form a Fe^{2+}–O_2–Al^{3+} complex which functions as an initiator of iron-mediated lipid peroxidation [3,26].

One of the main physiological features of Alzheimer's disease (AD) is the presence of senile plaques composed of β-amyloid aggregates and of neurofibrillary tangles within specific brain regions. Both aluminum and iron may be present within the neurofibrillary tangles (NFT) in the brains of Alzheimer's patients [17] and it has been proposed that these two metals could be associated with the formation of β-amyloid aggregates [24,42]. While the presence of excess levels of aluminum in NFT or else-

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0006-8993/99/$ - see front matter
PII: S0006-8993(99)01694-7

where in the Alzheimer brain has been repeatedly questioned [4,22], excess levels of intracerebral aluminum can undoubtedly cause neurotoxicity [20,33].

Amyloid plaques appear to be loci of oxidative stress [9], and thus the underlying mechanism of toxicity of amyloid may involve the generation of reactive oxygen species (ROS) produced by the β-amyloid peptides [32]. Our earlier studies supported this by showing that β-amyloid peptide (25–35), which has been reported to be active in promoting oxidative stress [10], is capable of enhancing iron-induced free radical production. Aluminum and β-amyloid can both stimulate Fe^{2+}-promoted ROS production [7].

Ascorbic acid is a well established powerful anti-oxidant, in terms of its ability to scavenge $^{\cdot}OH$ radicals. However, as a reducing agent, it has the ability to reduce ferric to ferrous salts and in the presence of hydrogen peroxide, it acts like O_2^- to reduce ferric ion to ferrous, and hence promoting the formation of $^{\cdot}OH$ radicals through the Fenton reaction. Thus, ascorbate in the presence of iron has significant pro-oxidant potential.

In this study, the stability of ferrous salts in the presence of aluminum sulfate, β-amyloid peptide fragment 25–35, and ascorbic acid, has been investigated. All of these agents were able to retard the oxidation of ferrous ion to insoluble ferric hydroxide thus potentially allowing facilitation of the Fenton reaction.

2. Materials and methods

2.1. Materials

Ferrous sulfate was purchased from Mallinckrodt (Paris, KY), while β-amyloid (25–35) peptide was obtained from Quality Controlled Biochemicals, (Hopkinton, MA). The mesoporous silicate containing functionalized monolayers was a gift from Dr. J. Liu, Pacific Northwest National Laboratory, Richland, WA. All other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Assay for ferrous iron

The measurement of the oxidation of ferrous to ferric was carried out by measuring the spectrophotometric absorbance of the formation the complex formed between ferrous ion and phenanthroline [43]. Twenty millimolar of Bis–Tris buffer (Bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane) was used at a pH of 5.8. All materials were dissolved in this buffer except for bathophenthroline which was dissolved in double distilled water. The selection of pH used in these incubations was based on a compromise between three factors. (i) It should be high enough to reflect a value that could occur physiologically in some cell compartments. The pH of neuronal lysosomes is around 4.6 while mature neuronal endosomes have a pH range of 5.2–6.0 [30]. (ii) It should be low enough so as to allow the basal rate of oxidation of ferrous iron to proceed over an appropriate time interval. (iii) It should be low enough to prevent the macro-precipitation of aluminum hydroxide. Finally, pH 5.8 has been reported to be the optimal for promotion of iron-induced lipid peroxidation of liposomes by aluminum [27]. After combining the necessary chemical agents, the absorbance at 530 nm was spectrophotometrically determined after adding 1.75 mM bathophenanthroline. Bathophenanthroline binds to Fe^{2+} to form a red colored complex with $\lambda_{max} = 530$ nm [40]. Bis–Tris with bathophenanthroline was used as a blank.

2.3. Statistical analysis

Each determination was derived from 4–6 separate assays. Differences between samples were assessed by One-way analysis of variance followed by Fisher's least significant difference test. The acceptance level of significance was $p < 0.05$ using a two-tailed distribution.

3. Results

Twenty micromolar $FeSO_4$ was rapidly oxidized to Fe^{3+} when incubated at 37°C. The proportion of iron present as Fe^{2+} fell to 60% of the original value within 30 min and was 4.6% after 24 h. This process was significantly slowed down in the presence of both 100 μM and 500 μM of $Al_2(SO_4)_3$ (Fig. 1). In a preliminary study, it was found that aluminum did not interfere with the stability of the binding of ferrous iron to bathphenathroline. The concentrations of $FeSO_4$ and $Al_2(SO_4)_3$ initially used were selected on the basis of prior studies seeking maximal potentiation of pro-oxidant interactions [6]. The higher concentration of aluminum substantially stabilized ferrous iron throughout the period of study. Even after 48 h, 30%

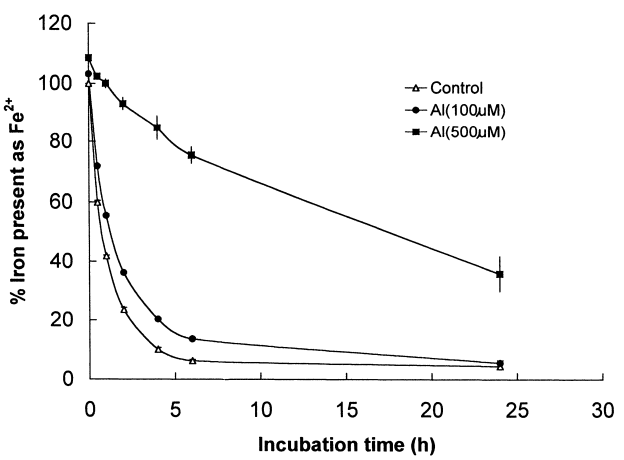

Fig. 1. Rate of oxidation of 20 μM $FeSO_4$ by 100 μM and 500 μM $Al(SO)_4$. Values are presented as percentage of iron remaining in the ferrous form after varying times. Each data point represents the mean ± S.E. of 4–5 individual determinations.

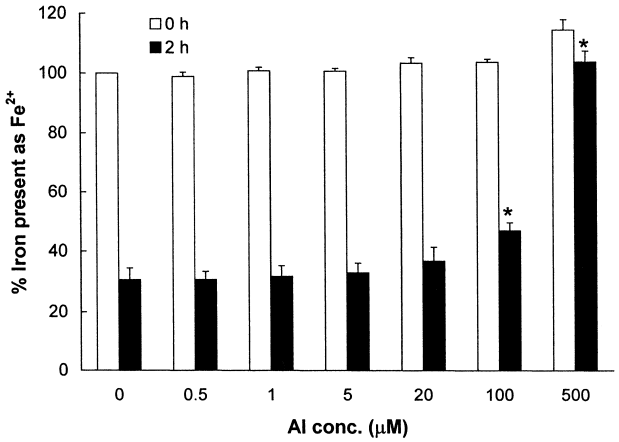

Fig. 2. Oxidation of 20 μM $FeSO_4$ in the presence of various concentrations of $Al_2(SO_4)_3$. The values presented are the percentages of original ferrous iron remaining. Each data point represents the mean ± S.E. of 4–5 individual determinations. * $p < 0.05$ that value differs from corresponding control level.

of the iron was present in its original ferrous form (data not shown). The stabilization affected by 100 μM Al was less substantial, but was significant for at least 6 h. A more detailed analysis using a range of aluminum concentrations was performed and the effect of aluminum in retarding the oxidation of $FeSO_4$ found to be dose-dependent (Fig. 2).

Two concentrations of β-amyloid peptide (25–35) were assayed (Fig. 3). At both concentrations, the proportion of ferrous ion remaining increased as the incubation time got longer. At 20 μM, β-amyloid significantly retarded the rate of oxidation of ferrous sulfate only at 48-h time point. One hundred micromolar of β-amyloid (25–35) stabilized ferrous iron at all three time points studied. Therefore, the pattern of stabilization by β-amyloid was different from that using $Al_2(SO_4)_3$. Both of the larger amyloid fragments β-amyloid (1–40) and β-amyloid (1–42) also slowed down the rate of loss of ferrous ion from solution (data not shown). In order to examine the specificity of the ferrous-

Table 1
Stability of ferrous iron in presence of various peptides

Additions	% Fe^{2+} remaining
None	18.7 ± 1.3
Aβ (25–35)	32.0 ± 2.8*
Aβ (35–25)	22.2 ± 0.2
Ser–Phe–Pro–Trp–Met–Glu–Ser–Asp–Val–Thr	22.6 ± 0.7
Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys (somatostatin)	14.8 ± 0.7
Arg–Gly–Asp–Ser–Pro–Ala–Ser–Ser–Lys–Pro	18.3 ± 0.8

Percentage of unoxidized Fe^{2+} remaining after a 2 h incubation at 37°C of 20 μM $FeSO_4$ together with 100 μM of various peptides. Data are derived from 3–6 individual determinations ± S.E.
* Significantly greater than control value ($p < 0.05$).

stabilizing properties of several amyloid components, the ability of a variety of unrelated peptides to retard the rate of ferrous oxidation was evaluated. None of the other peptides tested were able to slow the oxidation of ferrous sulfate (Table 1).

While the ferrous content of the solution fell to 23% of the original value after 2 h incubation of 20 μM $FeSO_4$, in the presence of ascorbate there was no perceptible loss of Fe^{2+}. Ascorbic acid also substantially conserved ferrous iron, after 6 h (Fig. 4). The ascorbate concentration used was 100 μM which is similar to that found in the cytosol [13]. After more extended incubation times, the proportion of Fe^{2+} remaining was reduced but remained above control values throughout the period investigated.

While it was not possible to directly test the binding of ferrous iron to a non-precipitable form of aluminum, this binding could be examined using particulate alumina. The ability of aluminum hydroxide to bind ferrous ion was examined by mixing 20 μM $FeSO_4$ with aluminum hydroxide powder. After centrifuging down the alumina, bathophenanthroline was used to determine the amount of

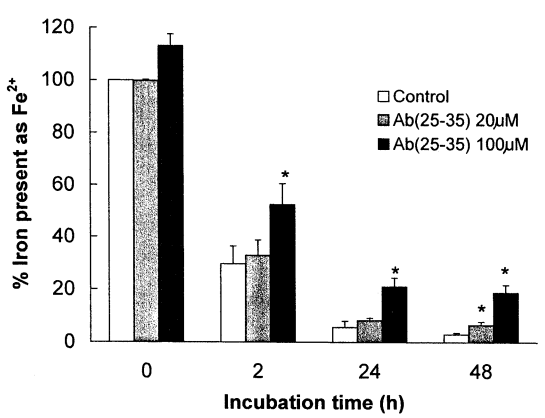

Fig. 3. Retardation of oxidation of 20 μM $FeSO_4$ in the presence of 20 μM and 100 μM β-amyloid (25–35) peptide. Each data point represents the mean ± S.E. of 4–5 individual determinations. * $p < 0.05$ that value differs from corresponding control level.

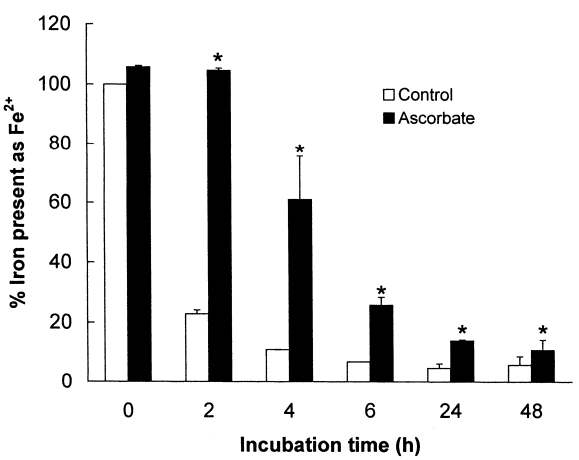

Fig. 4. Effect of 100 μM ascorbic acid on the rate of oxidation of 20 μM $FeSO_4$. Each data point represents the mean ± S.E. of 4–5 individual determinations. * $p < 0.05$ that value differs from corresponding control level.

ferrous ion left in the supernatant. This amount was found to be 2.6 ± 1.1 μM. Subsequent addition of ascorbate to the supernatant did not increase this value showing that results could not be attributed to formation of ferric ions during the procedure. Thus 87% of the $FeSO_4$ had been adsorbed by the alumina. Mesoporous silica, a synthetic mineral with a very large surface area, 900 m^2/g^{-1} [14] containing a monolayer of thiol groups, also retarded oxidation of ferrous iron. When 250 μg/ml of this compound was incubated for 2 h with 20 μM $FeSO_4$, the percentage of ferrous iron remaining was increased from $18.7 \pm 1.3\%$ to $48.4 \pm 6.0\%$.

4. Discussion

The three materials examined in this work, aluminum sulfate, β-amyloid, and ascorbic acid can all stabilize ferrous ion by reducing its rate of oxidation. Ferrous iron is potent in promoting the generation of oxidative species as it actively catalyzes the Fenton reaction [36]. It can spontaneously react with available H_2O_2 in the brain in forming the highly toxic hydroxyl radical, $^{\cdot}OH$. Ferric iron (Fe^{3+}), on the other hand can rapidly become insoluble at physiological pH and does not produce such toxic end-products. The ability of the agents under study to enhance free radical production may be due to the stabilization of iron in the ferrous form and consequent retardation of its deposition as ferric hydroxide. This stabilization would allow more interactions between ferrous iron and free longer-lived oxidative species, particularly H_2O_2. Strong iron chelators such as bathophenanthroline can be redox active and, in the presence of some intracellular components, can facilitate the reduction of protein-bound ferric iron [37]. Since bathophenanthroline was only added at the end of prolonged incubations, immediately prior to spectrophotometric analysis, and since no tissue preparations were used, such properties are unlikely to have confounded our data.

The study was performed at a low pH reflecting that found within endosomes or lysosomes [30]. There are reports that the properties of these organelles are altered by amyloid peptides and that their activation may play a role in the pathogenesis of Alzheimer's disease [11,39].

Brain tissues usually contain an extremely minute amount of aluminum. However, this concentration may be increased in the neurofibrillary tangles and plaques associated with Alzheimer's disease [15]. Any stabilization of Fe^{2+} by Al could permit more Fenton-type interactions between ferrous iron and H_2O_2. Aluminum may be able to potentiate the pro-oxidant capacity of iron both by direct competition at the binding sites within the iron-sequestering proteins and by retarding major conversion to ferric iron.

Ohyashaki et al. [27] reported that some aluminum compounds accelerate the oxidation of ferrous iron in the presence of liposomes. This was especially marked in the case of aluminum acetylacetonate which is organic and lipophilic, and was correlated with the intensity of lipid peroxidation of liposomes. We have confirmed this result but have found that sodium acetylacetonate could also catalyze this oxidation at a similar rate. Therefore, the organic moiety, acetylacetonate rather than aluminum is responsible for this promotion (data not shown). The paradoxical finding that aluminum can enhance iron uptake by neuroblastoma cells despite the fact that both metals compete for transferrin [1], may relate to the stabilization iron in the ferrous form. Ferrous iron is known to be more readily transported across cell membranes and this also allows ascorbate to facilitate iron uptake Han et al. [19].

One of the significant characteristics of AD brains is the existence of senile plaques, within which are amyloid peptides. It has been proposed that oxidative events arising from within plaques contribute toward the etiology of AD [36]. Several studies have shown that amyloid has the ability to promote ROS production [21,23,35]. Roskams and Connor [34] and Bondy et al. [7] have suggested that iron is an essential component of oxidative events associated with amyloid. The basis of such an interaction may be accounted for by the present finding that β-amyloid peptide fragments (25–35), and peptides β-amyloid (1–40) and β-amyloid (1–42) can prolong the existence of iron in its ferrous form. Since several other peptides, including the reverse 35–25 amyloid analog were unable to stabilize iron in its ferrous form such stabilization is unlikely to be due to a specific amino acid residue such as methionine. It is more likely that the ability of amyloid peptides to form colloidal micelles [31], accounts for their interaction with Fe^{2+}.

Ascorbic acid, as expected, maintained iron in the ferrous form for up to 48 h. It is known that ascorbic acid is a powerful reducing agent which can reduce ferric to ferrous iron and can stabilize ferrous iron at low pH [16]. Any material that prolongs the duration of free ferrous iron within cells, may extend the time available for redox cycling and generation of reactive oxygen species. The means whereby aluminum and β-amyloid enhance iron-related oxidative events, superficially resemble those occurring in the presence of ascorbate. However, unlike ascorbic acid, neither aluminum nor β-amyloid has significant reducing power, and their ability to prolong the lifetime of Fe^{2+} must be accounted for by mechanisms not involving direct reduction of ferric iron.

One possible alternate mechanism that may integrate some of our findings, is that both the aggregation of β-amyloid and the colloidal nature of aluminum at pH 5.8, constitute surfaces upon which the persistence of, and oxidant potential of, ferrous iron can be increased. At equilibrium, excess alumina, when in contact with a 20 μM $FeSO_4$ solution, was able to absorb $87 \pm 5\%$ of the soluble iron salt. The concept of colloidal aluminum binding ferrous iron and thus prolonging it's stability is also

supported by the finding that a more lipophilic aluminum salt, the acetylacetonate, which is truly soluble rather than colloidal at pH 5.8, was unable to retard the rate of oxidation of ferrous iron. The critical role of an insoluble matrix is further supported by the finding that mesoporous silica, a synthetic mineral with a very large surface area also retarded oxidation of ferrous iron (Table 1).

Finally, there are several biological examples of enhanced oxidant events, following the binding of iron to particulate surfaces [8]. Several soluble iron chelators with no redox-flux potential, such as ADP and EDTA are also able to promote the ROS generating capacity of iron by forming coordinates that bring the redox potential within the physiological range. Changes in ligand fields around the ferrous ion can allow a coordination site on the iron atom to remain open and unhindered, and this may be the common factor that enhances outer sphere iron-related ROS production [18].

In summary, the results obtained here may reflect a general mechanism underlying the iron-related production of free radicals within the central nervous system. All the three agents that were studied, reduced the rate of oxidation of ferrous iron. This stabilization is likely to involve more than a mere inhibition of transition between ferrous and ferric iron (which would effectively act in an antioxidant manner), but may also reflect a restraint, preventing ferric iron from leaving solution as a hydroxy-complex, and thus no longer able to participate in redox cycling. By this means, iron-catalyzed free radical production and lipid peroxidation could be significantly enhanced. Prolongation of the oxygen-radical generating properties of iron may in part account for the neurotoxicity of β-amyloid and aluminum and this may have relevance to Alzheimer's disease.

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