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Promotion of transition metal-induced reactive oxygen species formation by β-amyloid

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Abstract

β-amyloid protein appears to be involved in the neural degeneration associated with Alzheimer's disease. However, its mechanism of action is poorly understood. The ability of the neurotoxic peptide fragment (25–35) derived from β-amyloid, to promote the generation of reactive oxygen species (ROS) by a postmitochondrial fraction (P2) derived from rat cerebrocortex, has been examined. The peptide fragment, when incubated together with P2, did not cause excess ROS formation. However, 10 μM $FeSO_4$ or 10 μM $CuSO_4$ were able to enhance ROS production in the P2 fraction and this was increased further in the concurrent presence of the 25–35 fragment. The corresponding inverse sequence non-neurotoxic peptide (35–25) had no parallel ability to augment iron-stimulated ROS production suggesting a degree of specificity for the observed effect. There was no formation of excess ROS when the 25–35 peptide and 0.5 mM $Al_2(SO_4)_3$ were incubated with the P2 fraction. However in the presence of both aluminum and iron salts together with the 25–35 peptide, ROS production was augmented to a level significantly higher than that in the absence of aluminum. Polyglutamate, a peptide reported to mitigate aluminum toxicity had no effect on iron-related ROS generation but completely prevented its further potentiation by aluminum. The results indicate that β-amyloid is able to potentiate the free-radical promoting capacity of metal ions such as iron, copper and aluminum. Such potentiation may be a relevant mechanism underlying β-amyloid-induced degeneration of nerve cells.

Keywords: Amyloid; Iron; Reactive oxygen species; Transition metal; Aluminum

1. Introduction

Alzheimer's disease has become a major concern in aging populations, since the prevalence of this disorder in the very elderly is very high. A morphological characteristic of Alzheimer's disease is the appearance of amyloid plaques within specific cortical and limbic brain regions. The major protein component of these inclusions, β-amyloid, has been the subject of intense study but the mechanisms underlying β-amyloid neurotoxicity remain unclear. One focus of investigation has been the apparent capacity of amyloid peptides to enhance generation of reactive oxygen species in isolated cells of neural origin [22,33,44], although this view remains controversial [31]. The ability of β-amyloid to promote the appearance of free radicals has even been shown in cell free media [24]. The mechanism by which such free radicals are produced by β-amyloid acting on neural tissues may be mediated by the generation of nitric oxide [27] and may also involve excitotoxic events [22]. Oxidative damage to proteins has been reported in post mortem tissue from Alzheimer's patients [39,47,48,21,25,53], and thus pro-oxidant properties of β-amyloid may have clinical relevance. However a causal relation between oxidative damage and this disorder has not yet been unequivocally shown and remains controversial [4,23,52,41].

The current study was undertaken to gain further insight into the mechanism underlying the pro-oxidant properties of β-amyloid with especial reference to the role of metals in this process. The rationale for study of iron salts is that in the ferrous form, this metal is a powerful pro-oxidant and that the metabolism of iron and of iron complexing proteins such as transferrin, may be altered in Alzheimer's disease [11,17,32]. Copper, another transition metal with the ability to promote formation of oxidant radicals, has also been proposed as being implicated in amyloid-related

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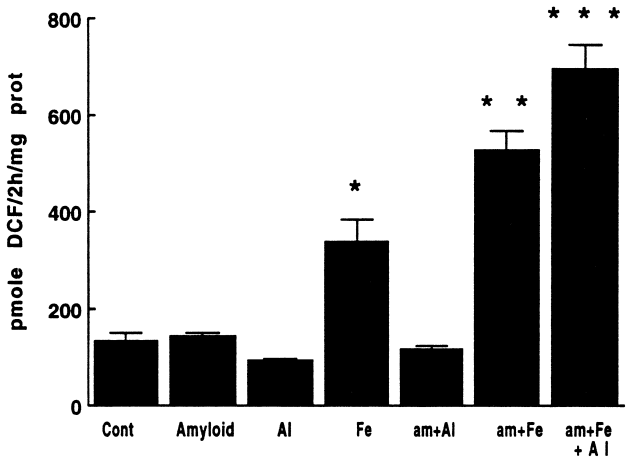

Fig. 1. Rates of generation of ROS in cortical P2 fraction, in the presence of 50 μM amyloid β peptide 25–35, aluminum (0.5 mM) or iron salts (10 μM), separately or together. Values are means ± S.E.M. ($n = 5$). ∗Value is significantly greater than control value; ∗∗Value is greater than corresponding value presence of iron and in the absence of amyloid peptide; ∗∗∗Value is greater than the corresponding value in the absence of aluminum but in presence of iron and peptide ($p < 0.05$).

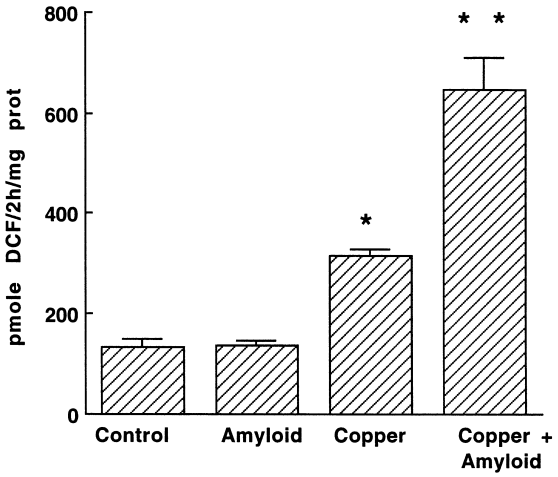

Fig. 2. Rates of generation of ROS in cortical P2 fraction, in the presence of 50 μM amyloid β peptide 25–35 and 10 μM $CuSO_4$, separately or together. Values are means ± S.E.M. ($n = 4$). ∗Value is greater than control value; ∗∗Value is greater than corresponding value presence of copper and in absence of amyloid peptide ($p < 0.05$).

toxicity [37]. Aluminum is a suspect in the etiology of Alzheimer's disease [37,18,50,36,1] although this concept has been challenged [5]. However, aluminum is clearly able to promote the free-radical generating capacities of iron in several experimental systems although the mechanistic basis of this property not known [19,38,6,7].

We have investigated the potential of the 25–35 β-amyloid fragment previously reported to be active in promoting oxidative stress [9], to enhance free radical generation in an isolated P2 mitochondrial–synaptosomal preparation. We now report that, while β-amyloid by itself does not promote pro-oxidant events, it can exacerbate free radical generation induced by several metals.

2. Experimental

2.1. Animals and tissue preparation

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 125–150 g were utilized. Rats were housed three per cage with wood chip bedding and maintained on a 12-h light/dark cycle in a temperature controlled (20 ± 1°C) room. Food and water were provided ad lib. Rats were decapitated and the brains were excised quickly on ice, and the cerebrocortex was dissected out. Tissue was weighed and homogenized in 10 volumes of 0.32 M sucrose and centrifuged at $1800 \times g$ for 10 min. The resulting supernatant fraction was then centrifuged at $31\,500 \times g$ for 10 min to yield the cerebral P2 faction. The P2 pellet was taken up in HEPES buffer to a concentration of 0.1 g-eq/ml. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and HEPES, 10; pH 7.4.

2.2. Assay for oxygen reactive species

Reactive oxygen species (ROS) were assayed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) [2]. DCFH is capable of being oxidized to the fluorescent 2′,7′-dichlorofluorescein by reactive oxygen. The utility of this probe in isolated cerebral subcellular cerebral systems represents a direct means of quantitating highly reactive oxidizing moieties and has a sensitivity that generally exceeds that of

Table 1
The effect of 10 μM ferrous sulfate on rates of generation of ROS in cortical P2 fraction, in the presence of 50 μM amyloid β peptide 25–35 or the inverted sequence peptide 35–25

Peptide additions	Nanomoles of DCF formed per milligram protein per 2 h	
	No added Fe	+10 μM $FeSO_4$
None	134 ± 17	339 ± 45
25–35 β-amyloid fragment	144 ± 7	528 ± 33∗
35–25 inverted sequence	147 ± 16	303 ± 22

Values are means ± S.E.M. ($n = 5$).
∗Value differs significantly from corresponding value in absence of added peptide ($p < 0.05$).

Table 2
Glutathione levels in cortical P2 fraction, following incubation at 37°C for 15 min in the presence of 50 μM amyloid β peptide 25–35 and/or 10 μM ferrous sulfate

Additions	Micromoles of GSH per milligram protein
None	9.1 ± 0.5
25–35 β-amyloid fragment	9.0 ± 0.2
$FeSO_4$	7.6 ± 0.2 *
$FeSO_4$ + β-amyloid fragment	7.1 ± 0.4 *

Values are means ± S.E.M. ($n = 5$).
* Value differs significantly from corresponding value in absence of any additions ($p < 0.05$).

more indirect markers such as lipid peroxidation [29]. P2 suspensions were diluted in 19 volumes of HEPES buffer. The diluted fractions were then loaded with 5 μM DCFH-DA (added from a stock solution of 0.5 mM in 10% ethanol) at 37°C for 15 min. After this incubation with DCFH-DA, the fractions were incubated for a further 2 h in the presence of various metal salts and peptides. The concentrations of $FeSO_4$ and $Al_2(SO_4)_3$ used were derived from an earlier study and represented those levels found to be optimal for promotion of ROS in P2 fractions [6]. Peptide solutions were maintained at −80°C and were only thawed once prior to use in order to ensure minimal aggregate formation. At the beginning and at the end of incubation, fluorescence was monitored on a Perkin-Elmer LS-5 fluorescence spectrophotometer, with excitation wavelength at 488 nm (bandwidth 5 nm), and emission wavelength at 525 nm (bandwidth 20 nm). Oxygen reactive species formation was quantitated from a 2′,7′-dichlorofluorescein (DCF) standard curve (0.05–1.0 mM) and results were expressed as nanomoles of DCF formed per 15 min per milligram protein. Protein concentration was assayed by the method of Bradford [8].

2.3. Glutathione

Glutathione (GSH) levels were determined within S2 fractions using a modification of the method of Shrieve et al. [45]. The principle behind the assay is that monochlorobimane (mBCl), a nonfluorescent compound, reacts with glutathione to form a fluorescent adduct. Protein thiols are not assayed by this procedure. mBCl was dissolved in ethanol to a concentration of 5 mM and stored at −10°C in the dark. mBCl was added to 2 ml of a S2 suspension to a final concentration of 10 μM, after which the suspension was incubated for 15 min at 37°C. The fluorescence of the supernatant was spectrofluorometrically determined at an excitation wavelength 395 nm and an emission wavelength 470 nm. The tissue GSH concentration was calculated using a GSH standard curve.

2.4. Statistical analyses

Differences between groups were assessed by one-way ANOVA followed by Fisher's Least Significant Difference Test. The acceptance level of significance was $p < 0.05$ using a two-tailed distribution.

2.5. Materials

2′,7′-Dichlorofluorescin was purchased from Molecular Probes (Eugene, OR), while DCF required for calibration was obtained from Polysciences (Warrington, PA). Other materials including peptides were from Sigma (St. Louis, MO).

3. Results

The basal level of ROS production by the P2 particulate fraction from rat cortex was considerably increased by the presence of 10 μM $FeSO_4$ (Fig. 1). The rate of generation of reactive oxygen species by this fraction was unaffected by 50 μM of the neurotoxic β-amyloid 25–35 peptide fragment, Aβ(25–35) (Table 1). Prior incubation of Aβ(25–35) for 24 h at 20°, did not result in this peptide acquiring the capacity to enhance ROS formation in the absence of a transition metal (data not shown). However, when the peptide and iron salt were present concomitantly, the ROS-promoting potential of $FeSO_4$ was enhanced by over 50%. When $Al_2(SO_4)_3$ was also present, this potentiation was further enhanced to a level that was greater than

Table 3
Effect of 50 units/ml SOD upon rate of DCF oxidation by P2 fraction in the presence of 10 μM $FeSO_4$ or 50 μM β-amyloid fragment 25–35

Additions	Nanomoles of DCF formed per milligram protein per 2 h	
	SOD not present	SOD present
None	160 ± 17	125 ± 7
25–35 β-amyloid fragment	163 ± 15	136 ± 11
$FeSO_4$	342 ± 53 *	287 ± 30 *
$FeSO_4$ + β-amyloid fragment	583 ± 47 * [a]	416 ± 33 * [a]

Values are means ± S.E.M. ($n = 4$–5).
* Value differs significantly from corresponding value in absence of any additions.
[a] Value differs from value in presence of iron and absence of amyloid ($p < 0.05$).

that attained by peptide + iron. The ability of 10 μM $CuSO_4$ to promote ROS generation was also increased in the concurrent presence of Aβ(25–35) (Fig. 2). Thus copper and iron had a similar effect in this regard.

Levels of glutathione (GSH) within the P2 fraction were used as another index of oxidant status. The GSH content of this fraction was unaltered by the presence of Aβ(25–35), and decreased in the presence of iron. When both the peptide and metal were present, the minor further decrease that was observed was not significant (Table 2).

An attempt was made to determine whether the excess ROS production in the presence of both iron and Aβ(25–35), involved superoxide anion as an intermediate. The rate of ROS formation when iron and Aβ(25–35) were present together, was greater than that produced in the presence of iron alone even when superoxide dismutase (SOD) (50 units/ml) was added (Table 3). A similar potentiation was obtained in the presence of 250 units/ml catalase (data not shown). Thus there was no evidence for superoxide or hydrogen peroxide as a critical intermediate of amyloid/iron interaction.

The non-neurotoxic peptide with inverted sequence of Aβ(25–35), namely Aβ(35–25), was unable to further promote the rate of Fe-induced ROS generation, suggesting a degree of specificity for the effect observed with the β-amyloid 25–35 peptide (Table 1).

A further experiment was designed to inquire as to the selectivity of the potentiation of metal related oxidative events by Aβ(25–35). This involved the use of a synthetic peptide, L-polyglutamate which has been reported to reduce the toxicity of aluminum in plants [42]. In this case Fe-induced ROS production was unaffected by the peptide but the aluminum related enhancement of this ROS production was greatly reduced (Fig. 3).

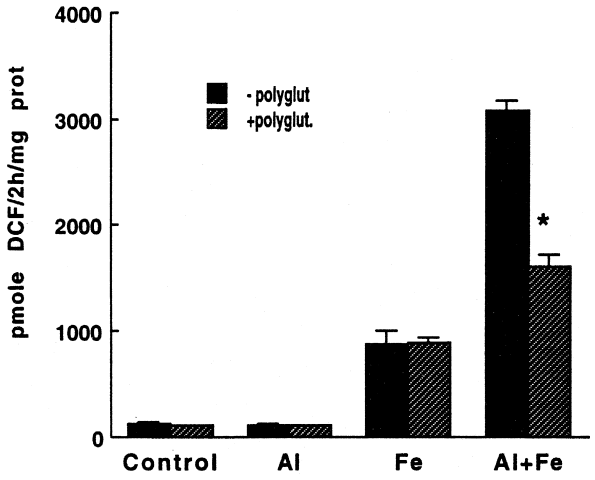

Fig. 3. Inhibition of aluminum enhanced Fe-related ROS generation in P2 fraction by 10 μM L-polyglutamate (MW = 15 000). Concentrations of $Al_2(SO_4)_3$ and $FeSO_4$ were 0.5 mM and 50 μM, respectively. Values are means ± S.E.M. ($n = 5$). * Value differs significantly from corresponding value in the absence of L-polyglutamate ($p < 0.05$).

4. Discussion

The results presented here suggest that the amyloid β peptide 25–35 has no intrinsic ROS-promoting potential in the absence of a transition metal ion. However, it can stimulate the production of ROS by an iron or a copper salt. The level of glutathione was unaffected by the presence of amyloid peptide but was depressed by $FeSO_4$. However, no significant interaction of amyloid with iron was found (Table 2). This may be to the relatively lower sensitivity of this more indirect means of evaluating oxidant status. There have been several reports that β-amyloid alone can promote oxidant events in isolated preparations and that 'aging' of the peptide can enhance this (see Section 1). We suggest that such results may be due to traces of iron salts in the preparations. The extended incubation required for 'aging' of amyloid may leach iron salts out of containing vessels and thus appear to further enhance oxidant potency of amyloid preparations. On occasion we have obtained a sample of amyloid β-peptide that exhibited intrinsic ROS-promoting activity. In such cases, even 1 μM deferoxamine was able to completely block this effect, suggesting that it was attributable to the presence of traces of iron (data not shown). Studies on the putative role of iron are confounded by the fact that the intracellular concentration of low molecular weight iron is both low and difficult to quantitate. Iron has been reported to be essential for even basal toxicity of β-amyloid [43] but it has also been found that 10 mM deferoxamine did not inhibit amyloid-related free radical formation [24]. These inconsistencies may relate to the fact that it is difficult to ensure that metal chelation invariably leads to successful sequestration of a metal, rendering it incapable of promoting free radical production. Deferoxamine, is a potent iron chelator at low concentrations, but under some conditions it is also capable of promoting pro-oxidant events both in isolated systems and in animals [28,51], and at high concentrations, can directly quench free radicals [20].

The mechanism underlying the enhancement of metal-induced ROS by amyloid, may relate to the fact that the iron-related promotion of pro-oxidant events is known to be potentiated at solid/liquid interfaces [16]. The formation of iron or copper complexes where the coordination sites of the metal are only partially occupied, may form the basis for increasing the pro-oxidant potency of transition metals. The surface of particles may be a critical site for appearance of free radical activity [13]. Such particulates include mineral fibers such as silica [12], carbide [30], or asbestos [35,46]. Catalytic particulates may also be of biological origin. For example, melanin, a complex indole polymer, provides a site for promotion of iron-related pro-oxidant events [15,4]. In a parallel manner, the tendency for β-amyloid peptides to aggregate and form micelles may provide key loci for iron-based ROS formation. Iron and copper have been reported to complex with the

histidyl and sulfhydryl residues respectively, within amyloid protein [37,49]. This binding may form the basis for the reduction of transition metals and consequent initiation of Fenton chemistry [26]. The reason why the reverse peptide did not potentiate iron may relate to the fact that only the 25–35 peptide is able to form high molecular weight β-pleated aggregate complexes [40] and these may be able to present key surfaces for the ionic iron attraction required prior to any covalent attachment.

Aluminum was clearly able to further potentiate the ROS produced as a result of the amyloid/iron interaction. This parallels the ability of aluminum salts to enhance iron- or copper-promoted oxidative events in the absence of amyloid peptide [7]. Aβ(25–35) is known to form oligomeric aggregates with 'aging', which, over a period of time may result in a more neurotoxic complex [31,40]. The ability of the toxic peptide to act as a surface for iron-related free radical activity may be enhanced by aluminum salts since these can promote β-amyloid aggregation [10,34] and may accelerate plaque deposition [14]. The finding that a peptide (polyglutamate) known to protect against aluminum toxicity, also blocked the aluminum effected enhancement of iron-induced oxidative events, suggests a relation between the ROS-potentiating properties of aluminum salts in the presence of iron, and their toxicity.

Taken together, the findings reported here indicate that β-amyloid is able to potentiate the free-radical promoting capacity of metal ions such as iron, copper and aluminum. Such interactions may constitute one of the processes underlying the neurotoxicity of β-amyloid.

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