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### Journal

Journal of Molecular Neuroscience, 27(2)

### ISSN

0895-8696

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### Publication Date

1996-02-01

### DOI

10.1007/bf02815093

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# The Promotion of Iron-Induced Generation of Reactive Oxygen Species in Nerve Tissue by Aluminum

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Received May 26, 1995; Accepted July 25, 1995

## ABSTRACT

Aluminum is suspected to play a role in several neurological disorders. Reactive oxygen species (ROS) lead to oxidative stress, which is thought to be a possible mechanism for neurological damage. Interactions between aluminum and iron, a known promoter of prooxidant events, were studied in cerebral tissues using a fluorescent probe to measure rates of generation of ROS.  $\text{Al}_2(\text{SO}_4)_3$  alone failed to stimulate ROS production over a wide range of concentrations (50–1000  $\mu\text{M}$ ). The aluminum-deferrioxamine chelate in the absence of iron could also not potentiate ROS formation. However,  $\text{Al}_2(\text{SO}_4)_3$  potentiated  $\text{FeSO}_4$ -induced ROS, with a maximal effect at 10  $\mu\text{M}$  Fe and 500  $\mu\text{M}$  Al. Kaolin, a hydrated aluminum silicate, did not potentiate iron-induced ROS formation. Ferritin had a minor stimulatory effect on ROS generation, but this was not potentiated by the concurrent presence of  $\text{Al}_2(\text{SO}_4)_3$ . Transferrin had no effect on basal rates of ROS generation, but when  $\text{Al}_2(\text{SO}_4)_3$  was also present, ROS production was enhanced. It is concluded that:

1. There is a potentiation of iron-induced ROS by aluminum salts;
2. Free or complexed aluminum alone is not a key producer of ROS; and
3. High rates of ROS production are unlikely to be owing to the displacement by aluminum iron from its biologically sequestered locations.

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**Index Entries:** Aluminum; iron; free radicals; reactive oxygen; synaptosomes; oxidative stress.

**Abbreviations:** DCFH-DA, 2', 7'-dichlorofluorescin diacetate; DCFH, 2', 7'-dichlorofluorescin; DCF, 2', 7'-dichlorofluorescein; ROS, reactive oxygen species.

## INTRODUCTION

The capacity of iron in a low-mol-wt form to stimulate biological lipid peroxidation is well known. This property has been reported to be enhanced within neural tissues by aluminum salts (Gutteridge et al., 1985; Aruoma et al., 1989; Fraga et al., 1990). The phenomenon is puzzling, in view of the inability of aluminum ions to undergo the changes in valency, characteristic of transition metals that accounts for their potential to promote excess pro-oxidant activity within cells. Neither does aluminum have a strong affinity for sulfhydryl groups. Although the intrinsic capacity of aluminum to induce the generation of free radicals has been reported, this may depend on the concurrent presence of iron (Fraga et al., 1990; Oteiza et al., 1993).

In view of the proposed association between Alzheimer disease and cerebral aluminum content (McLachlan et al., 1989), illumination of the effects of aluminum on cerebral metabolism may be important. The potentially pro-oxidant role of aluminum is especially relevant because of the reported presence of excess oxidative activity in both the aged brain, and more specifically, in those regions of the Alzheimer brain showing the most pronounced pathological changes (Smith et al., 1991). Iron and aluminum are present at high concentrations in neurofibrillary tangles (Good et al., 1992) and may cooperate in the promotion of physiological aggregation of the  $\beta$ -amyloid peptide (Shigematsu and McGeer, 1992; Mantyh et al., 1993). Such aggregation may be the result of oxidative events (Hensley et al., 1994). Lipid peroxidation rates are also elevated in autopsy samples of Alzheimer cortex (Subbarao et al., 1990), and there is other evidence of an association between this disease and oxidative stress (Pappola et al., 1992). Excess levels of iron together with aluminum have also been found in the substantia nigra of Parkinsonian patients (Hirsch et al., 1991), and aluminum accumulates within neurons in amyotrophic lateral sclerosis-Parkinsonian dementia of Guam (Perl et al., 1982).

The current study was designed both to confirm such interactions between iron and aluminum, and to understand the processes that may underlie this property. The direct assay of short-lived oxidative species was performed using a fluorescent probe, rather than measurement of more secondary indices of quantitation of damage to lipids or proteins. Issues that have been addressed include the potential of aluminum to displace iron from its intracellular binding sites, the ratio of iron to aluminum affecting such excess generation of reactive oxygen species (ROS), the

molecular form of aluminum producing nonadditive pro-oxidant effects with iron, and the role of membranes in catalyzing any interaction.

## MATERIALS AND METHODS

### *Tissue Preparation*

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 150–175 g were utilized. Rats were decapitated, the brains were excised quickly on ice, and the cerebrocortex was dissected out. Tissue was weighed and homogenized in 10 vol of 0.32 M sucrose and centrifuged at 1800g for 10 min. The resulting supernatant fraction was then centrifuged at 31,500g for 10 min to yield the crude cerebral synaptosomal pellet (P2). The P2 pellet was taken up in HEPES buffer to a concentration of 0.0037 g Eq/mL. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; MgCl<sub>2</sub>, 0.1; NaHCO<sub>3</sub>, 5.0; glucose, 6.0; CaCl<sub>2</sub>, 1.0; and HEPES, 10; pH 7.4.

### *Assay for ROS Formation*

ROS were assayed using 2', 7'-dichlorofluorescein diacetate (DCFH-DA), which is de-esterified within cells to the ionized free acid, dichlorofluorescein, DCFH. This is trapped within cells and thus accumulated (Bass et al., 1983). DCFH is capable of being oxidized to the fluorescent 2', 7'-dichlorofluorescein (DCF) by reactive oxygen. The utility of this probe in isolated subcellular cerebral systems has been documented (LeBel and Bondy, 1991). P2 or P3 suspensions were diluted in 9 vol of HEPES buffer. The diluted fractions were then incubated with 5 mM DCFH-DA (added from a stock solution of 1.25 mM in ethanol) at 37°C for 15 min. After this loading with DCFH-DA, the fractions were incubated for a further 15 min in the presence of various metallic compounds. At the beginning and at the end of incubation, fluorescence was monitored on a Farrand Spectrofluorometer, with excitation wavelength at 488 nm (bandwidth 5 nm), and emission wavelength 525 nm (bandwidth 20 nm). The rate of generation was found to be linear over the incubation period (LeBel and Bondy, 1990). Autofluorescence of fractions was corrected for by the inclusion in each experiment of parallel blanks with no DCFH-DA. The correction for autofluorescence was always <11% of the total. ROS formation was quantitated from a DCF standard curve (0.05–1.0 mM), and results were expressed as nmol DCF formed/15 min/mg protein.

### *Materials*

DCFH-DA was purchased from Molecular Probes, Inc. (Eugene, OR), whereas DCF required for calibration was obtained from Polysciences, Inc. (Warrington, PA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

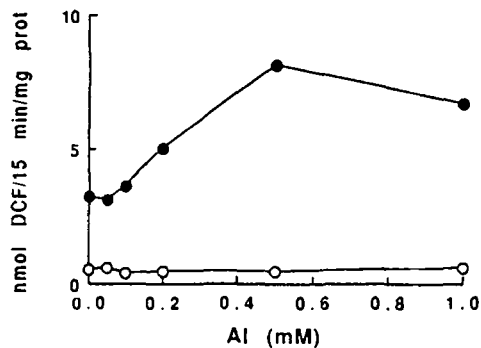


Fig. 1. The effect of varying concentrations of  $\text{FeSO}_4$  on generation of reactive oxygen species within the cerebrocortical P2 fraction in the presence or absence of  $500 \mu\text{M}$   $\text{Al}_2(\text{SO}_4)_3$ . Values represent the mean of 2-3 individual determinations, which differed by  $< 15\%$ .  $\circ$ , Al alone;  $\bullet$ , +  $20 \mu\text{M}$  Fe.

### Protein Determination

Protein concentration was assayed using the method of Bradford (1976).

### Statistical Analyses

Differences between groups 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.

## RESULTS

There was a clear enhancement of  $\text{FeSO}_4$ -induced rates of ROS generation within crude synaptosomes by  $\text{Al}_2(\text{SO}_4)_3$ . When the iron salt concentration was maintained at  $10 \mu\text{M}$ , maximal potentiation was observed in the presence of  $0.5$ – $1 \text{ mM}$  aluminum (Fig. 1). This amount of  $\text{Al}_2(\text{SO}_4)_3$  was insufficient to lower the pH of the HEPES-buffered incubation medium. In the absence of added iron,  $\text{Al}_2(\text{SO}_4)_3$  failed to stimulate ROS at all concentrations tested ( $50$ – $1000 \mu\text{M}$ ).

In the absence of aluminum, iron-stimulated ROS was maximal at  $5 \mu\text{M}$ . However, in the presence of  $0.5 \text{ mM}$   $\text{Al}_2(\text{SO}_4)_3$ , the ROS-enhancing capacity of  $\text{FeSO}_4$  was increased, and this was continued up to an iron concentration of  $50 \mu\text{M}$  (Fig. 2).

There has been speculation that aluminum may enhance the potency of iron-induced lipid peroxidation by way of subtle alterations of membrane structure (Gutteridge et al., 1985; Oteiza et al., 1993). For this reason, the potential for interaction between iron and aluminum in the absence of membrane structures was examined by use of the postmicrosomal high-speed supernatant fraction of cerebral cortex. In this case,

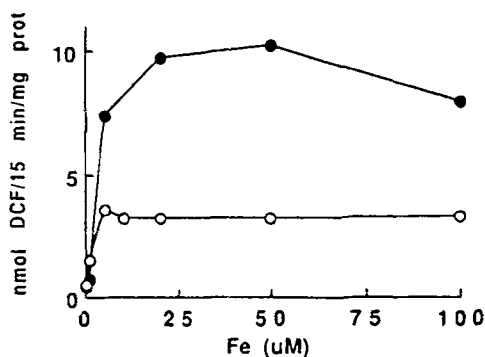


Fig. 2. Generation of ROS within the cerebrocortical P2 fraction in the presence or absence of  $10 \mu\text{M}$   $\text{FeSO}_4$  and varying concentrations of  $\text{Al}_2(\text{SO}_4)_3$ . Values represent the mean of 2 individual determinations, which differed by  $< 15\%$ . ○, Fe alone; ●, +0.5 mM Al.

Table 1  
Effect of Soluble Aluminum as the Soluble Sulfate or in Insoluble Mineral Form on Iron-Stimulated ROS Formation in Cortical Synaptosomes

Additions	nmol DCF/15 min/mg protein
None	$0.52 \pm 0.09$
$20 \mu\text{M}$ $\text{FeSO}_4$	$4.42 \pm 0.18^a$
Kaolin (170 mg/mL)	$0.67 \pm 0.14$
$20 \mu\text{M}$ $\text{FeSO}_4$ + Kaolin	$0.24 \pm 0.05$
0.5 mM $\text{Al}_2(\text{SO}_4)_3$	$0.47 \pm 0.09$
$20 \mu\text{M}$ $\text{FeSO}_4$ + 0.5 mM $\text{Al}_2(\text{SO}_4)_3$	$6.79 \pm 0.12^a$

Data represent means from 4-7 determinations  $\pm$  SEM.

<sup>a</sup>Significantly elevated relative to basal level.

basal rates of ROS production were very low, and these were not stimulated by addition of inorganic iron or aluminum compounds, or by the presence of both ions (data not shown). Thus, the presence of a membrane fraction is needed for the potentiation described.

A proposal has been made that aluminum-silicon complexes, such as zeolite or kaolin, may be the most potent accelerators of iron-based oxidative stress (Garrel et al., 1994) and that these may be related to the pathogenesis of Alzheimer disease (Evans et al., 1992). The formation of ROS within the cortical P2 fraction was inhibited by a colloidal suspension of kaolin at a concentration of  $170 \mu\text{g/mL}$ . This concentration was calculated to result in a aluminum concentration of 0.5 mM. The stimulatory effect of  $\text{FeSO}_4$  was inhibited rather than stimulated in the presence of kaolin (Table 1). Since kaolin did not inhibit basal rates of ROS generation, this result could not have been owing to the quenching of fluorescence. The ionic form of aluminum may be essential for the promotion of

Table 2  
Effect of Aluminum Sulfate and the Presence  
of Iron-Containing Proteins on Synaptosomal ROS Formation

Additions	nmol DCF/15 min/mg protein
None	0.54 ± 0.05
Ferritin (~ 50 mM Fe)	1.18 ± 0.13 <sup>a</sup>
Ferritin + 0.5 mM Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	0.61 ± 0.12
Transferrin (~ 60 μM Fe)	0.63 ± 0.03
Transferrin + Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	1.81 ± 0.30 <sup>a</sup>

Data represent means from 6-10 individual determinations ± SEM.

<sup>a</sup>Significantly elevated relative to basal level.

iron-induced ROS. Another issue that was addressed is whether the observed potentiation of ROS was the result of the gradual formation of an insoluble complex, perhaps of hydrated ions. The time-course of appearance of enhanced ROS generation by Fe + Al salts was determined. A freshly prepared mixture of the salts was just as active as the same mixture that had been allowed to stand for 2 d (data not shown). Centrifugation of the standard mixture of 50 μM FeSO<sub>4</sub> and 500 μM Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> did not alter the capacity of the mixture to promote excess ROS (data not shown). These data suggested that the promoting effect of aluminum on iron-induced ROS does not involve the formation of a high-mol-wt insoluble mineral complex.

It may be that the displacement by aluminum of iron from its biologically complexed forms can account for some of the observed interactions between these metals. To test this hypothesis, the effect of aluminum on ROS production was examined in the presence of ferritin, an iron storage protein, and transferrin, a protein involved in iron transport. Both proteins were in the iron-loaded form. Ferritin normally contains some complexed aluminum (Dedman et al., 1992), which can modulate its properties (Fleming and Joshi, 1991). Transferrin has a high affinity for, and may transport aluminum into the central nervous system (CNS) (Roskams and Connor, 1990). The concentrations of ferritin and transferrin used (13.5 μg/mL and 10 mg/mL for ferritin and transferrin, respectively) were calculated to give an iron concentration roughly equivalent to 75 μM iron. Ferritin stimulated ROS generation as has been previously described (Kukiella and Cederbaum, 1994), but this effect was not enhanced by the concurrent presence of aluminum (Table 2). Transferrin did not affect basal rates of ROS production by the crude synaptosomal fraction, but when aluminum sulfate was also present, there was a major elevation of ROS (Table 2), suggesting that iron may have been displaced from this protein by aluminum.

A final issue that has been addressed is the possibility that chelates of aluminum may be able to promote ROS formation even in the absence of iron; 500 μM aluminum sulfate potentiated the ROS generating capacity

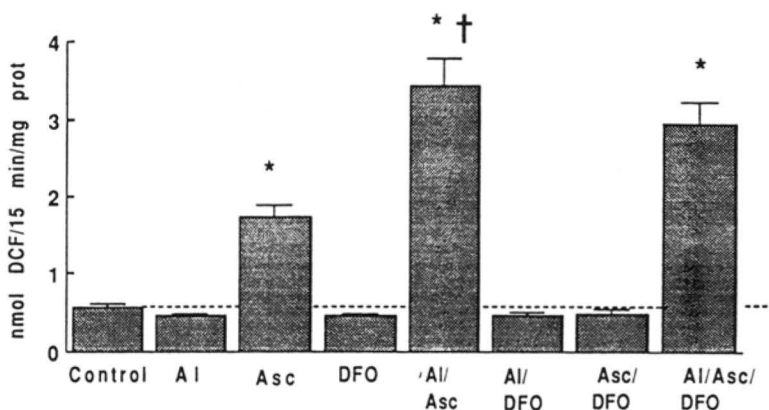


Fig. 3. The effect of 0.5 mM aluminum sulfate (Al), 1.0 mM ascorbic acid (Asc), and 10  $\mu$ M desferrioxamine (DFO) on ROS formation in cerebrocortical synaptosomes. Values represent the mean of determinations on 7 individual rats  $\pm$  SEM. \*  $p < 0.05$ —that value differs from untreated control level. †  $p < 0.05$ —that value is greater than that of ascorbate alone.

of 1 mM ascorbate. The stimulation of ROS by ascorbate alone at this concentration was completely abolished by 10  $\mu$ M desferrioxamine (Fig. 3). This low concentration of deferoxamine was intended to sequester iron selectively. Higher concentrations of this compound have an intrinsic ability to quench free radicals (Halliwell, 1989). In view of the high affinity of deferoxamine for iron, this was interpreted as demonstrating the requirement for the presence of trace amounts of iron for this pro-oxidant effect of ascorbate. In contrast, the promotion of ascorbate-induced ROS by aluminum was not prevented by 10  $\mu$ M desferrioxamine.

## DISCUSSION

The findings reported above suggest that the potentiation of iron-induced ROS generation by aluminum cannot be largely attributed to:

1. The presence of complex aluminum-silicon-based mineral surfaces on which iron catalysis of ROS synthesis can occur;
2. The intrinsic pro-oxidant properties of free aluminum salts in the absence of iron or chelators;
3. The displacement of iron from biologically complexed sites, causing its appearance in low-mol-wt forms. Although aluminum may bind to transferrin, the iron content of this protein is low (around 0.14%), and the respective affinity constants of the iron and aluminum complexes make this possibility unlikely.



The most promising remaining explanation of the synergistic interactions between these metals may then be the formation of iron-aluminum-hydroxide complexes. These may enhance the potency of iron by formation of a type 2 chelate, parallel to the sequestration of iron by ascorbate or EDTA. Another possibility is the destabilization of lipid bilayer structures by aluminum salts, making membranes more vulnerable to oxidative damage (Oteiza, 1994). In view of the relatively low penetrance of either metal ion across cell membranes, some of the effects described in this work may involve changes at the surface of the limiting plasma membrane of synaptosomes, which are then reflected by altered intracellular events.

Aluminum appeared able to promote ascorbate-induced ROS in the absence of unchelated iron. Thus, although not possessing an intrinsic capacity to stimulate ROS production by tissues, aluminum may be able to enable the generation of ROS by chelators, in the absence of metals with multivalent potential, such as iron. Aluminum can be weakly chelated by desferrioxamine (Kruck et al., 1993), and such complexing can enhance the rate of excretion of this metal (McLachlan et al., 1989). This chelator was only present at 1/100 of the aluminum concentration in this experiment, and results could thus not be attributed to sequestration of aluminum. However, the presence of a high concentration of aluminum may displace iron from its desferrioxamine chelate and allow iron-ascorbate interactions. This experiment therefore does not conclusively demonstrate an iron-independent aluminum-ascorbate ROS-promoting interaction.

These findings derived from an isolated system do not preclude the possibility that particulate aluminum-containing materials may gain access to the CNS, and thus have the potential to activate microglia or macrophages, which in turn could lead to deleterious oxidative events within the brain (Evans et al., 1991). Soluble aluminum may be transported to the CNS by formation of a complex with transferrin, which also chelates  $\text{Fe}^{3+}$  (Roskams and Connor, 1990). The similarity of the ionic radii of aluminum and ferric iron (9 Å) (Dean, 1979) may be relevant to the transport and release of unbound iron and aluminum in the brain.

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