Aluminum and copper in drinking water enhance inflammatory or oxidative events specifically in the brain

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Abstract

Inflammatory and oxidative events are up-regulated in the brain of AD patients. It has been reported that in animal models of AD, exposure to aluminum (Al) or copper (Cu) enhanced oxidative events and accumulation of amyloid beta (Aβ) peptides. The present study was designed to evaluate the effect of a 3-month exposure of mice to copper sulfate (8 μM), aluminum lactate (10 or 100 μM), or a combination of the salts. Results suggest that although Al or Cu may independently initiate inflammatory or oxidative events, they may function cooperatively to increase APP levels.

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1. Introduction

Cu is an essential metal and a component of many enzymatic reactions. However, this redox active metal can also mediate the formation of reactive oxygen species (ROS) and this can have adverse consequences. Al is a trivalent cation incapable of redox changes and unlike Cu, has no known biological role. Both metals have been associated with neurological impairments.

Al has been shown to play a causal role in dialysis encephalopathy (Alfrey et al., 1976) and epidemiological studies suggest a possible link between exposure to this metal and a higher prevalence of AD (McLachlan et al., 1996; Rondeau et al., 2000; Flaten, 2001). This association is dependent on the duration of Al exposure and only becomes significant if an individual has resided in an area with high Al in drinking water (>100 μg/L) for several years. Homeostatic alterations in brain Cu levels have also been implicated in the pathogenesis of several neurological disorders including Alzheimer’s, Parkinson’s and prion diseases (Torsdottir et al., 1999; Mercer, 2001; Brown and Kozlowski, 2004). The toxicity of excess Cu is best demonstrated by the heritable disorder, Wilson’s disease, in which the liver is unable to excrete the metal and thus there is tissue accumulation. In this condition, liver and brain are the most compromised areas (Kitzberger et al., 2005).

The present study investigated the potential synergistic effects of chronic exposure to Cu, Al or both metals in promoting inflammatory and oxidative events in mouse brains. The design was based on the following observations: Al present in the drinking water enhanced inflammatory markers in the CNS (Campbell et al., 2004). Furthermore, in transgenic mouse models of AD, dietary Al increased markers of lipid peroxidation and Aβ levels (Pratico et al., 2002a,b). In isolated systems, Al can potentiate the oxidative stress produced by transition metals such as iron (Bondy and Kirstein, 1996) or Cu (Bondy et al., 1998; 2001).
Becaria, A., Schreurs, N. (2003) and Cu present in trace amounts (0.12 ppm) in the drinking water has been implicated in the accelerated deposition of Aβ and promotion of oxidative events in the brain of a rabbit model of AD (Sparks and Schreurs, 2003).

Animals were exposed for 12 weeks to Al lactate (10 or 100 μM), Cu sulfate (8 μM), or both metals concomitantly, and oxidative and inflammatory markers were evaluated. To determine if exposure to the metals contribute to potentiation of AD pathology, levels of APP, and its constituent peptides, Aβ1–40 and Aβ1–42, were also determined. The lower Al exposure is in the range of concentrations reported to be associated with an increased risk of AD. The present EPA maximum recommended standard for Cu in drinking water is 1.3 ppm (Buchanan et al., 1999). However, levels as high as 7.8 ppm has been found in tap water (Spitalny et al., 1984). The copper (8 μM) exposure used in this study is equivalent to 2 ppm.

While Al caused an increase in CNS proinflammatory cytokines, both metals, independently, enhanced oxidative markers in the brain. Except for an enhancement of APP levels, there was no other synergistic interaction between the two metals.

2. Materials and methods

Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis, MO).

2.1. Animal maintenance and exposure

Two-month-old male B6C3F1 mice (a hybrid between C57BL/6 and C3H) were obtained from Charles River Laboratories (Wilmington, MA). They were housed four per cage and maintained on a 12-h light/dark cycle in a temperature controlled (20±1 °C) room. Food and water were provided ad lib. Mice were divided randomly into 6 groups: (1) control, (2) copper sulfate (8 μM), (3) aluminum lactate (10 μM), (4) aluminum lactate (10 μM) plus copper sulfate (8 μM), (5) aluminum lactate (100 μM), and (6) aluminum lactate (100 μM) plus copper sulfate (8 μM). The metal salts were freshly prepared and added to double-distilled water twice weekly. The drinking water of the control animals was also double distilled. The animals were exposed for 12 weeks. The weight of the animals or the amount of water consumed was not different between groups.

2.2. Preparation of samples

Animals were anesthetized with pentobarbital (65 mg/kg, i.p.) and then perfused transcardially with saline, followed by 4% paraformaldehyde. The brains were excised and placed in 4% paraformaldehyde. The tissue was prepared as previously described (Campbell et al., 2004). For nNOS staining, the sections were stained free-floating. After hydrogen peroxide treatment, samples were blocked in serum, and then immunostained with a 1:300 dilution of primary rabbit anti-nNOS antibody (Immunostar; #24431), a goat anti-rabbit secondary antibody, and finally an avidin–biotin–HRP complex (Elite Vectastain ABC kit, Vector, Burlingame, CA). Incubation times were 24 h for the primary antibody, 30 min for the secondary antibody, and 1 h for the avidin–biotin–HRP complex. Sections were centrifuged at 1000×g for 10 min and the serum was collected and stored at −80 °C.

Cytoplasmic fractions were prepared using the method of Lahiri and Ge (2000). Tissue from each animal was weighed and homogenized in 2 ml of an ice-cold buffer consisting of (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 0.5% NP-40). The suspension was incubated on ice for 10 min and centrifuged (1500×g) at 4 °C for 1 min. The supernatant containing the cytoplasmic constituents was collected and 200 μl of 5× protease inhibitor cocktail was added. Samples were aliquoted and stored at −80 °C.

2.3. Competitive enzyme immunoassay

Levels of TNF-α or IL-1α were determined using a competitive enzyme immunoassay kit from Neogen Corp. (Lexington, KY) for the detection of the total protein in the cytoplasmic tissue fractions. Briefly, 100 μl of the brain or spleen cytoplasmic fraction, along with murine TNF-α or IL-1α antibody were added to plates precoated with goat anti-rabbit antibody and incubated at room temperature for 3 h. Murine TNF-α or IL-1α conjugate was added and incubated for 30 min. The plate was washed and incubated for another 30 min with streptavidin–alkaline phosphatase. Then 200 μl of chromogen was added to the plate and the color generated was determined with a spectrophotometric plate reader set at 490 nm. Levels of TNF-α or IL-1α were determined in the serum using sandwich enzyme immunoassay kits (Neogen Corp., Lexington, KY) for the detection of free forms of the proteins.

Levels of IL-4 were measured in the brain, serum, or spleen using a kit from Biosource (Camarillo, CA). Samples (50 μl) were added to plates coated with a monoclonal antibody against mouse IL-4. After addition of a biotin conjugate, plates were incubated for 2 h. The plate was washed and streptavidin–HRP was added to the plate. After 30-min incubation, the plate was washed and chromogen was added. The color generated was measured at 450 nm.

2.4. Immunohistochemistry assay

Animals were anesthetized with pentobarbital (65 mg/kg, i.p.) and then perfused transcardially with saline, followed by 4% paraformaldehyde. The brains were excised and placed in 4% paraformaldehyde. The tissue was prepared as previously described (Campbell et al., 2004). For nNOS staining, the sections were stained free-floating. After hydrogen peroxide treatment, samples were blocked in serum, and then immunostained with a 1:300 dilution of primary rabbit anti-nNOS antibody (Immunostar; #24431), a goat anti-rabbit secondary antibody, and finally an avidin–biotin–HRP complex (Elite Vectastain ABC kit, Vector, Burlingame, CA). Incubation times were 24 h for the primary antibody, 30 min for the secondary antibody, and 1 h for the avidin–biotin–HRP complex. Sections were...
treated with hydrogen peroxide with DAB to visualize the antibody binding sites and mounted on gelatinized glass slides. The nNOS images were captured by a Nikon Dxm1200F HIQE Color CCD camera attached to an Eclipse TE2000 fluorescent microscope.

2.5. Malondialdehyde (MDA) assay

The levels of MDA were determined spectrophotometrically by following a modified protocol developed by Gerard-Monnier et al. (1998). This procedure measures free MDA as opposed to the commonly used TBARS (thiobarbituric acid-reactive substances) method which measures total MDA in biological samples. The modified protocol is specific for this lipid peroxidation product because 4-hydroxyalkenals do not produce significant color under the conditions of the assay. To further minimize their reaction, an antioxidant was added. 150 μl of brain cytoplasmic fractions, 10 μl of the antioxidant probucol and 690 μl of the chromogenic reagent phenylmethyl indole were added together. Hydrochloric acid was then added to the mixture and the tubes were incubated for 1 h at 45 °C. A clear supernatant was obtained by centrifugation at 10,000×g for 10 min. The supernatant was transferred to a cuvette and absorbance was measured at 586 nm.

2.6. Assay for Aβ Levels

A sensitive ELISA test was used for measuring levels of Aβ1-40 and Aβ1-42 by using a previously described assay (Lahiri et al., 1998). Highly specific antibodies were used to detect Aβ1-40 and Aβ1-42 species. An affinity-purified anti-Aβ rabbit IgG was used as a capture antibody and an affinity-purified HRP-conjugated anti-Aβ rabbit IgG Fab fragment was used as a detection antibody. Tetramethyl-benzidine (TMB) was used as the chromogen.

2.7. Assay for APP levels

APP was measured by Western blotting technique as previously described (Lahiri et al., 2004). Briefly, 30 μg of total brain protein was separated on a 12% polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE). Proteins from the gel were electrophoretically transferred to a Nytan membrane. Each immunoblot was independently probed with a mouse monoclonal antibody, 22C11, which was raised against the N-terminus of APP, and mouse monoclonal antibody against the beta-actin protein. The 22C11 clone identifies all forms of APP found in cell membranes and the APP-like proteins (APLP1 and APLP2). A biotinylated horse antimouse IgG was used as the secondary antibody. Immunoreactive detection was performed and density of each immunoreactive band was measured by using UVP-GDS5000 Gel Documentation system (San Gabriel, CA, USA). Signals from APP bands were normalized with beta-actin bands. Data was analyzed using Scion Image software (Scion Corporation, Frederick, MD, USA).

2.8. Statistical analysis

The difference among groups was assessed using one-way ANOVA, followed by the Tukey test. Results were considered significant at P ≤ 0.05.

3. Results

3.1. Cytokine levels

The levels of TNF-α were elevated in the brains of animals exposed to Al (100 μM) or Al (100 μM)+Cu (8 μM) and/or copper sulfate in drinking water for 12 weeks [Con=Control; Cu=Cu (8 μM); Al10=Al (10 μM); Al10+Cu=Al (10 μM) plus Cu (8 μM); Al100=Al (100 μM); Al100+Cu=Al (100 μM) plus Cu (8 μM)]. Levels of TNF-α and IL-1α were greatest in the Al100 and Al100+Cu exposed animals. The levels of IL-4 were increased in the Al10, Al10+Cu and Al100+Cu groups. After exposure to Cu alone, levels of IL-4 were not significantly altered. Bars represent mean of six individual determinations±S.E. (P ≤ 0.05).
but there was no significant difference between these two treatment groups (Fig. 1A). To determine whether this response was mediated by systemic pro-inflammatory changes, serum and spleen levels were also measured (Figs. 2A and 3A). In the serum, TNF-α levels decreased in all metal-exposed groups compared to the control animals. This effect was not significant in the Al (10 μM) + Cu (8 μM) group, which had high variance (Fig. 2A). Following Al treatment, levels of TNF-α in spleen decreased in a dose-dependent manner. Exposure to Al together with Cu further enhanced the decrease in TNF-α in this tissue (Fig. 3A). The IL-1α response in the brain was similar to the TNF-α response in that the levels of this cytokine were greater after exposure to Al (100 μM) or Al (100 μM) + Cu (8 μM). Again, there was no significant difference between the two treatment groups (Fig. 1B).

In contrast to TNF-α, levels of IL-1α dose-dependently increased in the serum of Al-treated animals and co-exposure to Cu did not modulate this effect (Fig. 2B). Basal IL-1α levels were greater in the spleen than in serum or brain. Exposure to Cu, Al (10 μM) or Al (100 μM) did not significantly affect the levels of the cytokine in this tissue. However co-exposure with Cu and Al increased the levels of IL-1α at both concentrations used. Exposure to Al (100 μM) together with Cu (8 μM) caused a greater response compared to treatment to either Cu or Al (100 μM) alone (Fig. 3B).

Levels of the anti-inflammatory cytokine IL-4 were also determined in the brain, blood and spleen. The levels were undetectable in the serum. Compared to control, brain levels significantly increased after exposure to Al (10 μM), Al (10 μM) + Cu (8 μM) or Al (100 μM) + Cu (8 μM). After exposure to Cu or Al (100 μM), there was no μM) but there was no significant difference between these two treatment groups (Fig. 1A).
significant increase in IL-4 levels in the brain compared to control. Treatment with Cu in combination with Al (100 μM) significantly increased the levels of this cytokine relative to exposure to Al (100 μM) alone (Fig. 1C). In the spleen, levels of IL-4 decreased after exposure to Al at both concentrations. This response was not modulated by the further addition of Cu to the drinking water (Fig. 3C).

3.2. Oxidative markers

The lipid peroxidation end product malondialdehyde (MDA) was significantly elevated in the brain tissue after exposure to Cu (8 μM), or Al (100 μM), or Al (10 μM) + Cu (8 μM), or Al (100 μM) + Cu (8 μM). Al (10 μM) did not significantly increase the levels of MDA but together with Cu caused a response similar to that found after exposure to Cu alone. MDA levels were greatest after exposure to Al (100 μM) and co-exposure with Cu significantly decreased this response (Fig. 4).

The number of nNOS-immunoreactive neurons increased in the frontal cortex of mice exposed to Cu (8 μM), or Al (100 μM), or Al (100 μM) + Cu (8 μM). Treatment with Al and Cu in combination decreased the response relative to exposure to Al (100 μM) alone (Fig. 5).

3.3. APP and Aβ levels

Aluminum (100 μM) or copper (8 μM) exposure did not lead to an increase in the levels of Aβ1–40 and Aβ1–42 (Fig. 6). In contrast, levels of APP were significantly increased.

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**Fig. 4.** Brain lipid peroxidation. MDA levels in brain cytoplasmic fraction after exposure to aluminum lactate and/or copper sulfate in drinking water for 12 weeks [Con = Control; Cu = Cu (8 μM); Al10 = Al (10 μM); Al10 + Cu = Al (10 μM) plus Cu (8 μM); Al100 = Al (100 μM); Al100 + Cu = Al (100 μM) plus Cu (8 μM)]. MDA levels were increased after exposure to Cu, Al10 + Cu, Al100, or Al100 + Cu. The Al10 + Cu group had higher levels of MDA than that of the group receiving 10 μM Al alone. In contrast, the Al100 + Cu group had a lower MDA content than that of the Al100 group. Bars represent mean of six individual determinations ± S.E. (P < 0.05).

**Fig. 5.** nNOS immunoreactivity. (A) nNOS positive neurons in the cortex of mice after treatment with aluminum lactate and/or copper sulfate in drinking water for 12 weeks. Bar = 100 μm. (B) Quantification of the nNOS immunoreactive neurons [Con = Control; Cu = Cu (8 μM); Al100 = Al (100 μM); Al100 + Cu = Al (100 μM) plus Cu (8 μM)]. The number of nNOS immunoreactive neurons was elevated in the Cu, Al100, and Al100 + Cu groups. The number of nNOS positive neurons was greater in the Al100 group relative to the Al100 + Cu group. Bars represent mean of three individual determinations ± S.E. (P < 0.05).

**Fig. 6.** Brain Aβ levels. Levels of Aβ1–40 (A) or Aβ1–42 (B) in the brain of mice exposed to copper, aluminum, or both salts [Con = Control; Cu = Cu (8 μM); Al100 = Al (100 μM); Al100 + Cu = Al (100 μM) plus Cu (8 μM)]. Bars represent mean of six individual determinations ± S.E.
further enhanced this effect (Fig. 7).

This is illustrated by the finding of increased TNF-α levels in CSF of AD patients compared to controls (Campbell, 2004). The level of IL-1β was also increased in primary cultures of human microglia exposed to Al (100 μM) and Cu (8 μM). An increase in IL-1β was also observed after exposure to Al (100 μM) plus Cu (8 μM). App levels were elevated in the Al100 and Al100+Cu-treated animals. The levels of the protein were greatest in the Al100+Cu group. Bars represent mean of six individual determinations ± S.E. (P ≤ 0.05).

after exposure to Al (100 μM) and copper co-exposure further enhanced this effect (Fig. 7).

4. Discussion

Inflammatory events are present in neurodegenerative disorders and appear to contribute to progression of the disease (Campbell, 2004). This is illustrated by the finding of significant increase in CSF TNF-α levels precedes development of AD in patients with mild cognitive impairment (Tarkowski et al., 2003). We have previously reported that Al present in drinking water dose-dependently increased TNF-α and IL-1α levels selectively in mouse brains (Campbell et al., 2004). Furthermore, in primary human neural cells, Al exposure induces expression of inflammatory genes (Lukiw et al., 2005). Since both Al and Cu have been implicated in the pathology of AD (Pratico et al., 2002a,b; Sparks and Schreurs, 2003), we investigated whether co-exposure to both metals in the drinking water further exacerbates the effects of individual metals. Cu did not modulate the Al-induced pro-inflammatory events in the brain and this was paralleled by similar findings using an isolated human glioblastoma cell line (Becaria et al., 2003).

The elevation in TNF-α appears to be unique to brain tissue since the concentrations in both serum and spleen decreased after exposure to the metal salts. This provides evidence that the inflammatory response within the CNS consequent to chronic treatment with low levels of Al differs qualitatively from that of the systemic immune response. Furthermore, the Al-induced neuroinflammatory response cannot be attributed to penetration of plasma TNF-α into the brain. In contrast, the Al-induced enhancement of IL-1α levels in the brain may be influenced by systemic events since serum concentrations of this cytokine were also increased after exposure to Al. The spleen of the mice contained the greatest concentration of IL-1α (75–107) compared to the serum (0.8–1.9) or brain (2.3–4.7) levels (values are expressed as (ng/ml)/mg protein and are in the range of lowest to highest concentrations found). Thus, IL-1α may have a homeostatic function in the spleen. After exposure to Al and Cu, levels of this cytokine were increased in cytoplasmic fractions from this organ.

Al hydroxide (alum) is the most commonly used adjuvant in vaccines. Intraperitoneal injection of alum caused spleen accumulation of IL-4 producing cells. These cells expressed granulocyte, monocyte/macrophage markers (Jordan et al., 2004). Since activated microglia also express IL-4 (Park et al., 2005), the present study investigated how a different duration and route of exposure to Al would affect the levels of this cytokine in the brain and spleen. Chronic exposure to low levels of Al lactate in drinking water decreased the levels of IL-4 in the spleen. Cu did not have an effect and did not modulate the response to Al. In the brain, IL-4 has been shown to function as an anti-inflammatory cytokine that reduces mediators of brain inflammation such as TNF-α, IL-1β and iNOS (Ledeboer et al., 2000). There was a significant increase in IL-4 levels after exposure to Al (10 μM), or to Cu plus Al (10 or 100 μM). The increase in IL-4 may be attenuating TNF-α and IL-1α levels after exposure to low levels of Al (10 μM). However after exposure to higher concentrations of Al (100 μM) plus Cu, the enhanced IL-4 response was not able to mediate the increase in the pro-inflammatory cytokines. The interplay between pro- and anti-inflammatory cytokines released by CNS cells may determine the over-all inflammatory milieu of the brain after an environmental exposure to metals.

Although oxidative and inflammatory events are independent phenomena, they are related. During inflammation, an increase in NO production may increase the oxidative status of the CNS. The neuronal isoform of nitric oxide synthase (nNOS) is highly responsive to changes in oxidative stress (Liu et al., 2002). Perinatal exposure to Al (5 mM) in the drinking water increased the number of nNOS immunoreactive neurons in the frontal cortex of rat pups (Kim, 2003). In the brain tissue, MDA and nNOS levels were elevated after exposure to either Al (100 μM) or Cu (8 μM). Al and other trivalent metals which have no redox capacity in biological systems may stimulate lipid peroxidation by interacting with membrane phospholipids, causing changes in the physical properties of the membrane such as rigidification and phase separation (Verstraeten et al., 1997).

Although in vitro, Al interacts with Cu to increase its oxidative potential (Bonten et al., 1998; Becaria et al., 2003; Di and Bi, 2003) this was not the case in the present study. After exposure to Al (100 μM) plus Cu, MDA and nNOS levels decreased compared to the response obtained with Al (100 μM) alone and were similar to those of the Cu-exposed group. This may be due to the dual nature of Cu. Although it is a redox-active metal, it is also a cofactor in the antioxidant enzyme Cu/Zn superoxide dismutase.
To determine to what extent the inflammatory and oxidative changes may influence amyloid pathology, the levels of APP, Aβ₁₋₄₀, and Aβ₁₋₄₂ were determined. Al has been shown to aggregate Aβ in vitro (Exley et al., 1993; Kawahara et al., 1994; Bondy and Truong, 1999) and in vivo (Miu et al., 2003). Dietary exposure to the metal exacerbates oxidative stress, Aβ deposition, and plaque formation in the brain of transgenic mice over-expressing APP (Pratico et al., 2002a,b). The binding of Cu to Aβ also promotes amyloid aggregation (Atwood et al., 2000) and trace amounts of Cu in the drinking water led to accelerated deposition of Aβ and promotion of oxidative events in the brain of a rabbit model of Alzheimer’s disease (Sparks and Schreurs, 2003). In the brain of these animals, fibrillar forms of Aβ increased predominantly (Sparks, 2004). In the present study, the concentrations of Aβ peptides were not significantly altered after exposure to the metals. However, Al (100 μM) increased levels of APP and co-exposure with Cu further enhanced this response. This finding is in agreement with a previous study showing that in primary human neural cells, Al or iron exposures up-regulated gene expression for APP and that the combination of the metals was more effective compared to the response to the metals individually (Alexandrov et al., 2005).

The events leading to the common sporadic form of AD are unknown. The pathophysiology of AD is complex and most likely involves multiple distinct and overlapping pathways of neuronal damage. In addition to the presence of the pathological hallmarks of the disease (senile plaques and neurofibrillary tangles), AD brains exhibit other abnormalities: loss of synapses, gliosis, microglial activation, signs of inflammation and oxidative damage (Pratico et al., 2002b). Current evidence suggests that both oxidative stress and neuroinflammation are early events in the pathogenesis of AD. The relationship between inflammatory responses and free radical generation is complex and although they may be separate events, the two are correlated. Thus, in a transgenic animal model of AD, long-term treatment with the anti-inflammatory drug indomethacin, in combination with the anti-oxidant, vitamin E, improves amyloid plaque pathology much more efficiently than the individual compounds (Yao et al., 2004).

In the present study, it was postulated that Al and Cu co-exposure would lead to exacerbation of oxidative and inflammatory factors. While in CNS, Al, at the higher concentration, acted as a pro-oxidant and a pro-inflammatory agent, the administration of the metal with Cu did not synergistically modulate the response obtained with Al alone. However, Cu did potentiate the Al-induced increase in brain APP levels. Thus, the results suggest that in the CNS, the oxidative stress and inflammation caused by Al or Cu may be, to some extent, separate events. However, the metals may work in concert to increase levels of APP. To what extent this increase may contribute to the pathological cascade associated with AD is at present unknown. Since both Cu and Al have been shown to accelerate amyloid plaques in animal models of AD, the increase in APP may eventually lead to exacerbation of amyloid pathology.

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