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Aluminum and copper interact in the promotion of oxidative but not inflammatory events: Implications for Alzheimer's disease

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Abstract. The etiology of Alzheimer's Disease (AD) is multifactorial. It has been suggested that transition metals such as copper (Cu) and iron (Fe) as well as aluminum (Al) may be involved in the pathogenesis of the disorder. While Cu and Fe are redox-active, Al only exists in the trivalent form and is redox-inert. We previously demonstrated that Al exposure causes an increase in inflammatory parameters in human glioblastoma T98G cells. In the present study we further demonstrate that co-exposure with Cu exacerbates the oxidative but not inflammatory effects of Al in this cell line. While Cu-induced reactive oxygen species (ROS) production was greatly enhanced in the presence of Al, TNF-α secretion induced by either metal was not further potentiated by simultaneous exposure to Al and Cu. Furthermore, exposure to both metals reduced the individual Al and Cu-induced activation of the immune-related transcription factor NF-κB. Therefore, while synergistic interaction between the two metals increases oxidative events, this does not lead to potentiation of Al-induced inflammation. Thus the ability of aluminum to promote inflammatory processes does not depend on an increase ROS production induced by interaction with transition metals.

Keywords: Neuroinflammation, cytokines, NF-κB, oxidative stress

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease, characterized by three main features: senile plaques, neurofibrillary tangles and neuroinflammation [26]. The mechanism for the pathogenesis of this disease has not yet been fully established [43]. Aluminum (Al) has been suggested to accelerate or play a causal role in the pathogenesis of AD. The metal is found in the brain of patients with this disorder [10,39,54]. However, other studies have been unable to confirm these findings [5,23,31]. Furthermore, several epidemiological studies indicate a positive relationship between AD frequency and Al concentration in drinking water [2,32,36,42]. However, others have not found such an association [28,51,52]. Therefore, the controversy regarding the role of aluminum in AD is still ongoing and unresolved. However, a recent study showing that Al exposure leads to enhanced amyloid β (Aβ) production and plaque formation in transgenic mice over-expressing human amyloid β precursor protein (AβPP) renews the argument that the metal may play a role in AD [40].

Existing evidence suggests that Al is capable of causing inflammation systemically and in the central nervous system. Adults vaccinated with Al-containing vaccines developed inflammatory nodules [11,35] and low doses of Al present in parenteral nutrition formula produced marked portal inflammation [12]. In cells of glial origin Al causes an activation of the immunologically relevant transcription factor nuclear factor kappa B (NF-κB) and increases levels of tumor necrosis factor alpha (TNF-α) secretion [9]. Furthermore, chronic Al lactate treatment of rabbits increases glial fibrillary

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acidic protein concentrations in the cerebral cortex [56] and the metal can induce the production of nitric oxide by microglial cells [14].

Copper is an essential yet potentially harmful metal that is implicated in the pathogenesis of several neurological disorders including Alzheimer's, Parkinson's and prion disease [33,49]. Levels of copper and iron are increased at the edges of AD senile plaques [25] and the presence of these redox reactive metals in both plaques and neurofibrillary tangles may result in oxidative damage [44]. AβPP is present on most cell surfaces and is cleaved by specific secretases to yield different sizes of Aβ peptides. Abnormal processing of AβPP may lead to overproduction of Aβ and its consequent aggregation into senile plaques. Copper has been shown to bind to AβPP which then reduces the metal from Cu(II) to the ROS generating Cu(I) [34]. Aβ can bind to and reduce redox active metals such as iron and copper and by doing so, produce hydrogen peroxide [19].

In isolated biological systems, Al markedly promotes the capacity of copper to produce reactive oxygen species (ROS) [4]. Oxidative stress is elevated in neurodegenerative disorders such as AD [7]. Since Al also exacerbates events connected to ROS formation, it may enhance the adverse events already existent in the diseased brain. In the present study we used human glioblastoma T98G cells to evaluate the effect of 500 μM aluminum sulfate and 5 μM copper sulfate on oxidative and inflammatory indices and test whether Al-induced inflammation is exacerbated by a redox active metal.

2. Materials and Methods:

2.1. Materials

Human glioblastoma (T98G) cells were purchased from ATCC (Rockville, MD). All tissue culture supplies were obtained from Gibco (Grand Island, NY). Cell viability and cell proliferation kits were purchased from Molecular Probes (Eugene, OR). The enzyme immunoassay used for detection of the cytokines was from Neogen (Lexington, KY). All other chemicals used were from Sigma (St. Louis, MO).

2.2. Methods

2.2.1. Cell preparation

Human glioblastoma cells were grown in minimum essential medium (MEM) with a modification consisting of nonessential amino acids, sodium pyruvate, lipoic acid, vitamin B12, biotin and ascorbic acid. The media also contained Earle's salts and L-glutamine. It was supplemented with 10% fetal bovine serum (heat-inactivated). Cells (0.4 million) were seeded and incubated at 37°C in a humidified atmosphere of 5% CO_2. Once the cells were confluent, they were treated with the salts. The aluminum (500 μM) and copper (5 μM) solutions were prepared in the cell media and immediately sterile-filtered prior to the first dosing. The Al concentration used was based on a previous study showing that at this level the metal causes an inflammatory response in this cell line [9]. The concentration of Cu was chosen based on dose-response assays showing that at 5 μM, the metal caused a pronounce effect on ROS production without changing cell viability (data not shown). The solutions along with the media, were then incubated with the treated cells to insure parallel conditions for the aging of the Al salt. Since it is postulated that colloidal Al may produce the observed inflammatory response and aged aluminum salts are known to aggregate [47], the effects of Al and Cu was studied for 3 days of exposure.

2.2.2. Cell proliferation assay

Cells were frozen overnight at −70°C and the number was determined using the CyQuant cell proliferation assay kit (Molecular Probes). Briefly, cells were thawed at room temperature and 200 μl of the dye, diluted in cell lysis buffer was added. The dye rapidly binds to nucleic acids and exhibits strong fluorescence when bound. The cells were incubated at room temperature for 5 min and the fluorescence was measured at excitation 480 nm and emission 520 nm using a FL600 microplate fluorescence reader. A standard of cells counted with the hemacytometer was used to determine the actual cell number.

2.2.3. Cell viability assay

A viability/cytotoxicity kit (Molecular Probes) was used to measure cell survival after treatment. This assay is based on the simultaneous measurement of the fluorescence of two dyes. Live cells were determined by the retention of the calcein AM dye, which is non-fluorescent and upon enzymatic conversion by ubiquitous intracellular esterase activity, becomes intensely fluorescent. The EthD-1 dye can only enter cells with damaged membranes and there it binds to nucleic acids and produces a strong red fluorescence. A final concentration of 1 μM calcein AM and 2 μM EthD-1 was used in the assay. Upon addition of the dye, samples

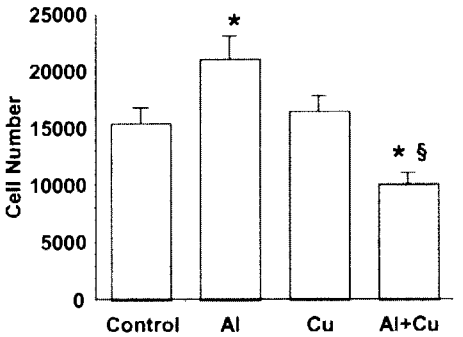

Fig. 1. Cell proliferation in human T98G cells treated with 500 μM aluminum sulfate and/or 5 μM copper sulfate for 3 days. The rate of cell proliferation was determined using the CyQuant cell proliferation assay kit from Molecular Probes. *Value is significantly different ($P < 0.05$) than individual Al and Cu exposure. Bars represent mean of eight individual deterinations $\pm$ S.E. Values are based on two experiments conducted on two different days.

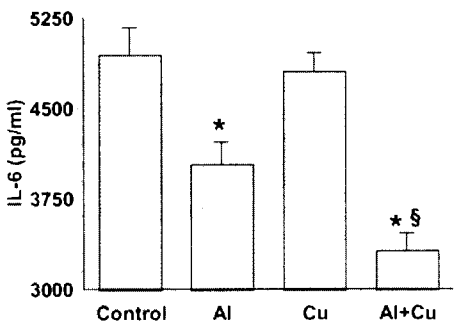

Fig. 2. IL-6 secretion in T98G cells treated with 500 μM aluminum sulfate and/or 5 μM copper sulfate for 3 days. *Value is significantly different ($P < 0.05$) than the control. §Value is significantly different ($P < 0.05$) than individual Al and Cu exposure. Bars represent mean of eight invdividual determinations $\pm$ S.E. Values are based on two experiments conducted on two different days.

were incubated at room temperature for 45 min. The fluorescence due to calcein was measured at excitation 485 nm and emission 530 nm, while that of EthD-1 was measured at excitation 530 nm and emission 645 nm using a FL600 microplate fluorescence reader. The percentage of live and dead cells was calculated based on the intensity of fluorescence using a standard of live and dead cells. Cells were killed by treatment with 0.25% digitonin for 10 min. Cell viability and proliferation studies were performed to detect any direct toxic effects.

2.2.4. *Protein immunoassay*

Levels of TNF-α and IL-6 were determined using sandwich enzyme immunoassay kits from Neogen (Lexington, KY) for the detection of free forms of

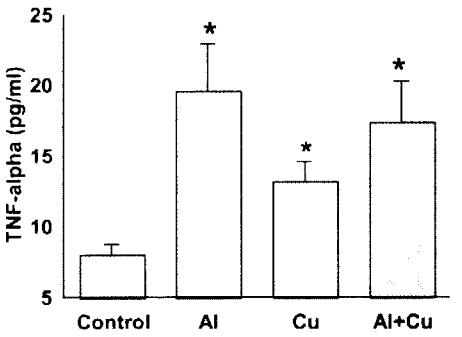

Fig. 3. TNF-α secretion in human glioblastoma T98G cells treated for 3 days with 500 μM aluminum sulfate and/or 5 μM copper sulfate. *Value is significantly different ($P < 0.05$) than the control. Bars represent mean of eight individual determinations $\pm$ S.E. Values are based on two experiments conducted on two different days.

the proteins in cell culture supernatants. Briefly, 100 μl of the cell supernatant was incubated with rabbit anti-human IL-6 or TNF-α polyclonal antibody for 3 h. The plate was washed and incubated for 45 min with goat anti-rabbit-conjugated alkaline phosphatase. Then, 200 μl of the color reagent solution was added to the plate and the color generated was determined with a spectrophotometric plate reader set at 490 nm. For the IL-6 assay, the samples were diluted 10$\times$ because the level of this cytokine in the cell culture supernatant was too concentrated for the detection range of the assay.

2.2.5. *Electrophoretic mobility shift assay*

The gel shift assay was utilized to determine the extent of NF-κB activation in the human glioblastoma (T98G) cells using a protocol developed by Promega (Madison, WI). After treatment, the media was removed and the cells were washed with Tris-HCl/0.7% NaCl (pH 7.4) buffer. Preparation of nuclear extracts was performed according to a protocol developed by Lahiri et al. [22]. Briefly, one to three million cells in 0.4 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 and 0.5 mM PMSF, 0.5% NP-40, was incubated for ten minutes and then centrifuged (1500 $\times$ g) at 4°C for 1 min. The nuclear pellet was resuspended in 50 μl of a buffer consisting of 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF. The samples were shaked for 15 minutes on ice and then centrifuged at 11,000$\times$ g for five minutes at 4°C. The supernatant, which is the nuclear fraction, was carefully extracted and stored at $-80°$. The amount of protein in 1 μl of each sample was determined by the BCA protein assay from Pierce (Rockford, IL) and 20 μg of each

sample, incubated with ^{32}P–labeled oligonucleotides containing the NF-κB consensus sequence, was loaded onto the gel. Double stranded oligonucleotide were end–labeled with [^{32}P] ATP with T4 polynucleotide kinase following the protocol provide by the manufacturer Promega (Madison, WI). A negative control containing no cell extract, as well as competitor reactions were run simultaneously with the samples. The specific competitor contained unlabelled NF-κB consensus nucleotide while the nonspecific competitor contained unlabelled AP-1 consensus oligonucleotide. The competitor reactions also contained 20 μg of HeLa extract. X-ray films were manually developed and the intensity of each band was measured and quantitated using the image analyzer, Eagle Eye, from Strategene (San Diego, CA).

2.2.6. *Assay for reactive oxygen species formation*

Reactive oxygen species (ROS) were assayed using 2',7'-dichlorofluorescin diacetate (DCFH-DA). This dye rapidly enters cells and is desterified to the ionic dichlorofluorescin (DCFH) [24]. DCFH is oxidized to the fluorescent 2',7'-dichloro-fluorescein by reactive oxygen species. Assays were carried out in a FL600 microplate fluorescence reader. The microplate reader allowed for fast light excitation and precise fluorescence capturing, thus minimizing photo-oxidation of the probe. Excitation and emission were set respectively at 485 nm and 530 nm.

2.3. *Statistical analysis*

The difference among groups was assessed using one-way analysis of variance followed by the Tukey test.

3. Results

3.1. *Cell viability and proliferation*

After 3-day exposure to Al (500 μM) and Cu (5 μM), cell proliferation was decreased compared to control and treatment with the individual metals (Fig. 1). However 3-days of treatment with Al alone produced an increase in the rate of cell proliferation. There was no significant change in cell viability throughout the study.

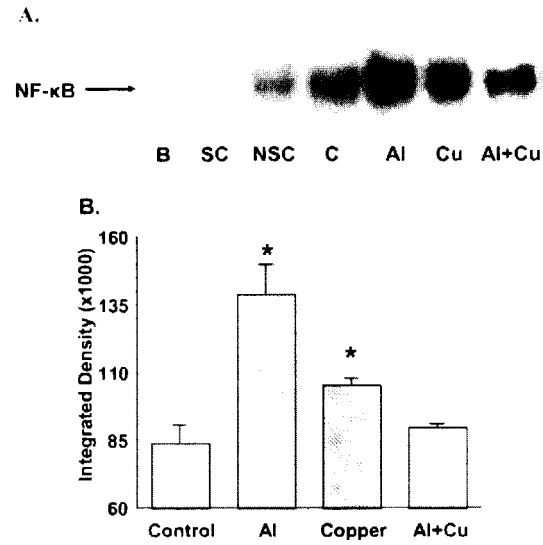

Fig. 4. The effect of treatment with 500 μM aluminum sulfate and/or 5 μM copper sulfate on NF-κB activation in T98G cells. Nuclear extracts were incubated with ^{32}P-labeled oligonucleotides containing the NF-κB consecsus sequence and the shifted bands are shown. (A) T98G cells were treated for 3 days. B, blank; SC, specific competitor (unlabelled NF-κB consensus nucleotide and HeLa extract); C, control; Al, aluminum sulfate; Cu, copper sulfate; Al+Cu, aluminum sulfate and copper sulfate. (B) The integrated density of the shifted band. Bars represent mean of four individual determinations $\pm$S.E.

3.2. *Cytokine secretion*

The level of secreted interleukin-6 (IL-6) decreased after 7-hour treatment with Al (500 μM), Cu (5 μM) or Al (500 μM) plus Cu (5 μM)(data not shown). After 3-days of treatment the level of IL-6 in the co-exposed group continued to decrease (Fig. 2). Levels of TNF-α secretion were significantly increased in all groups after treatment for 3 days (Fig. 3).

3.3. *NF-κB activation*

Exposure of the glioblastoma cells for 7 h to Al (500 μM) and/or Cu (5 μM) did not cause any significant activation of the transcription factor (data not shown). 3 days of treatment with Al or Cu caused an increase in the intensity of the shifted band. However, at this time-point the concomitant exposure to Al and Cu led to levels that were not significantly different from control values (Fig. 4).

3.4. *Reactive oxygen species formation*

After 3 days of exposure to Cu there was a dramatic increase in the production of ROS compared to control

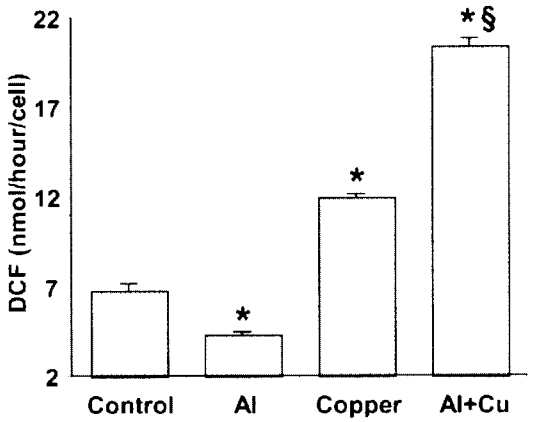

Fig. 5. Rate of ROS formation in human T98G cells treated for 3 days with 500 μM aluminum sufate and/or 5 μM copper sulfate. * Value is significantly different (P<0.05) than the control. §Value is significantly different (P<0.05) than individual Al and Cu exposoure. Bars represent mean of eight invdividual determinations ± S.E. Values are based on two experiments conducted on two different days.

values. Treatment with Al did not produce a parallel increase; in fact ROS levels were depressed. 3-day exposure to both metals simultaneously resulted in a further increase in the level of ROS production compared to the effect of Cu alone (Fig. 5).

4. Discussion

Microglial cells become progressively more active with normal aging [48]. Extended production of chemotactic factors by these cells can result in cytotoxicity because they activate macrophages that produce ROS [13]. In the present study, a 3-day exposure to Al increased the rate of proliferation of human glioblastoma cells. This Al-induced increase was also reported after 6 days of treatment in this same cell line and this effect may be due to increased activation of the cells [9]. Co-exposure of cells to Al and Cu produced a synergistic decrease in cell proliferation of human glioblastoma cells. Since cell viability was not affected, it appears that the metals in combination arrest cell division in this human CNS-derived cell line. These cells are transformed and thus capable of multiplying for a long period of time, it is not clear whether such an arrest may lead to eventual cell death.

Cytokines such as IL-1β, IL-6 and TNF-α are upregulated in AD. TNF-α is elevated in AD serum, CSF, cortex and glial cell cultures after exposure to Aβ [1]. In the present study, human glioblastoma cells exposed for 3 days to Al or Cu showed a significant increase in TNF-α secretion. These findings are consistent with previous studies in which this cytokine increased after a 6-day exposure to Al sulfate in this same cell line [9]. TNF-α mRNA has also been reported to be increased in the brain of mice chronically exposed to Al in drinking water [50] and following Cu treatment in human peripheral blood leukocytes [46].

Excess levels of IL-6 are generally detrimental and can add to the pathology associated with several CNS disorders. There are contradictory results regarding the levels of IL-6 in cerebral spinal fluid of AD patients. Increased [55], decreased [29] or unchanged [17] IL-6 levels have been reported. In the present study, Al or Al and Cu together caused a decrease in IL-6 secretion in human glial cells after a 3-day exposure while TNF-α was increased at this same time-point. These findings are consistent with those reported previously by our laboratory, where Al caused a decrease in IL-6 secretion after a 6-day treatment [9].

Studies of post-mortem brain from AD patients have found an increase in NF-κB activity. Immunoreactivity to p65, a member of the rel family of proteins composing the activation factor, was also elevated in neurons and astrocytes surrounding amyloid plaques [30]. TNF-α is a potent stimulator of NF-κB, which plays an integral role in the innate immune response. Our results show that Al and Cu cause a time dependent increase in NF-κB activation. The effect of Al on NF-κB may be due to the precipitation of Al in solution and the formation of colloidal species [9]. These large colloids of aluminum may be a source of Al^{+3} species. Both the colloidal complexes and the increased amount of toxic Al species may be responsible for the inflammogenic effect of Al in this cell line. The same may be true for Cu-mediated induction of the transcription factor since it has been shown that NF-κB is activated by Cu present as a particulate in air pollution [21].

NF-κB is activated by ROS [45] and this effect can be inhibited by antioxidants [18,41]. Therefore, it was postulated that Cu treatment would lead to a robust induction of the transcription factor. Although we did see an increase in ROS formation with Cu but not Al, it was the latter metal that had the most profound effect on NF-κB activation. This suggests that there are other mechanisms involved in this process other than an increase in ROS formation. Indeed, a recent study shows that thalidomide (a drug with anti-inflammatory properties) while suppressing hydrogen peroxide and TNF-α induced NF-κB activation, had no effect on lipopolysaccharide, phorbol ester or ceramide-enhanced activation of the transcription factor [27]. This selectivity indi-

cates that other inflammatory pathways which are not modulated by oxidative events, exist. The present study suggest that Al induction of NF-κB may be through such alternate pathways. Co-exposure to Al and Cu led to potentiation of ROS formation, but to an unexpected decrease in activation of the transcription factor. This observation is in agreement with another study which showed that co-exposure of rat brain capillary endothelial cells to hydrogen peroxide and TNF-α blocks NF-κB translocation to the nucleus. In contrast, exposure to the cytokine alone produced a pronounced increase in the activation of the transcription factor [15].

In isolated systems, Al potentiates the oxidative stress produced by Fe [3] and Cu [4]. In the present study it was found that Al causes the same effect on Cu-related ROS production in cell culture. Colloidal Al may bind these metals and thus modulate their ability to promote metal-based oxidative events [8]. This may give further impetus to the concept of the production of ROS by colloidal compounds that complex pro-oxidant metals and thus allow them to participate in Fenton reactions for a longer period [55]. It has been proposed that metals without redox capacity such as Al can make fatty acids more available to attack by free radicals, thus facilitating the propagation of lipid peroxidation [37, 38]. Since Al is present in the brain [6] and the level of metal increases with age [16], this effect may be important in age-related neurological disorders such as AD.

Although both inflammatory and oxidative events are proposed to play a role in AD, and both processes are intricately associated with each other, the present study shows that, this correlation is incomplete. Thus, oxidative stress does not necessarily lead to an exacerbation of inflammation and vice versa. The fact that both of these events are present in the AD brain has raised the issue of whether these processes are pathological in nature or are protective. ROS formation during preconditioning of hippocampal cell cultures by exposure to xanthine/xanthine oxidase or iron sulfate has been shown to be neuroprotective in staurosporine–induced toxicity and this was attributed to NF-κB activation [41]. Furthermore, low doses of amyloid β activate NF-κB and protect neurons against subsequent exposure to toxic levels of the protein [20]. Therefore, It seems that oxidative and inflammatory events may initially be protective mechanisms and only after chronic activation do they lead to exacerbation of already existent pathological processes leading to neurodegeneration.

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