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Elevation of cerebral proteases after systemic administration of aluminum

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Abstract

The levels of three proteases in the cerebral cortex of rats following a three week exposure to aluminum, were measured. The activity of apopain (CPP32), an interleukin 1 β converting enzyme (ICE)-like cysteine protease specifically associated with apoptosis, was increased following dosing with aluminum. The activity of calcium-activated neutral protease, calpain, was also increased. However, the enzyme activity of trypsin-like serine protease, known to be elevated by oxidative events, was unchanged. Since aluminum is suspected as a possible factor in the pathogenesis of Alzheimer's disease and other neurological diseases, it is speculated that changed levels in proteolytic enzymes may relate to the neurotoxicity of aluminum. © 1998 Published by Elsevier Science Ltd. All rights reserved.

Key words: Aluminum; Proteases; Apoptosis; Reactive Oxygen Species; Alzheimer's disease

1. Introduction

Aluminum is a neurotoxin which has been proposed to be associated with neurodegenerative diseases such as Alzheimer's disease (AD) (Crapper et al., 1973; Perl and Brody, 1980; Trapp et al., 1978) and amyotrophic lateral sclerosis (ALS) (Garruto et al., 1986; Perl et al., 1982), but such a relationship has hitherto not been clearly established. AD is pathologically characterized by the appearance of senile plaques, neurofibrillary tangles (NFT), and loss of neuronal cells. Accumulation of proteins or peptides, specifically β -amyloid and tau, within these lesions are hallmarks of AD and may be, in part, due to dysregulation of protease enzymes. Certain proteases may play important roles in AD. Modifications of chymotrypsin-like proteases and trypsin-like serine proteases have been shown to be involved in accumulation of β -amyloid and tau protein in AD (Smith et al., 1993). Calpain has been reported to be activated in AD (Saito et al., 1993). This enzyme is suspected to be involved in cytoskeletal pathology and neurofilament accumulation associated with AD and aged brains (Banay-Schwartz et al., 1994; Nixon et al., 1994) and calpain-mediated

proteolysis of neurofibrillary tangles has been shown to be altered by aluminum (Nixon et al., 1990; Shea et al., 1992; Zatta et al., 1993).

Cellular proteases have several functions, including participation in apoptotic pathways, and may be relevant to learning and memory processes (Chinnaiyan et al., 1996; Nicholson et al., 1995). Apopain is essential for apoptosis by cleaving and activating poly (ADP-ribose) polymerase (Nicholson et al., 1995) and while changes in apopain activity have not been reported in AD, an association between apoptosis and AD has been suggested by many previous studies (Gorman et al., 1996; Kusiak et al., 1996).

In a search for a possible mechanistic basis for aluminum neurotoxicity, we have examined the effects of aluminum exposure upon levels of several relevant proteases. Chymotrypsin-like protease was reported to be increased in vitro, by aluminum salts (Clauberg and Joshi, 1993; Joshi et al., 1994), but an opposite result was found by Zatta (1993). Trypsin-like serine protease activity was also reported to be elevated in isolated systems by aluminum (Joshi et al., 1994). However, there are no reports on levels of cerebral proteases following aluminum dosing of intact animals. Another reason for carrying out this study is that several proteases are induced by the presence of oxidatively damaged proteins (Dean, 1987; Davies, 1993) and we have found that

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aluminum exposure at the dosing levels used here, can promote pro-oxidant events within the brain (Bondy et al., in press, 1998).

Iron is a very potent pro-oxidant and aluminum is known to potentiate iron-related reactive oxygen species formation in isolated systems (Otieza, 1994; Bondy and Kirstein, 1996). For this reason, the current study also used groups of rats treated with iron, both alone and combined with aluminum, in order to determine whether aluminum modulates iron-induced ROS production in living animals.

2. Materials and methods

2.1. Materials

CPP32 substrate (Ac-asp-glut-val-asp-aminomethylcoumarin) was obtained from Calbiochem. (La Jolla, CA). Fluorescein-12-ddUTP and terminal transferase were from Boehringer Mannheim (Indianapolis, IN). Rhodamine 110, bis-(benzyloxycarbonyl-L-isoleucyl-L-prolyl-L-arginine amide) and *tert*-butoxycarbonyl-leu-met amide 7-amino-4-chloromethylcoumarin were purchased from Molecular Probes, Inc. (Eugene, OR).

Aluminum lactate was from Pfaltz and Bauer, Inc. (Waterbury, CT). Protein Assay dye was from Bio-Rad Laboratories (Hercules, CA). Other materials were from Sigma Co. (St Louis, MO).

2.2. Methods

2.2.1. Animals and dose schedule

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 100–150 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 ($20 \pm 1^\circ\text{C}$) room. Food and water were provided ad lib. until the start of the feeding protocol.

Rats received one of two diets ad lib. for 21 days. One of these groups received an iron-containing diet containing 0.3% (v/v) of ferrocene, prepared by Dyets Inc. Bethlehem, PA (Florence et al., 1992) while the other received the same diet without added ferrocene. Half of the rats from each diet group were injected intraperitoneally, on every third day with aluminum gluconate (0.6 ml/rat of a 184 mM solution = 3 mg Al) prepared as described by Florence et al. (1995). 184 mM aluminum chloride was mixed with 560 mM D-gluconic acid (sodium salt) to yield 184 mM aluminum gluconate. There were four groups of rats with eight animals in each final group (control, Al, Fe, Al + Fe). Body weights were monitored and recorded every third day.

2.2.2. Tissue preparation

Rats weighing 100–200 g were decapitated; the cerebral cortex were excised quickly. Tissue was weighed and

homogenized in 10 vol. of 0.32 M sucrose and centrifuged at 1800 g for 10 min. The crude nuclear pellet (P1) was resuspended in 10 vol. of Tris-HCl (40 mM, pH 7.4). The resulting supernatant fraction was then centrifuged at 31,500 g for 10 min to yield a final supernatant (S2) fraction. Protein concentration was assayed using the method of Bradford (1976).

2.2.3. Apopain activity

(Ac-asp-glut-val-asp-aminomethylcoumarin) was used to measure apoptotic activity since it is specifically cleaved by apopain to form a fluorescent derivative (Nicholson et al., 1995). This substrate was dissolved in ethanol and added at a final concentration of 10 μM to 1.8 ml of 40 mM Tris-HCl and 0.2 ml cortex S2 suspension. Incubation was at 22°C and fluorescence was measured after 10 and 30 min using excitation and emission wavelengths of 380 nm and 480 nm respectively. The difference between these values was used as the index of enzyme activity.

2.2.4. Fluorophore end-labeling of DNA fragmentation

Cortical nuclear (P1) DNA fragmentation following apoptosis was measured by enzymatic labeling of 3'-OH ends of DNA with fluorophore-labeled dideoxynucleotide. Proteinase K (20 μg), fluorescein-12-ddUTP (4.1 μM) and terminal transferase (5.7 units), were incubated with 40 μl of a P1 suspension for 35 min at 37°C . Following this, DNA was precipitated with 10 volumes of ethanol at -80° and the suspension was centrifuged. Fluorescence was read in the resuspended pellet at 490 nm excitation and 515 nm emission (Patel et al., 1995).

2.2.5. Calpain-mediated proteolysis

A specific calpain substrate, *tert*-butoxycarbonyl-leu-met amide 7-amino-4-chloromethylcoumarin (Rosser et al., 1993), was added at a final concentration of 10 μM along with 1 mM dithiothreitol, 5 mM CaCl_2 , 0.2 mM EDTA, 0.1% Triton X-100, to 1.8 ml of 40 mM Tris-HCl at pH 7.4 and 0.2 ml cerebral cortex S2. The fluorescence was recorded before and after incubation at 37°C for 1 h, using a 380 nm excitation wavelength and 460 nm emission wavelength. Non-specific protease activity was deducted from this value by performing a parallel incubation in the absence of CaCl_2 .

2.2.6. Trypsin-like serine protease

Serine protease activity was measured by utilizing the cleavage of a synthetic peptide whose hydrolytic cleavage leads to the appearance of rhodamine which is quantified by fluorescent assay (Leytus et al., 1983). Rhodamine 110, bis-(benzyloxycarbonyl-ile-pro-arg amide), dissolved in ethanol was added at a final concentration of 10 μM to 1.8 ml of 40 mM Tris-HCl at pH 7.4 and 0.2 ml of cortex S2 suspension, and incubated for 1 h at 37°C .

Fluorescence was read before and after incubation at 492 nm excitation and 523 nm emission wavelengths.

2.2.7. Statistical analysis

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.

3. Results

3.1. Apopain protease activities

Using Ac-asp-glu-val-asp-aminomethylcoumarin as a specific apopain (caspase 3) substrate, aluminum treated animals had significantly elevated apopain activities in the brain, while iron-treated rats showed no significant change (Fig. 1). Following combined treatment with aluminum and iron, there was a significant elevation of apopain activity that mirrored the changes found following exposure to aluminum alone.

To test the possibility that elevated apopain activity was associated with apoptosis, we measured cellular DNA fragmentation by assaying fluorescently labeled enzymatically incorporated dideoxynucleotide at 3'-OH ends of fragmented DNA. However, no significant changes between control animals and aluminum treated animals were found (data not shown).

Fig. 1. Levels of apopain in cerebral cortex of rats treated with aluminum, iron or salts of both metals together. Values represent mean $\pm$ SE from 8 individual animals/group. * signifies that value differs from that of control group ($P < 0.05$).

3.2. Calpain proteases

Using *tert*-butoxycarbonyl-leu-met amide 7-amino-4-chloromethylcoumarin as a model calpain substrate, total calpain activity in brains of rats treated with aluminum for three weeks was significantly elevated in comparison to control values (Fig. 2). No significant differences were found in rats treated with iron alone, while combined treatment with aluminum and iron significantly increased total calpain activity relative to controls in a manner parallel to changes found using aluminum alone. When calpain was assayed in the absence of EDTA and in the presence of $50 \mu\text{M Ca}^{2+}$, activity was too low to allow accurate determination (data not shown). Thus the calpain subclass that was studied here was almost solely calpain II.

3.3. Trypsin-like serine proteases

The activities of trypsin-like serine proteases in rat brain were slightly elevated in the aluminum, iron and aluminum with iron treated animals relative to the control group as judged by rates of hydrolysis of rhodamine 110, bis-(benzyloxycarbonyl-ile-pro-arg amide). However, these elevations were not statistically significant (Fig. 3).

3.4. Addition of aluminum to cerebral preparations in vitro

Aluminum sulfate was added directly to isolated cerebral S2 fractions and protease activity was determined. As the concentrations of aluminum tested ($1\text{--}50 \mu\text{M}$), there was no detectable effect on the levels of any of the three proteases assayed (data not shown). Thus the enzymatic changes found in dosed animals reflected a metabolic

Fig. 2. Levels of calpain in cerebral cortex of rats treated with aluminum, iron or salts of both metals together. Values represent mean $\pm$ SE from 8 individual animals/group. * signifies that value differs from that of control group ($P < 0.05$).

Fig. 3. Levels of serine protease in cerebral cortex rats treated with aluminum, iron or salts of both metals together. Values represent mean $\pm$ SE from 8 individual animals/group. * signifies that value differs from that of control group ($P < 0.05$).

response rather than a primary effect of aluminum upon an enzyme.

4. Discussion

4.1. Apopain

Apoptotic pathways have been suggested to have a role in neurodegenerative disease, such as AD (Gorman et al., 1996; Kusiak et al., 1996). This protease has been shown to activate PARP (poly (ADP-ribose)-polymerase), which in turn recognizes DNA strand breaks (such as those that occur during the controlled degradation of DNA when cells undergo apoptosis). Apopain has been implicated as the capsase which best fits the evidence for a central role in apoptosis (Mukasa et al., 1997). It may be relevant that both of the proteases found to be elevated in this study, (calpain and apopain) are associated with apoptotic cells and appear to operate in conjunction (Nath et al., 1996). Aluminum treatment significantly increased apopain activity relative to controls while iron treatment had no effect on activity levels of the enzyme. The combination of aluminum and iron also had a significant effect on activity elevation which closely paralleled the increase due to aluminum exposure alone. Thus aluminum, administered systemically, may modulate cortical apoptotic pathways.

4.2. Calpain

Since the level of calpain assayed in the presence of a low calcium concentration (50 μ M) was very low, the calpain subclass assayed in this study was predominantly calpain II which is known to be activated under pathological conditions. The role of calpain protease in the patho-

physiology of neurodegenerative disease has been the subject of several studies (Ostwald et al., 1993; Saito et al., 1994). In the current study, calpain activity increased following aluminum but not iron treatment. The combination of aluminum and iron also resulted in an increase that most likely solely reflected the contribution of aluminum alone since the aluminum and iron level was roughly equal to aluminum alone.

Subsequent to intracellular Ca^{2+} elevation, calpain undergoes autolysis and becomes activated (Shea et al., 1996). Protein kinase C (PKC) is a substrate for calpain and becomes constitutively activated as the catalytic domain is cleaved from the regulatory domain of the PKC. This activation has been shown to result in hyperphosphorylation of the cytoskeletal protein tau in human neuroblastoma cells, which renders tau a less efficient substrate for proteolysis by calpain with subsequent abnormal accumulation of tau (Shea et al., 1995). Aluminum has previously been found to increase the resistance of a variety of calpain substrates to hydrolysis (Nixon et al., 1994). The presence of aluminum may exacerbate these events by inducing high intracellular Ca^{2+} levels (Julka and Gill, 1996), as well as directly interacting with hyperphosphorylated tau (Guy et al., 1991; Savory et al., 1995), thereby making tau even more resistant to proteolysis by calpain. However, since saturating concentrations of calcium were used in our assay, the increase in calpain activity described here cannot reflect alteration of intracellular Ca^{2+} levels in the brain. Aluminum did not directly stimulate calpain in vitro, and so the effect of this metal in vivo, involved more complex mechanisms than direct interactions with proteins. In the absence of added calcium, calpain II has previously been found to be inhibited by millimolar concentrations of aluminum chloride added in vitro (Zhang and Johnson, 1992) but such concentrations are unlikely to be encountered in intact tissues.

4.3. Trypsin-like serine proteases

The trypsin-like serine protease group is one of the most abundant protease classes found in the brain. It has approximately 5-fold higher activity in cortical tissue than in rat liver (unpublished results). However, treatment of rats with aluminum, iron, or the two metals together had no significant effect on the activity of this protease in rat brain. This enzyme is known to be upregulated following oxidative damage to proteins (Dean, 1987; Davies, 1993). Despite reports suggesting that pro-oxidant status may be enhanced following treatment with aluminum salts (Ohtawa et al., 1983; Fraga et al., 1990; Gupta and Shukla, 1995; Bondy et al., submitted, 1997), there was no evidence of induction of serine proteases following oxidative damage to proteins. However, rabbits treated chronically with aluminum have elevated levels of ubiquitin, suggesting increased proteolytic activity in

this aluminum-susceptible species (Saito et al., 1993). Several specific cerebral serine proteases such as neurotrypsin and neuropsin, have recently been characterized by molecular techniques. (Chen et al., 1995; Gschwend et al., 1997). The influence of aluminum exposure upon these proteases, confined to limbic and hippocampal regions would be of interest. However, highly selective substrates with which to evaluate their activity are not currently available.

Although the activity of apopain and calpain was elevated in aluminum treated rats, in no case did concurrent treatment with iron potentiate the effect of aluminum dosing. This parallels the finding that aluminum dosing can promote pro-oxidant events in vivo, independently of the presence of iron (Bondy et al., 1998). This is in contrast to the situation in isolated preparations where iron is required in order to demonstrate the pro-oxidant capacity of aluminum (Bondy and Kirstein, 1996; Otieza, 1994).

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