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Aluminum induces lipid peroxidation and aggregation of human blood platelets

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Abstract

Aluminum (Al^{3+}) intoxication is thought to play a major role in the development of Alzheimer's disease and in certain pathologic manifestations arising from long-term hemodialysis. Although the metal does not present redox capacity, it can stimulate tissue lipid peroxidation in animal models. Furthermore, *in vitro* studies have revealed that the fluoroaluminate complex induces diacylglycerol formation, 43-kDa protein phosphorylation and aggregation. Based on these observations, we postulated that Al^{3+}-induced blood platelet aggregation was mediated by lipid peroxidation. Using chemiluminescence (CL) of luminol as an index of total lipid peroxidation capacity, we established a correlation between lipid peroxidation capacity and platelet aggregation. Al^{3+} (20-100 µM) stimulated CL production by human blood platelets as well as their aggregation. Incubation of the platelets with the antioxidants nor-dihydroguaiaretic acid (NDGA) (100 µM) and *n*-propyl gallate (NPG) (100 µM), inhibitors of the lipoxygenase pathway, completely prevented CL and platelet aggregation. Acetyl salicylic acid (ASA) (100 µM), an inhibitor of the cyclooxygenase pathway, was a weaker inhibitor of both events. These findings suggest that Al^{3+} stimulates lipid peroxidation and the lipoxygenase pathway in human blood platelets thereby causing their aggregation.

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- Chemiluminescence
- Platelet aggregation

Introduction

Aluminum (Al^{3+}) overload is frequently associated with neurological disorders such as Alzheimer's encephalopathy, amyothrophic lateral sclerosis and aging (1,2). Al^{3+} accumulation is also claimed to be related to renal impairment, anemia and other clinical complications in patients on hemodialysis (3). Furthermore, these patients present enhanced levels of plasma lipid peroxidation in comparison to healthy individuals (4) and atherosclerosis in prolonged hemodialysis (5). Despite the fact that Al^{3+} is a non-redox metal, it has been reported that mice chronically fed with this metal presented high levels of brain tissue lipid peroxidation (6). The end products of lipid peroxidation are cytotoxic and may cause platelet dysfunction. Nevertheless, reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide and the hydroxyl radical, well-known stimulators of lipid peroxidation, are generated by platelets (7,8). These species have been shown to be modulators of platelet adhesion and aggregation, directly or through

effects on vascular endothelial cells which generate prostacyclin and nitric oxide (9).

Previous observations in the literature have accounted for the activation of phospholipase A_2 by ROS (10). Furthermore, Al^{3+} in combination with fluoride, forming the fluoroaluminate complex (11), activates phospholipase A_2, phospholipase C and ß-thromboglobulin release in human blood platelets (12). The enhancement of phospholipase A_2 and phospholipase C activities promotes the mobilization of arachidonic acid from platelet membrane phospholipids resulting in increased synthesis of eicosanoids (13,14). In addition, the generation of oxygen metabolites during prostaglandin synthesis and the lipoxygenase-dependent arachidonic acid turnover is accompanied by light emission from platelets in the presence of luminol (15,16). Luminol-dependent chemiluminescence (CL) is generated through its peroxidase-catalyzed oxidation, and has been used to estimate the oxidative stress status in a number of systems (17).

In the present report we describe Al^{3+}-dependent human blood aggregation mediated by lipid peroxidation. The effects of Al^{3+} on lipid peroxidation in human blood platelets and on their aggregation were evaluated by using luminol-dependent CL and turbidometric aggregometry.

Platelet-derived CL was stimulated by Al^{3+} with the concomitant occurrence of platelet aggregation. Both events were strongly inhibited by the potent inhibitors of the lipoxygenase system nor-dihydroguaiaretic acid (NDGA) and *n*-propyl gallate (NPG) (18) and, to a lesser extent, by the cyclooxygenase inhibitor acetyl salicylic acid (ASA) (19).

Material and Methods

Reagents

ASA, NDGA, NPG and luminol were purchased from Sigma Chemical Co. (St. Louis, MO). Aluminum salt ($AlCl_3$) was obtained from Fluka (Buchs, Switzerland). All other chemicals were of the purest grade available and were obtained commercially.

Subjects

The study included 25 healthy volunteers (mean age, 38 years), who gave informed consent before participating in this study.

Isolation of platelets

Human blood platelets were obtained from healthy, drug-free individuals and collected in 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifuging the citrated blood at room temperature for 6 min at 100 *g*. The pH of the platelet suspension was adjusted to 6.1 by the addition of 1 M citric acid. PRP aliquots were then centrifuged in an Eppendorf microcentrifuge for 15 s at 2,000 *g*. Platelets were washed twice with 140 mM NaCl and 2.1 mM KCl in 3.8 mM HEPES, pH 6.1, supplemented with 5 mM EGTA and 1 mM prostacyclin and resuspended and adjusted to 3×10^8 cells/ml in 3.8 mM HEPES, pH 7.4, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

Treatment of platelets

Platelets were incubated with inhibitors or vehicle (0.2% ethanol) for 5 min at 37°C prior to the addition of Al^{3+}. Subsequently, platelet aggregation and CL production were determined.

Measurement of platelet aggregation

Platelet aggregation was determined by the turbidometric method of Born and Cross (20), using a dual channel Chronolog 660 Lumi-aggregometer. Aliquots of 400 µl of washed platelets were pipetted into a small siliconized cuvette and stirred at a constant speed of 1,000 rpm at 37°C. Al^{3+} solutions

(20-100 µM, final concentrations) were added and aggregation was recorded continuously for 5 min after addition of the agonist. The extent of aggregation (%) was recorded 5 min after aluminum addition.

Formolization of platelets

Platelet agglutination was evaluated according to MacFarland et al. (21). PRP was submitted to treatment with 2% formaldehyde for 16 h at 4ºC. Platelets were washed and centrifuged as described previously and 400-µl aliquots of platelets were stimulated with Al^{3+} (100 µM). Ristocetin was used as positive control.

Measurement of chemiluminescence

Luminol-dependent CL was determined according to Dahlgren (22). A platelet suspension containing 3×10^8 cells/ml was maintained at 37ºC under stirring. Luminol (0.2 mM) dissolved in 2.0% dimethyl sulfoxide was added to the cell suspension prior to the addition of Al^{3+}. CL measurements started by the addition of Al^{3+} solutions (20-100 µM) to the cells. CL intensity was measured with a Chronolog 660 Luminometer equipped with a sample stirrer. The CL signal is reported as mV/gain.

Release of lactate dehydrogenase

As a parameter of cytoplasmic leakage, lactate dehydrogenase (LDH) activity was measured spectrophotometrically (Celm, São Paulo). Aliquots were taken from the incubates 5 min after the addition of Al^{3+} and the supernatant was obtained by centrifugation for 2 min at 2,000 *g* in an Eppendorf microcentrifuge. LDH activity in the supernatant was compared with total LDH activity of control platelets after lysis with Triton detergent (23).

Statistical analysis

Data are reported as means ± SEM. The Student *t*-test was employed to estimate differences between groups. Differences were considered to be significant when the probability was $P<0.05$. The statistical program Instat-2 was utilized.

Results

Human platelets were stimulated with Al^{3+} (20-100 µM) at 37ºC for 5 min. Significant platelet aggregation was observed after incubation of the cells with 50 µM Al^{3+}. The maximum response was obtained after incubation of the cells with 100 µM Al^{3+} (Table 1).

Peroxidation levels were determined using the luminol-dependent CL technique. Similar to the pattern observed for aggregation, lipid peroxidation capacity in platelets was linearly dependent on Al^{3+} concentration (Table 2). To rule out the possibility that the effect of aluminum was due to platelet lysis, we measured LDH activity in platelet supernatants after the reactions were carried out; 5% or less LDH was measurable under these circumstances, indicating that no significant lysis had taken place (data not shown).

Table 1 - Effect of increasing concentrations of aluminum on human blood platelet aggregation.

Platelets were incubated at 37ºC, Al^{3+} solutions were added and aggregation was recorded for 5 min. Data are reported as mean ± SEM of 10 independent determinations.

Al^{3+} (µM)	Aggregation (%)
20	4.4 ± 1.0
50	43.6 ± 5.5
100	83.3 ± 1.8

Table 2 - Effect of different concentrations of aluminum on chemiluminescence intensity.

Platelets were incubated at 37ºC, Al^{3+} solutions were added and chemiluminescence intensity (mV/gain) was recorded for 5 min. Data are reported as mean ± SEM of 10 independent determinations.

Al^{3+} (µM)	Chemiluminescence (mV/gain)
20	21.8 ± 1.5
50	69.9 ± 5.3
100	148.4 ± 14.1

Table 3 - Effects of acetyl salicylic acid (ASA), nor-dihydroguaiaretic acid (NDGA), and *n*-propyl gallate (NPG) on human platelet aggregation and chemiluminescence stimulated with 100 µM Al^{3+}.

Platelets were preincubated with NDGA, NPG or ASA for 5 min at 37ºC before stimulation. Each value represents the mean ± SEM of 10 independent experiments. *P<0.05 compared to control (Student *t*-test).

Treatment	Aggregation (%)	Chemiluminescence (mV/gain)
Al^{3+} (control)	79.4 ± 1.2	142.0 ± 17.1
Al^{3+} + 1 µM ASA	79.5 ± 1.4	140.6 ± 17.3
Al^{3+} + 10 µM ASA	79.0 ± 1.1	110.0 ± 16.2*
Al^{3+} + 100 µM ASA	78.2 ± 1.1	71.2 ± 18.3*
Al^{3+} + 1000 µM ASA	66.2 ± 2.2*	48.4 ± 11.3*
Al^{3+} + 1 µM NDGA	79.2 ± 2.3	39.3 ± 4.5*
Al^{3+} + 10 µM NDGA	78.6 ± 2.8	2.1 ± 0.36*
Al^{3+} + 100 µM NDGA	50.6 ± 2.1*	0.92 ± 0.11*
Al^{3+} + 1000 µM NDGA	7.9 ± 2.3*	0.0*
Al^{3+} + 1 µM NPG	78.5 ± 1.4	49.7 ± 14.0*
Al^{3+} + 10 µM NPG	77.0 ± 2.5	18.0 ± 6.14*
Al^{3+} + 100 µM NPG	49.0 ± 2.7*	0.39 ± 0.09*
Al^{3+} + 1000 µM NPG	1.3 ± 1.3*	0.0*

Subsequently, we examined the effect of the compounds that modulate eicosanoid synthesis in platelets, NDGA and NPG (inhibitors of the lipoxygenase pathway), and ASA (inhibitor of the cyclooxygenase pathway). Table 3 shows the effects of ASA, NDGA and NPG on Al^{3+}-induced platelet aggregation and chemiluminescence. At a lower ASA dose (1 µM) no inhibition of aluminum-dependent aggregation was observed. At the highest dose tested, ASA inhibited platelet aggregation by only 13%, whereas NDGA and NPG completely blocked the process at the same concentrations. Furthermore, Al^{3+}-dependent lipid peroxidation evaluated by CL of platelets was partially inhibited by ASA (1 mM). In contrast, 100 µM NDGA and NPG completely prevented Al^{3+}-dependent chemiluminescence.

Finally, to evaluate whether platelets undergo Al^{3+}-induced aggregation or agglutination, we determined the effect of Al^{3+} on formolization of platelets (Table 4). No platelet agglutination induced by Al^{3+} was observed under the experimental conditions.

Table 4 - Effects of aluminum (100 µM) and ristocetin (1 mg/ml) on platelet agglutination.

Data are reported as means ± SEM of 5 determinations. Cells were stimulated for 5 min at 37ºC with aluminum or ristocetin. For further details see Material and Methods.

Stimulus	Agglutination (%)
Ristocetin (1 mg/ml)	28.2 ± 2.4
Aluminum (100 µM)	0.0

Discussion

The objective of the present report was to evaluate the relationship between Al^{3+}-dependent human blood platelet aggregation and lipid peroxidation capacity. Earlier, Rendu et al. (11) reported that Al^{3+} at concentrations similar to those present in the blood circulation potentiated NaF-elicited platelet aggregation, as well as diacylglycerol and 43-kDa phosphorylation. These authors proposed that the fluoroaluminate complex, formed upon the association of NaF and $AlCl_3$, may be responsible for the observed effects. We have shown here that Al^{3+}, at concentrations higher than those used by Rendu et al. (11), promoted platelet aggregation and peroxidation in the absence of NaF. Recently, Al^{3+} has been shown to induce lipid peroxidation in brain tissue of mice chronically exposed to the metal (6). Gutteridge and co-workers (24) demonstrated that Al^{3+} salts do not directly stimulate lipid peroxidation, but instead accelerate iron- and hydrogen peroxide-dependent lipid peroxidation in phospholipid liposomes and human erythrocyte membranes. On the other hand, superoxide and hydrogen peroxide are known to induce platelet aggregation and they can be produced by these cells (8). Although Al^{3+} has no redox capacity, the metal can cause alterations at the membrane level facilitating iron-initiated lipid peroxidation. Oteiza (25) showed that Al^{3+} increases the packing of fatty acids, thus favoring the propagation of lipid peroxidation. Here, we show that lipid peroxidation capacity, estimated as luminol-dependent CL in human blood platelets, was progressively stimulated by increasing concentrations of Al^{3+}. There is experimental evidence that lipid peroxides increase platelet sensitivity to agonists (26,27). Furthermore, early reports indicated that CL in platelets arises from prostaglan-

din synthesis and lipoxygenase activity (15,16). Worner (15) reported that the chemiluminescence response of platelets exposed to arachidonic acid is dependent on the prostaglandin and lipoxygenase pathways. Accordingly, the inhibition of platelet CL measured here in the presence of antioxidants indicates metabolization of arachidonic acid under our experimental conditions. It was reported that ASA, at low concentrations, promotes specific acetylation of the cyclooxygenase activity component of prostaglandin synthesis, causing irreversible inhibiton of this activity in platelets from PRP (28). Nevertheless, partial inhibition by 1 mM ASA of Al^{3+}-dependent aggregation and CL arising from washed platelets incubated with the metal suggested a secondary role for the cyclooxygenase pathway in this process. On the other hand, NDGA and NPG, antioxidants and potent inhibitors of the lipoxygenase pathway, strongly inhibited Al^{3+}-dependent platelet aggregation and CL. Therefore, Al^{3+}-dependent platelet aggregation and associated CL most likely reflect a lipoxygenase-mediated event. Lipid peroxides have been shown to be positive cofactors of lipoxygenase activities in a number of systems (29). Thus, stimulation of lipid peroxidation in platelets by Al^{3+} would build up a concentration of peroxide activators, which in turn activate the lipoxygenase pathway in these cells, resulting in their aggregation. Alternatively, the metal itself may function as a positive cofactor for the lipoxygenase activity in platelets.

In conclusion, although Al^{3+} has no redox capacity, it is tempting to propose that this metal induces oxidative stress in platelets, stimulating lipoxygenase activity in these cells and promoting their aggregation.

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