Original Article

The RAS/PI3K Pathway is Involved in the Impairment of Long-term Potentiation Induced by Acute Aluminum Treatment in Rats

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Abstract

Objective To explore the role of RAS/PI3K pathway in the impairment of long-term potentiation (LTP) induced by acute aluminum (Al) treatment in rats in vivo.

Methods First, different dosages of aluminum-maltolate complex [Al(mal)₃] were given to rats via acute intracerebroventricular (i.c.v.) injection. Following Al exposure, the RAS activity of rat hippocampus were detected by ELISA assay after the hippocampal LTP recording by field potentiation technique in vivo. Second, the antagonism on the aluminum-induced suppression of hippocampal LTP was observed after the treatment of the RAS activator epidermal growth factor (EGF). Finally, the antagonism on the downstream molecules (PKB activity and the phosphorylation of GluR1 S831 and S845) were tested by ELISA and West-blot assays at the same time.

Results With the increasing aluminum dosage, a gradually decreasing in RAS activity of the rat hippocampus was produced after a gradually suppressing on LTP. The aluminum-induced early suppression of hippocampal LTP was antagonized by the RAS activator epidermal growth factor (EGF). And the EGF treatment produced changes similar to those observed for LTP between the groups on PKB activity as well as the phosphorylation of GluR1 S831 and S845.

Conclusion The RAS→PI3K/PKB→GluR1 S831 and S845 signal transduction pathway may be involved in the inhibition of hippocampal LTP by aluminum exposure in rats. However, the mechanisms underlying this observation need further investigation.

Key words: Aluminum; Long-term potentiation; RAS; PKB; AMPA receptor

INTRODUCTION

Aluminum (Al) is one of the most abundant metals in the earth's crust. Because of its ubiquitous distribution in nature and wide application in industry, human consumption of Al in daily life appears to be inevitable. However, it has no known physiological function in biological systems, and it is well-established that Al is neurotoxic to mammals[1]. Al has also been identified as a potential contributing factor in various cognitive dysfunctions and neuronal diseases, such as Alzheimer's disease[1-2].

Synaptic plasticity is believed to underlie key aspects of learning and memory. Hippocampal long-term potentiation (LTP) has been widely used as a neuronal model of synaptic plasticity in
experiments designed to elucidate the synaptic mechanisms involved in the loss of learning and memory\cite{3-5}. A number of studies indicate that the trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazoloproponionic acid (AMPA) receptor from intracellular pools to synaptic sites alters synaptic strength, and this trafficking has been recognized to be a central mechanism of LTP\cite{6-9}. Our previous study showed that Al dose-dependently suppressed LTP in the rat hippocampal CA1 region in vivo, and that this suppression may be related to the altered trafficking of AMPA receptor subunits\cite{10}. However, the biochemical pathways linking the impairment of LTP caused by Al treatment to AMPA receptor trafficking are poorly understood.

In recent years, many scholars have shown that the small GTPase RAS signaling pathway plays important roles in LTP and in formation and the consolidation of memories in the brain\cite{11-12}. The RAS and its downstream phosphatidylinositol-3-kinase (PI3K) signaling cascades control the synaptic trafficking of AMPA receptor: the RAS-PI3K-PKB pathway and the downstream phosphorylation of GluR1 S831 and S845 drive GluR1 into synapses\cite{13-17}. However, whether aberrant RAS signaling is responsible for the impairment of LTP caused by Al treatment has not previously been investigated.

On the basis of previous studies of the effects of Al on AMPA-Rs trafficking in LTP and the possible involvement of the RAS/PI3K pathway in LTP, we tested the hypothesis that the suppression of RAS→PI3K→PKB→GluR1 S831 and S845 signal transduction pathway by Al leads to the impairment of hippocampal LTP. First, to explore the involvement of small GTPase RAS in Al-induced LTP suppression, different doses of Al were administered to rats, and the hippocampal RAS activity was determined after LTP. Second, to further confirm the hypothesis, the RAS activator epidermal growth factor (EGF) was used to antagonize the Al-induced suppression of hippocampal LTP. Furthermore, the antagonism on the downstream signaling molecules (the PKB activity and the phosphorylation of GluR1 S831 and S845) were observed at the same time.

**MATERIALS AND METHODS**

**Al(mal)$_3$ Preparation**

The aluminum-maltolate complex [Al(mal)$_3$]$_3$ was prepared according to the procedures described in our previous publications\cite{10,18-19}. AlCl$_3$·6H$_2$O (Sigma Chemical Co, MO, USA) and maltolate (Sigma Chemical Co, MO, USA) were dissolved in distilled water and phosphate buffered saline (PBS), respectively. Al(mal)$_3$ was freshly prepared for each experiment by mixing these solutions in equal volumes, adjusting the pH to 7.4 with NaOH, and filtering with 0.22 µmol/L syringe filters.

**Animal Models and Treatments**

The experiments were performed on 48 adult male rats (the Laboratory Animal Center, Shanxi Medical University, Taiyuan, China) that were kept under constant temperature and humidity conditions with free access to food and water. First, to study the effects of acute Al exposure on RAS activity, the rats were randomly divided into four groups (6 rats/group): control group, 2.43 µg Al group, 12.15 µg Al group and 60.75 µg Al group. The rats (weight 220-250 g) received one dose of 5 µL of saline (control group) or a single dose of Al(mal)$_3$ (2.43, 12.15, or 60.75 µg Al) over 5 min via intracerebroventricular (i.c.v.) injection. Second, to determine if RAS activator EGF antagonized the Al-induced suppression of hippocampal LTP and the downstream signaling molecules, the rats were randomly divided into four groups (6 rats /group): the control group, EGF group, EGF+Al group and Al group. The rats received one dose of 5 µL of saline (control group), 60 ng of EGF (EGF group), 12.15 µg of Al+60 ng of EGF (EGF+Al group), or 12.15 µg of Al (Al group) over 5 min via intracerebroventricular (i.c.v.) injection. Thirty minutes later, electrophysiological measurements were performed. At the end of electrophysiological test, the rats were decapitated and the hippocampi were rapidly removed and stored at -80 °C until used for biochemical analyses.

The present study was approved by the Ethics Committee for Animal Studies of Shanxi Medical University. All efforts were made to minimize the number of animals used and their suffering.

**Electrophysiological Measurements**

**Surgical Procedure**

The surgical procedure was performed according to the methods of Zhang and our previous publication\cite{10,20}. The rats were anesthetized with an intraperitoneal (i.p.) injection of 1.5 g/kg urethane (Sigma Chemical Co, MO, USA), and the head was fixed in a stereotaxic apparatus (Narishige Group, Japan). The skulls were exposed and the animals’ body temperature was monitored. Three holes were drilled in the skull at the appropriate positions for inserting stimulating, recording electrodes and a guide cannula (1.3 mm
posterior to bregma, 0.8 mm lateral to the midline and 4.0 mm below the dura mater) for i.c.v. injection. A concentric bipolar stimulating electrode (FHC, Ino, USA) was placed in the Schaffer collateral commissural pathway (4.2 mm posterior to bregma, 3.8 mm lateral to the midline), and a monopolar recording electrode (FHC, Ino, USA) was positioned in the stratum radiatum of area CA1 (3.8 mm posterior to bregma, 2.9 mm lateral to the midline). The depth of the electrodes was adjusted such that the field excitatory postsynaptic potentia (fEPSP) amplitude was maximal. The isolated pulse stimulator (2100) and AC/DC differential Amplifier (3000) were manufactured by A-M Systems, Inc., USA. The high-speed data acquisition system (Micro 1401) was produced by CED, UK.

**Electrophysiological Recordings** In each recording session, the stimulus intensity selected for the baseline measurement was adjusted to yield about 50% of its maximum amplitude. Baseline recordings were monitored for at least 30 min followed by application of the high-frequency stimulus (HFS) protocol composed of a train of 20 pulses at 200 Hz that was repeated three times at an interval of 30 s. The baselines recordings were tested again for 1 h after HFS. The average value of the fEPSP amplitude of baseline recording was defined as 100%, and all of the recorded fEPSPs were standardized to this baseline value.

**Biochemical Analysis**

**Production of Total Protein Extracts** The total protein of rat hippocampus was extracted using a Tissue Protein Extraction Kit (CoWin Biotech Co., China). Hippocampal tissue was homogenized in ice-cold Tissue Protein Extraction Reagent (containing a protease inhibitor mixture) provided by the kit and kept on ice for 20 min. The tissue homogenate was centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was obtained on ice cold and used as the total hippocampal protein extract. The protein concentrations of the extracts were measured using a BCA (bicinchoninic acid) assay (CoWin Biotech Co., China) and the extracts were stored at -80 °C until use.

**RAS Activity Assay** The activity of RAS was determined using a RAS Activation ELISA ASSAY Kit (Millipore, USA). The glutathione-coated wells were pre-rinsed twice with 200 μL of Wash Buffer (Tris Buffered saline Tween, TBST). Raf-1-RBD (2.5 μL of 2 μg/μL) was added to 47.5 μL of Binding/Blocking Buffer, and 50 μL was added to each well. The wells were covered and incubated at 4 °C with rocking for 1 h, and then washed 3 times with 200 μL of Wash Buffer after removing the previous solution. Up to 200 μL of the total protein extracts were added to each well to obtain total amount per well of 10-100 mg of cell lysate. The wells (sample lysate, positive control, and negative control) were incubated at room temperature (RT) with mild agitation for 1 h and washed 3 times with 200 μL of Wash Buffer after removing the previous solution. Primary Antibody Solution (50 μL) was added to the wells, and the plates were incubated at RT for 1 h with mild agitation and washed 3 times with 200 μL of Wash Buffer after removing the previous solution. The procedure for incubating the Secondary Antibody Solution was the same as that performed for the Primary Antibody Solution. Finally, each well was rinsed twice with 200 μL of TBS, and 50 μL of the Chemiluminescent substrate was added. The results were read using a luminometer (SpectraMax M2, Molecular Devices, USA) between 5-60 min after adding substrate.

**PKB Activity Assay** The activity of PKB was determined using the PKB/Ark Activation ELISA Assay Kit (Enzo Life Sciences, USA). Each well of the PKB Substrate Microtiter Plate was soaked with 50 μL of Kinase Assay Dilution Buffer at RT, and the buffer was carefully aspirated 10 min later. Thirty microliters of each of purified active PKB, samples, blank and negative control was added to the appropriate wells. The reaction was initiated by adding 10 μL of diluted Adenosine Triphosphate (ATP) to each well, except the blank well. The wells were covered and incubated at 30 °C with gentle shaking every 20 min. Ninety minutes later, the reaction was stopped by emptying the wells. The phosphospecific Substrate Antibody was added to each well, except the blank well. The wells were covered and incubated at RT for 60 min with gentle shaking every 20 min and washed 4 times with Wash Buffer. Forty microliters of diluted Anti-Rabbit IgG:HRP Conjugate was added to each well, except the blank well. The wells were covered and incubated at RT for 30 min with gentle shaking every 10 min and washed 4 times with Wash Buffer. The tetramethylbenzidine (TMB) Substrate (60 μL) was added to each well. The wells were incubated at RT for 30-60 min (the incubation time was monitored by the investigator to obtain the appropriate color development), then, 20 μL of the Stop Solution 2 was added to each well in the same order that the TMB Substrate was added. The absorbances were read using a Microplate.
Aluminum impaired the LTP by the RAS/PI3K pathway

Reader (SpectraMax M2, Molecular Devices, USA) at the 450 nm wavelength.

**Western Blotting** The phosphorylation of GluR1 S831 and S845 were measured by western-blot analysis. Samples (30 µg per lane) were run by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% Tris-glycine polyacrylamide gels and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% fat-free milk at 37 °C for 1 h and incubated overnight at 4 °C in one of the following primary antibodies: rabbit anti-GluR1 S831, rabbit anti-GluR1 S845 (1:1000, Millipore, USA), or mouse anti-β-tubulin (the protein loading control, 1:4000, CoWin Biotech Co., China). After four times wash for 1 h in Phosphate Buffered saline Tween (PBST), the blots were incubated for 90 min in one of the following HRP-conjugated antibodies: anti-rabbit IgG (1:2000, CoWin Biotech Co. China) or anti-mouse IgG (1:2000, CoWin Biotech Co. China). The immunolabeled protein bands were detected using the ECL western blot detection kit (CoWin Biotech Co. China). The protein levels were quantified using densitometry analysis. All of the experiments were independently performed in triplicate, and the average was used for comparison.

**Data Analysis**

The data are presented as mean±SD and analyzed by one-way ANOVA analysis of variance followed by LSD test as a post hoc test for comparison between two groups and using the Pearson Correlation analysis, the correlation between the RAS activity and LTP was evaluated. *P*<0.05 was considered to be statistically significant. All data analyses were performed with the SPSS 22.0 for Windows statistical analysis software (SPSS Inc., Chicago, IL, USA).

**RESULTS**

The Effects of Acute Al Exposure on LTP

The effects of acute Al exposure on LTP were investigated before the RAS activity assay. As shown in Figure 1A, with increasing Al concentrations, the average value of the fEPSP amplitude gradually decreased. As shown in Figure 1B, the fEPSP amplitudes at three time points were chosen for statistical analysis. The fEPSP amplitudes of the control group (n=6) were 1.98±0.14, 1.85±0.19, and 1.78±0.19 at 1, 30, and 60 min after HFS, and the average fEPSP amplitudes of the low-dose Al group (2.43 µg, n=6) were 1.57±0.11 at 30 min and 1.52±0.10 at 60 min after HFS, which represented a slight but significant decrease compared with the control group (*P*<0.05); these values dropped to 1.71±0.13, 1.51±0.12, and 1.41±0.11 in the medium-dose Al group (12.15 µg, n=6, *P*<0.05) and further decreased to 1.50±0.08, 1.34±0.06, and 1.20±0.05 in the high-dose Al group (60.75 µg, n=6, *P*<0.05). Notably, the difference of LTP was apparent between the Al exposure groups and control group (*P*<0.05), and further suppression was apparent with increasing Al concentrations.

![Figure 1](image-url). The effects of acute Al exposure on LTP in rats. (A) Acute Al exposure resulted in a depression in HFS-induced LTP. (B) The effects of different concentrations of Al on LTP at three time points (1 min, 30 min, and 60 min). a: *P*<0.05 vs. the control group, b: *P*<0.05 vs. the 2.43 µg group, c: *P*<0.05 vs. the 12.15 µg group.
The Effects of Acute Al Exposure on RAS Activity

To investigate the involvement of RAS in the LTP suppression induced by Al, the RAS activity of hippocampal tissue was examined after the electrophysiological recordings. As shown in Figure 2, increasing the Al concentrations caused a greater decrease in the RAS activity. The statistical results showed that compared with the control group, the RAS activity of the 12.15 µg Al group and the 60.75 µg Al group were significantly decreased (P<0.05). The value of 60.75 µg Al group was much lower than the 12.15 µg Al group (P<0.05).

The Correlation of RAS Activity and LTP

As shown in Figure 3, the analysis of Pearson correlation revealed that RAS activity had positive correlation with standardized fEPSP amplitudes at 60 min after HFS (r=0.73, P<0.05).

The Antagonism on Al’s Effects by the RAS Activator EGF

The Antagonism on Al-induced LTP Suppression

To explore the antagonism on Al-induced hippocampal LTP by RAS activator, the rats received one dose of 5 µL of saline (control group), 60 ng of EGF (EGF group), 12.15 µg of Al+60 ng of EGF (EGF+Al group), or 12.15 µg of Al (Al group) via acute i.c.v. injection. As shown in Figure 4A, the RAS activator EGF antagonized the early suppression of hippocampal LTP induced by Al. Figure 4B showed the histograms of the amplitudes of the fEPSPs in different groups at five time points. The fEPSP amplitudes of the control group (n=6) were 1.91±0.25, 1.79±0.22, and 1.56±0.27 at 1, 30, and 60 min after HFS, respectively. The average fEPSP amplitudes of the Al group (n=6) were 1.6±0.04, 1.32±0.15, and 1.15±0.12 at the same time points, which represented a statistical decrease compared with the control group (P<0.05). The average fEPSP amplitudes of the EGF group (n=6) were 2.24±0.28 and 1.89±0.14 at 1 and 20 min after HFS, respectively, which represented a statistical increase compared with the control group (P<0.05), but no difference was found at 30, 40, and 60 min after HFS (P>0.05). The average fEPSP amplitudes of the EGF+Al group (n=6) were 1.83±0.05 and 1.58±0.15 at 1 and 20 min after HFS, respectively, which represented a statistical return compared to the Al group (P<0.05), but no difference was found at 30, 40, and 60 min after HFS (P>0.05).

The Antagonism on Al-induced Suppression of RAS Activity

To confirm the EGF antagonism on Al’s effects on RAS, the RAS activity in the hippocampus was measured after electrophysiological recordings. As shown in Figure 5A, compared with the control group, the RAS activity was significantly decreased by treatment with Al alone (P<0.05) and increased by treatment with EGF alone (P<0.05); the RAS activity clearly returned with the combined use of Al and EGF compared with Al group (P<0.05).

The Antagonism on PKB Activity

The activity of PKB, a downstream signaling molecule of RAS, was measured after LTP recordings in the hippocampus. As shown in Figure 5B, compared with the control group, the PKB activity was significantly decreased by treatment with Al alone (P<0.05) and increased by treatment with EGF alone (P<0.05); the PKB activity was clearly returned by the combined use of Al and EGF compared with Al group (P<0.05).

The Antagonism on the Phosphorylation of GluR1 S831 and S845

Phosphorylation of the
substrates of PKB in the hippocampus, GluR1 S831 and S845, was also measured. As shown in Figure 6, compared with the control group, the expression of GluR1 S831 was significantly decreased by treatment with Al alone ($P<0.05$) and significantly increased by treatment with EGF alone ($P<0.05$). The expression of GluR1 S831 was clearly returned by the combined use of Al and EGF compared with Al group ($P<0.05$). Similar changes were found in the expression of GluR1 S845 ($P<0.05$).

**Figure 4.** The antagonism on Al-induced suppression of hippocampal LTP by RAS activator EGF. (A) EGF antagonized the early suppression of hippocampal LTP induced by Al. (B) The amplitude of fEPSPs in different groups at five time points. a: $P<0.05$ vs. the control group, b: $P<0.05$ vs. the 12.15 µg Al group.

**Figure 5.** (A): RAS activity in the hippocampus after different treatments. (B): PKB activity in the hippocampus after different treatments. a: $P<0.05$ vs. the control group, b: $P<0.05$ vs. the 12.15 µg Al group.

**Figure 6.** The expression of phosphorylated GluR1 S831 and S845 in the hippocampus after different treatments. a: $P<0.05$ vs. the control group, b: $P<0.05$ vs. the 12.15 µg Al group.
The synaptic delivery of AMPA receptor, which depends on small GTPase RAS signaling, plays key roles in synaptic transmission and potentiation\cite{20, 21, 22, 23, 24}. Accordingly, a number of diseases that cause cognitive impairment are associated with aberrant RAS signaling\cite{22, 25, 26}. Al exposure leads to similar cognitive dysfunctions, suggesting that malfunctions of RAS signaling may be a common possible cause of Al-induced and disease-induced cognitive impairment. AMPARs with long cytoplasmic termini, including GluR1- and GluR2L-containing AMPARs, are normally restricted from synapses and are driven into synapses by RAS signaling during activity-induced synaptic enhancement\cite{21, 22, 13, 14, 15, 16, 17, 18, 19}. RAS activity via the PI3K-PKB signaling pathway controls the phosphorylation of GluR1 S845 and S831, which are the key steps leading to synaptic insertion of the receptor\cite{21, 22, 13, 14, 15, 16, 17, 18, 19}.

The present study explored this possible link between the disruption of LTP and the RAS signaling pathway by examining RAS activity in the rat hippocampus after LTP recording. We found that acute Al treatment produced suppression of hippocampal LTP, and RAS activity was similarly inhibited. The correlation analysis suggest that changes in activity of the small GTPase RAS may be related to the LTP impairment caused by Al. However, the results in the study of Cui Xin, et al.\cite{27} showed that chronic Al exposure increased the expression of RAS in the hippocampus, while the expression decreased in the Al exposure groups. We propose the following explanations for the differences between our study and Cui Xin’s: First, in our study, we measured the activity instead of the expression of RAS. Second, in our acute Al-exposure experiment, the short duration of exposure may have inhibited RAS’s activity due to more direct actions of the Al\(^{3+}\) ions. And, in Cui Xin’s study, the long-term oral administration of Al to rats may have initiated some mechanisms (e.g. biological adaptation, biological compensation or negative feedback) to increase the RAS expression as a response to the decrease of RAS activity. The mechanism responsible for the inhibition of RAS activity by Al after acute exposure is not fully elucidated. One important aspect of this mechanism is that Al has a high affinity for the metal binding sites in enzymes, due to its small size and near maximal charge, and this affinity causes it to disrupt critical enzymatic reactions. For example, the Al\(^{3+}\) affinity for the Mg\(^{2+}\)-binding site in a G protein is approximately 10\(^7\) times higher than the affinity of Mg\(^{2+}\), and the dissociation rate of Al\(^{3+}\) from biological ligands is 10\(^5\) times more slowly than Mg\(^{2+}\) and 10\(^6\) times more slowly than Ca\(^{2+}\)\cite{21, 22, 23, 24, 25, 26}. So, as a Mg\(^{2+}\)-dependent protein, RAS may be disrupted by Al\(^{3+}\) competitive replacement.

To further confirm that the RAS/PI3K pathway is involved in Al-induced LTP impairment, the antagonism by the RAS activator EGF on Al-induced hippocampal LTP suppression and on downstream signaling molecules were investigated, respectively. We found that the early suppression of hippocampal LTP by Al could be antagonized by EGF, despite not return to control levels, and similar changes were observed in the effects of EGF treatment on PKB activity and the phosphorylation of GluR1 S831 and S845. The results indicated that RAS activity via the PI3K-PKB signaling pathway controls the phosphorylation of GluR1 S845 and S831, which may be a possible mechanism leading to LTP impairment induced by Al. However, the effect of antagonism disappeared in the later stages of LTP, and the reason for this disappearance was unclear. It may be related to the dose of EGF, the duration of EGF and multiple regulations.

In summary, the down regulation of RAS activity combined with the antagonism on Al-induced LTP impairment by the RAS activator EGF and the effects of EGF treatment on downstream signaling molecules, suggest that Al exposure affects the normal function of the RAS/PI3K pathway. Thus the RAS/PI3K pathway may serve as one possible mechanism underlying the early impact of Al exposure on cognitive function, especially on learning and memory. This conclusion raises the possibility that RAS may be a useful molecular event contributing to preventing the impact of learning and memory induced by Al to a certain degree and to exploring some valid targets to treat diseases correlating to Al.

In addition, inhibiting the RAS-PI3K-PKB signaling pathway is thought to be a useful therapeutic approach for treating cancer, and a number of compounds that inhibit PI3K-PKB signaling are currently in the late preclinical development or the early phases of clinical trials\cite{28}. However, our findings raise some concern about the unwanted side effects of these PI3K-PKB signaling inhibitors, and underscore the importance of developing tissue- or cell-specific therapeutic approaches to treat cancer or other diseases.
Aluminum impaired the LTP by the RAS/PI3K pathway

Received: March 30, 2016; Accepted: November 3, 2016

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