Arsenic down-regulates the expression of Camk4, an important gene related to cerebellar LTD in mice

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1. Introduction

Arsenic (As) is one of the most common heavy metal contaminants found in the environment, particularly in water. Its toxicity is a global health problem affecting tens of millions of people. The neurological system is a major target of As. Epidemiological studies revealed that chronic exposure to inorganic As via drinking water resulted in a dose-dependent reduction in intellectual functions in children [33,35]. Also, in animal experimental studies, deficits in learning tasks [19] as well as behavioral alterations have been observed in rats following sodium arsenite treatment [37,25]. It was reported that As exposure caused defects in cerebellum [20]. Our previous study [22] also showed that As inflicted damage to cerebellar neurons. Some literatures have documented that cerebellum contributes not only to motor function but also to some sensory, cognitive, linguistic, and emotional aspects of behavior [12–14, 28–30]. The researches above indicated that cerebellum may be involved in the impairment of learning and memory by As. However, the molecular mechanism by which As adversely affects intelligence is poorly understood.

It is commonly thought that a persistent change in the efficacy of the synaptic transmission is the basic mechanism underlying learning and memory. The cerebellum, key structure of the motor function, exhibits a synaptic plasticity named cerebellar long-term depression (LTD) which plays the central role in motor learning [9]. The cAMP response element-binding protein (Creb) activation is essential to the maintenance of LTD, during which Creb needs to be phosphorylated by Ca2+/calmodulin dependent protein kinase IV (Camk4), PKA, ERK and ERK-stimulated RSK and MSK kinases or MAP kinase-activated protein kinase 2 (Mapkapk2) [2,27,12,23,26,15]. The above literatures indicated that these kinases contribute to activating Creb and are associated with the formation of long-term memories in central nervous system. It has been known that As-intake can affect learning and memory. Are these genes involved in LTD the targets of As?

The amount of toxicants in target organs is the substance basis of toxic effects. However, there were few reports on the accumulation of As in cerebellum. Therefore, in the present study, concentrations of As in cerebellar tissue of mice exposed to 1 ppm and 4 ppm As2O3 subchronically were determined by ICP–MS and neurobehavioral change associated with learning and memory was examined by the Morris Water Maze test. To further elucidate the molecular mechanism underlying adverse effect of As on learning and memory, the expression

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ABSTRACT

To elucidate the molecular mechanism of arsenic (As) on motor learning and memory, concentration of As in cerebellar tissue of mice exposed to 1 ppm and 4 ppm As2O3 subchronically was determined by ICP–MS, neurobehavioral changes associated with memory was examined by the Morris Water Maze test, and the critical gene expression profiles related to the Creb-dependent phase of cerebellar long-term depression (LTD) were analyzed by GeneChip. Our results showed the increased level of As concentration in cerebellum of the exposed mice in a dose-response manner, longer escape latency in the experimental group than controls and the down-regulated expression of Ca2+/calmodulin dependent protein kinase IV (Camk4), a very important regulator in the LTD pathway. We further analyzed the influence of As on cerebellar Camk4 expression by Western blot. The quantity of Camk4 band in the group exposed to 4 ppm As2O3 significantly decreased compared to the control group, agreeing well with gene microarray results. It is indicated that the accumulated As induced learning and memory impairment and impeded the Camk4 expression. Therefore, the Camk4 may be target of As-induced neurotoxicity. Furthermore, the intervention of antioxidants taurine or vitamin C did not prevent Camk4 from down-regulation, indicating that the down-regulation of Camk4 expression by As may be via an oxidation-independent way.

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profiles of genes related to the Creb-dependent phase of cerebellar LTD were analyzed by GeneChip and Western blot. Elevation of reactive oxygen species (ROS) level is supposed to be a major cause of As neurotoxicity. In order to explore whether the differential expression of the Camk4 were resulted from the increasing As-induced oxidative stress induced, we examined also intervention of antioxidants taurine and vitamin C (Vit C) in the cerebellar tissue of mice exposed to As.

2. Materials and methods

2.1. Chemicals

As2O3, HNO3, H2O2, taurine and Vit C were purchased from Sigma Chemical Company (St. Louis, USA). When used, As2O3 was weighed and dissolved in dilute NaOH solution, and then the pH of the 40 ppm As2O3 stock solution was adjusted to 7.2. Affymetrix GeneChip Arrays (Mouse Genome 430 2.0 Array) and related kits were from Affymetrix (Santa Clara, USA).

2.2. Animals and treatment

SPF mice (age 9 wk) weighing 26.3–30.9 g were purchased from Experimental Animal Center, Dalian Medical University. The animals were maintained on a standard diet and water ad libitum. They were caged under a 12 h dark–light cycle in standard conditions of temperature (18–22 °C) and humidity (50%). These mice were randomly divided into five groups. Group 1 received drinking water alone as control. Group 2 and Group 3 received 1 ppm and 4 ppm As2O3, respectively [31,7,4]. Group 4 and Group 5 received both of 4 ppm As2O3 and 150 mg/kg taurine [10] or 45 mg/kg Vit C [21], respectively. As2O3 was given through drinking water for 60 days. Taurine and Vit C were administered by gavage twice a week. The animal experiment was performed in accordance with the Animal Guideline of Dalian Medical University and in agreement with the Ethical Committee of Dalian Medical University.

2.3. Treatment of test samples

Sixty days after As2O3 administration, cerebellar tissue sample (0.2–0.3 g, wet weight) was taken from each mouse brain by microbalance (10 samples for each group). The samples were soaked in 10 ml HNO3 (ultra-pure, Kanto chemicals) for 1 day, and then initial acid digestion were carried out on an oven for 1 h. After the HNO3 digestion, the samples were cooled at room temperature and 10 ml H2O2 (Atomic absorption spectroscopy grade, Wako chemicals) was added into the HNO3 predigested samples. These samples were further digested for 2 h, and then were concentrated up to 10 ml. Finally, sample volume was adjusted to 20 ml with Milli-Q water.

2.4. Analysis of As

ICP–MS (Agilent 7500CQE) was used for an analysis of As (M/z = 75) with chemical reaction mode. He(4.5 ml/L) was used as reaction gas. By this reaction gas mode, the concentration of As was satisfactorily measured without interference by ICP–MS. Ga (1 mg/L, M/z = 71) was used as an internal standard in this mode instead of common Y or In. The calibration range of As was from 0 to 20 μg/L.

2.5. Procedure of the Morris Water Maze test

The apparatus of Morris Water Maze consisted of a circular pool located in a test room with white walls with several cues on them. The pool was virtually divided into 4 quadrants and the platform placed at a fixed position in the centre of a quadrant. A video camera above the centre of the pool was connected to a computerized tracking system that recorded and analyzed animal behavior (Institute of Materia Medical, Chinese Academy of Medical Sciences DMS-2) and escape latency to find the hidden platform was measured by it. In the present study, mice underwent 5 trial training sessions separated by 24 h for five consecutive days. Mice in the group received 4 ppm As2O3 and control group (10 each) were conducted for their spatial learning ability using the Morris Water Maze test on the 0th, 7th, 14th, 21st, 28th, 35th, 42nd, 49th, 56th and 63rd days after As administration [18].

2.6. Affymetrix GeneChip

On the 60th day after As2O3 administration, cerebellum tissue from six mice in each group was pooled to be a sample and to use single GeneChips [24]. Total RNA was extracted from the mice cerebellum by using the Trizol reagent (Invitrogen, San Diego, CA, U.S.A.) according to the manufacture’s recommendations. The purity of total RNA was photometrically tested. RNA with a 260:280 nm ratio of 1.8 or higher was used to generate the biotinylated cRNA target for the Mouse Genome 430 2.0 Array GeneChip (Affymetrix). All of these procedures were carried out as described by Affymetrix. After hybridization, the array was washed, stained with streptavidin–phycoerythrin using the Affymetrix GeneChip Fluidics Workstation 450, and scanned on a GeneArray™ scanner (Affymetrix).

2.7. Data handling and bioinformatic analysis

After the arrays were scanned, the generated signals were determined and analyzed by Microarray Suite Version 5.0 software. Single array analyzes were used to build databases of gene expression profiles. The transcript was assigned a present call (reliably detected) if user-definable p-value was below 0.04 and an absent call (not detected) if p-value was above 0.06. The p-value between 0.04 and 0.06 means marginal. On the basis of present calls, the signal log ratio estimates the magnitude and direction of change of a transcript when two arrays are compared (experiment versus baseline). Thus, a signal log ratio of 1.0 indicates an increase of the transcript level by 2 fold and −1.0 indicates a decrease by 2 fold. A signal log ratio of zero would indicate no change. The bioinformation about genes expressed differentially was obtained by using NetAffx (www.affymetrix.com).

2.8. Western Blot

Cerebellar protein of 6 mice from control group and 1 and 4 ppm As2O3 groups respectively were homogenized in a buffer containing 10 mM Tris, 50 mM NaCl, 50 mM EDTA, 1% Triton X-100, 1 mM phenylmethyl Sulfonyl fluoride (PH = 7.5). Protein-matched samples (BCA assay) were electrophoresed (SDS–PAGE), transferred to polyvinylideene fluoride membranes, and subjected to an immunoblot with a Camk4 antibody (1:1000, Cell signal, USA). Anti-rabbit IgG, conjugated with horseradish peroxidase, was used as secondary antibody (1:5000, Sigma, St., Santa Cruz Biotechnology, Inc). The bands containing Camk4 were detected with enhanced chemiluminescence (ECL) visualized on films.

2.9. Statistical analysis

Data were presented as mean ± standard deviation (SD). All data were analyzed with SPSS 11.0 for windows. Difference in mean values between groups was tested with the one-way ANOVA and LSD test. P values less than 0.05 were considered significant.

3. Results

3.1. Influence of As on learning and memory in mice

The influence of As on motor learning and memory task was examined by Morris Water Maze test. On the 63rd day after As
administration, the mice in experimental groups exhibited longer escape latency than control mice \((p < 0.05)\) and delay in escape latency increased in a dose-dependent way (Fig. 1A). Moreover, as shown in Fig. 1B, escape latency in control group was shortened gradually during observation for 63 days. While the escape latency in the experimental group began to increase gradually from the 28th day after As administration and there were significant differences compared to controls \((p < 0.05)\).

### 3.2. Concentrations of As in the cerebellar tissue of mice

The concentrations of As in the cerebellar tissue of mice were 4.53, 20.74 and 29.97 ng/g in the control group, 1 and 4 ppm As\(_2\)O\(_3\) groups, respectively (Fig. 2). There were significant differences in concentration of As between the experimental groups and control group \((p < 0.01)\) and the concentrations of As in the experimental groups increased in a dose–response manner.

### 3.3. The expression profiles of genes related to the cerebellar LTD in mice exposed As

The critical gene expression profiles related to the Creb-dependent phase of cerebellar LTD were analyzed by GeneChip. Among 25 genes selected, we found that Camk4, Fos and Jun in cerebellar tissue of mice were significantly down-regulated in the 4 ppm As\(_2\)O\(_3\) group administration.
3.4. Influence of As on cerebellar Camk4 expression in mice exposed to As

Among the genes involved in the Creb-dependent phase of LTD, Camk4 received the most attention, since it was down-regulated by nearly 4 fold in the 4 ppm group compared to the control group, and it directly phosphorylates Creb, which then activates the transcription of Fos and Jun (the other 2 considerably down-regulated genes) in the LTD pathway. We further analyzed the influence of As on their expression in the cerebellar tissue of mice. Western blot of mice cerebellum using antibodies against Camk4 showed double bands, corresponding to the $\alpha$ and $\beta$ isoforms of Camk4 protein respectively. The quantities of Camk4 doublet bands in the 4 ppm group both decreased significantly compared to the 1 ppm and control groups, especially the $\beta$ isoform exhibited a more remarkable decrease, while the quantity of the two bands in the 1 ppm group showed no significant difference compared to the control group (Fig. 4). The Western blot results agreed well with the gene microarray results.

3.5. Influence of taurine and Vit C with the down-regulation of Camk4 by $\mathrm{As}_2\mathrm{O}_3$ in mice

We examined the expression of cerebellar Camk4 protein of mice exposed to 4 ppm $\mathrm{As}_2\mathrm{O}_3$ intervened with taurine and Vit C as antioxidants. The quantities of doublet bands with the $\alpha$ and $\beta$ isoforms of Camk4 protein decreased significantly in the groups received 4 ppm $\mathrm{As}_2\mathrm{O}_3$ alone and coadministered taurine or Vit C compared to control group ($p<0.05$), while there were no significant differences in the quantities of their bands between these 3 treated groups ($p>0.05$) (Fig. 5).

4. Discussion

As is a notorious pollutant impairing the life quality of the occupants living in the contaminated regions. Many investigations have demonstrated that As causes neurotoxicity including impairment of learning and deterioration in pattern memory in humans and animals [33,3,6]. In our previous study, As exposure resulted in damage to cerebellar neurons including Purkinje cells [22]. Kato et al [11] also observed the above same morphological changes in cerebellar tissue of mice exposed to As. These results implied that cerebellum is also a target of As-induced neurotoxicity.

Experimental studies have shown that residual As concentration is elevated in various body tissues, including the kidneys, liver, muscle, and spleen, in laboratory rodents dosed with arsenate and arsenite [32,36,34]. However, there are few studies on As accumulation in cerebellum. In our study, the concentration of As was significantly higher in the cerebellar tissue of the exposed mice than that in control group ($p<0.01$) and increased in the experimental groups in a dose-response manner. It indicated that As may accumulate in cerebellum, which was responsible for its neurotoxicological effects. Meanwhile, neurobehavioral change associated with learning and memory was examined by the Morris Water Maze test. Our results showed that the mice in the groups exposed to As exhibited longer escape latency to find the hidden platform than control group ($p<0.05$) and the escape latency increased in exposure time-dependent way, confirming the results reported by Miyagawa et al. [17]. It suggests that As intake may induce marked impairment in motor learning and memory.

LTD is a form of synaptic plasticity and plays an important role in motor learning and memory. Thus, it is an interesting question whether the poisoning of As against memory and learning is via LTD or
not. However, no relevant study has been reported up to date. In the present study, the critical gene expression profiles related to the Creb-dependent phase of cerebellar LTD were analyzed by GeneChip. Our results showed that Camk4 gene was down-regulated by more than 3 fold in the 4 ppm As2O3 group, while the expression levels of PKA, ERKs, RSKs, MSks and Mapkapk2 which can also phosphorylate Creb were not significantly disturbed. Camk4 is critical to the cerebellar LTD. It was reported that the maintenance of cerebellar LTD was abolished in mutant mice lacking Camk4[5,8].

The influence of As on Camk4 was further certified by Western blot analysis. We found that both α and β isoforms of Camk4 protein were decreased. As can inhibit Camk4 expression at both mRNA and protein levels. Maybe Camk4 is a critical target of As. Since Camk4 is a key protein in the LTD pathway, it implies that the neurotoxicity of As may be partially repressed by Camk4 gene expression.

Elevation of ROS level is supposed to be a major cause of As neurotoxicity[16]. Another study done in our lab on the influence of As over mitochondria complex II Sdh showed that As could reduce Sdhα expression and this impairment could be mitigated by administering antioxidants taurine and Vit C (under peer review). In order to explore whether the down-regulation of Camk4 mRNA was related to the oxidative stress induced by As, we examined also intervention of antioxidants taurine and Vit C in Camk4 expression in cerebellar tissue of mice exposed to As. It was found that the repression of Camk4 by As cannot be rescued by taurine or Vit C, indicating that the As-induced down-regulation of Camk4 may be through an oxidation-independent way. Above all, it is strongly suggested that subchronic exposure to As may accumulate in cerebellum, impair motor-learning and memory and repress the LTD pathway via downregulating Camk4 expression. Further investigations are under the way in our lab to clarify the definite role of Camk4 in LTD and to uncover the exact mechanism of As-induced neurotoxicity.

Conflict of interest statement

Nothing declared.

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