REDUCTION OF CaMKII EXPRESSION IN THE HIPPOCAMPUS OF RATS FROM INGESTION OF FLUORIDE AND/OR LEAD

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SUMMARY: Co-existing as environmental pollutants in certain areas of China where lead (Pb) is mined, fluoride (F) and Pb pose serious risks to the human central nervous system (CNS). Calcium/calmodulin-dependent protein kinase II (CaMKII) expression, which is involved in the process of learning and memory, has an important role in CNS functioning. Here, in order to verify whether F and/or Pb affect CaMKII expression, we determined the CaMKII expression level in the hippocampus of rats administered 150 mg sodium fluoride/L and/or 300 mg lead acetate/L in their drinking water for 30 days. Through quantitative positioning analysis by western blotting and immunofluorescence, respectively, CaMKII expression levels in the F, Pb, and F plus Pb groups were found to be significantly depressed compared with controls. Interestingly, the western blotting technique, but not the immunofluorescence results indicated greater depression in the Pb group than in either the F or the F+Pb group. Overall, these findings may be helpful to gain a better understanding of the mechanism underlying F and Pb combined neurotoxicity.

Keywords: Calcium/calmodulin protein kinase II (CaMKII); Fluoride neurotoxicity; Hippocampus; Lead neurotoxicity; Rat hippocampus.

INTRODUCTION

Epidemiological studies in China have demonstrated that children living in high fluoride (F) areas present a lower intelligence.[1,2] In this connection, there is concern that some local areas with lead (Pb) mining operations[3] have both high F and Pb pollution.[4] It is also well known that Pb as well as F is a potent neurotoxicant, but what can be said about how combined exposure to F and Pb might affect intelligence? The detailed mechanism is still unknown.

Accumulated investigations indicate that the calcium/calmodulin-dependent protein kinase II (CaMKII) is an important participant in behavioral memory formation[5] and the hippocampal synaptic function,[6] which have been primary models for the study of the cellular and molecular basis of cognition.[7] A recent report confirmed that developmental Pb^{2+} exposure caused deficits in spatial learning and a decreased CaMKII expression level.[8] Therefore, could CaMKII be a target enzyme of both F and Pb toxicity? On the basis of the above considerations, we designed the present study to investigate the effect of co-exposure of rats to F and Pb on CaMKII expression in their hippocampus.

MATERIALS AND METHODS

Animal protocol and F and/or Pb exposure: Forty 50-day-old adult Wistar albino rats weighing 180±10.0 g were obtained from the Experimental Animal Center of Shanxi Medical University. They were housed under climate-controlled conditions with a 12-hr light/dark cycle and provided with standard laboratory diets and

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ultrapure water (Milli-Q Water purification system). An acclimation period of one week for adaptation of the rats to the new animal housing was provided before initiating the experiment. All protocols were approved by the Institutional Animal Care and Use Committee of China. Rats were exposed to F and Pb as previously described.[9] They were divided randomly into the following four groups of 10 rats, all with a 1:1 male/female ratio: (1) Control group receiving ultrapure water; (2) F group receiving 150 mg NaF/L ultrapure water, (3) Pb group receiving 300 mg lead acetate ($Pb(Ac)_2$)/L ultrapure water; (4) F+Pb group receiving the 150 mg NaF plus 300 mg $Pb(Ac)_2$/L ultrapure water.

Tissue preparation: After 30 days the rats were anesthetized with 4.5% amobarbital sodium (80 mg/kg i.p.), followed by perfusion transcardially with phosphate-buffered saline (PBS). The brains were quickly removed, and the left hemispheres of the hippocampus were separated and stored at –80ºC for western blotting. The right hemispheres were fixed in 4% paraformaldehyde in PBS at 4ºC, After 16 hr, they were again rinsed with distilled water, dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin for immunofluorescence examination.

Hippocampus extraction and preparation for Western blotting: The frozen hippocampus tissues were homogenized in RIPA buffer (Sigma, USA), and the homogenates were centrifuged at 1,000 g for 5 min at 4ºC. The supernatant was then centrifuged at 12,000 g for 5 min and harvested as the protein extract. Samples containing equivalent amounts of protein were applied to 10% acrylamide denaturing gels (SDS-PAGE). The proteins were then transferred to a nitrocellulose (NC) membrane for 80 min at 120V. Membranes were blocked in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (TBS-T) containing 5% fat-free milk powder for 1 hr at room temperature and then probed with anti-CaMKII (1:500, Santa Cruz Biotechnology, USA) overnight at 4ºC. [10] After washing, NC membranes were incubated for 60 min at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in TBS-T. The immunoreactive protein on the membrane was visualized with diaminobenzidine (DAB).

Fluoimmunoassay: With a rotary microtome, right hippocampus hemispheres were cut into 5-μm sections. After deparaffination, these sections were probed with anti-CaMKII (1:500, Santa Cruz Biotechnology, USA) overnight at 4ºC and then incubated with secondary fluorescent antibody away from light for 15 min at room temperature. After PBS washing, the immunofluorescence was visualized using an upright microscope (model: BX51, OLYMPUS, Japan), and images were captured by digital camera (model: PM-C35DX, OLYMPUS, Japan).

Statistical analysis: Experimental data were expressed as mean values±SEM. With the use of SPASS 11.5 statistical software, differences between groups were evaluated by independent-samples t-test. With $p<0.05$, differences were considered to be statistically significant.

RESULTS

Western blotting for CaMKII: The effect of F and/or Pb exposure on the level of CaMKII expression in the left hippocampus hemisphere of the rats was analyzed by western blotting. As shown in Figure 1, compared with the control, the protein expression was significantly reduced by exposure of the rats to F, Pb, and F+Pb.

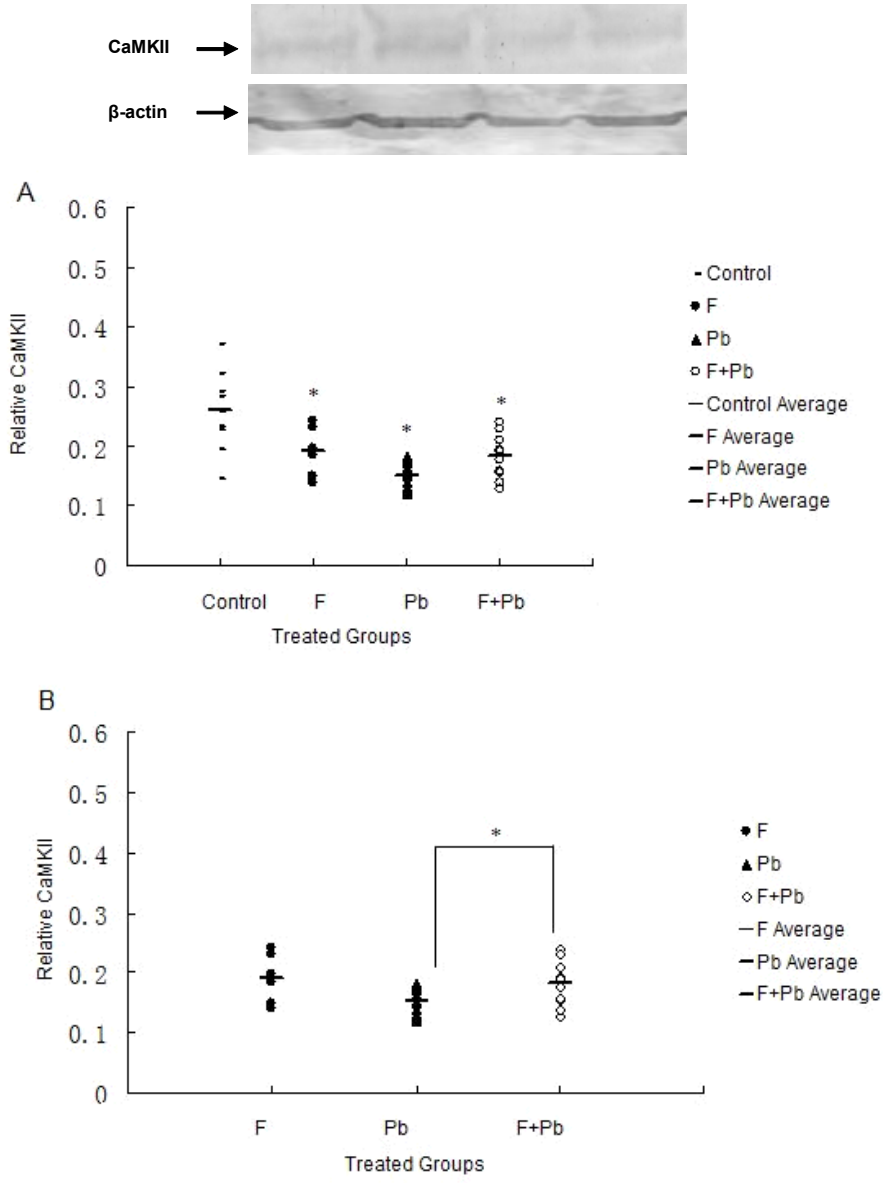

Figure 1. Western Blot: Effect of F and/or Pb on CaMKII expression in the left hemisphere hippocampus of rats (n=10 per group) following *in vivo* administration of NaF and $Pb(Ac)_2$ in the drinking water. The intensities of CaMKII are normalized to that of β-actin. (A): The protein expression of CaMKII in rat hippocampus exposed to F, Pb, and F+Pb were significantly reduced compared with control group. (B): The protein expression of CaMKII in the hippocampus of rats exposed to F+Pb presented a smaller change than with Pb only. The bar labeled with the asterisk indicates a significant difference ($p<0.05$) between the Pb and F+Pb groups by independent-sample t test.

Although not significantly different from the F group, the protein expression decrease of CaMKII in the Pb group was significantly greater than in the F+Pb group.

Immunofluorescence for CaMKII: The immunofluoresence in Figure 2 shows the CaMKII expression in the right hemisphere hippocampus of rats exposed to F and/or Pb for 30days, with the green fluorescence regarded as the protein expression of CaMKII. Compared with the control group (A), the F group (B), Pb group (C), and F+Pb group (D) exhibit weaker staining intensity, accounting for the decrease in CaMKII expression.

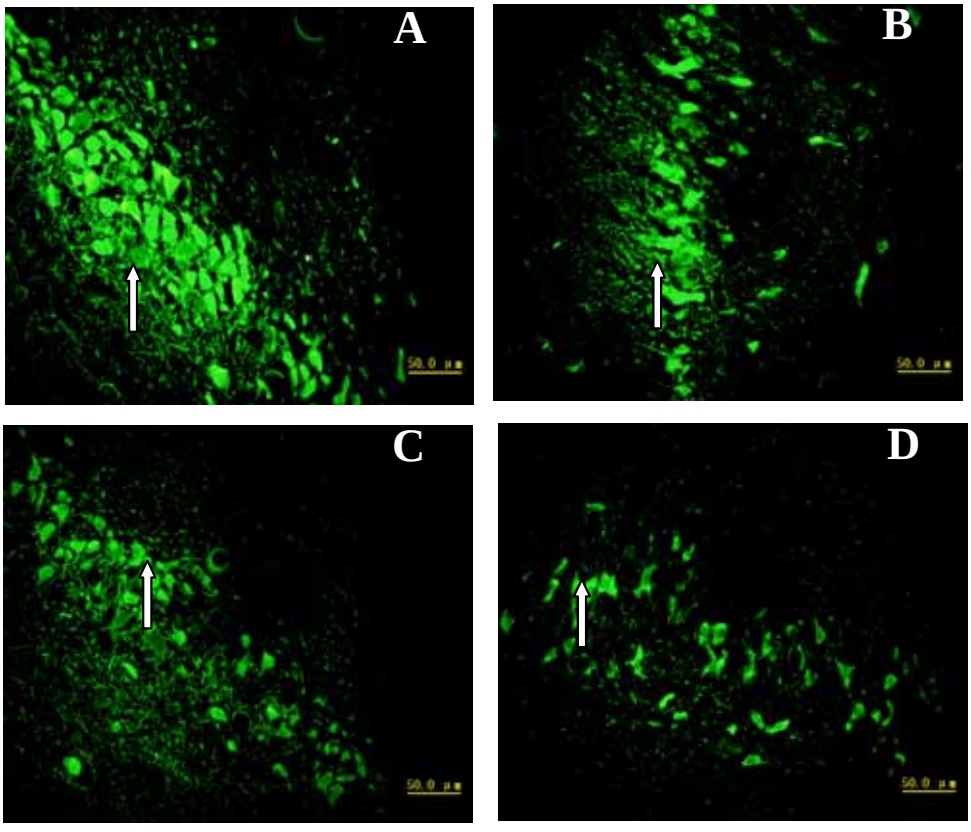

Figure 2. Immunofluorescence: Green fluorescence is regarded as the protein expression of CaMKII showing effect of F and/or Pb on CaMKII in the right hemisphere hippocampus of rats (n=10 per group) following *in vivo* administration of NaF and $Pb(Ac)_2$ in the drinking water. The arrows indicate CaMKII localization with fluorescent labeling. Compared to the control group (A), the intensity was less in the F group (B), Pb group (C), and F+Pb group (D).

DISCUSSION

Previously, in our laboratory, we have determined that combined F and Pb exposure can influence spontaneous behaviors and lower the learning and memory ability of rats before the appearance of dental lesions.[12] CaMKII is an abundant synaptic signaling enzyme in the hippocampus and frontal cortex[13] involved in the process of learning and memory.[14] Recently, Yasuda et al. found that expression of a constitutively active mutant form of CaMKII in the cortex disrupted spatial memory.[15] Thus CaMKII activity is highly associated with memory formation and

function. Moreover, Toscano et al. have reported Pb^{2+}-exposed rats have reduced CaMKII activity and expression in the hippocampus.[8] Given the above findings, we propose a novel hypothesis that CaMKII represents a valuable protein candidate for further study of combined F and Pb toxicity.

In the present study, the expression of CaMKII in both the left and right hippocampus hemisphere of rats exposed to F, Pb, and F+Pb was significantly reduced. As others have reported, decreased CaMKII expression directly affects memory formation and the induction of synaptic potentiation.[6,16] A very recent study in rats by Zhu et al. showed that increased F concentration gradually affected synaptic membrane physiological function, resulting in a significant CNS damage, including the hippocampus.[17] Meanwhile, we have found that spontaneous and conditioned-response behaviors of both adult and young rats in response to exposure to F and/or Pb were inhibited,[12,18] in support of the current work. The results with the Pb group in the present study also agree with the report by Toscano et al., which demonstrated the influence of Pb^{2+} on CaMKII activity and affinity for substrate alterations in the hippocampus of rats.[8] Another recent study showed that acute Pb exposure caused differential expression patterns of Ca^{2+}/calmodulin-dependent enzymes along the dorsoventral axis of the hippocampus.[11] Taken together, these studies indicate that CaMKII may be used as a of biological marker of neurotoxicity and for elucidating the possible mechanism by which F and Pb exert their neurotoxic effects.

Immunofluorescence staining also revealed a marked decrease in fluorescence in the F and Pb groups, indicating a low CaMKII expression. Referring to the western blotting results, these changes in CaMKII immunoreactivity seem to exhibit that combined exposure to F and Pb has a greater effect on CaMKII expression in the rat hippocampus than exposure to F or Pb separately.

On the other hand, as seen in the western blotting results in Figure 1, Pb is slightly stronger at inhibiting CaMKII expression than either F or F+Pb combined. Thus it appears that F may decrease the CaMKII inhibiting ability of Pb. Why would this occur? Perhaps the most likely reason for this is that the interaction of F^{-} and Pb^{2+} under the experimental conditions of dosage. This interaction might affect each other's absorption, metabolism, and accumulation.[19] For example, Sawan, et al. showed that F consistently increased blood Pb and the levels of Pb in calcified tissues in rats exposed to low levels of Pb intake.[19] In addition, Masters et al. found that children living in communities with water supplies fluoridated with hydrofluorosilicic acid had elevated Pb levels compared to children in nonfluoridated communities.[20]

Consistent with these findings, our studies have shown that F increases Pb levels by exposure to F and Pb.[18] Interestingly, the total Pb content may not be the same as the amount of available or "free" Pb. It appears likely that the combination of F and Pb reduces the amount of free Pb able to exert its toxic effect. Consequently, it will be worth further study on the amount of Pb biologically available during combined exposure to F and Pb. From the present results, we deduce that the CaMKII expression level in the F+Pb group is less reduced than in the Pb group

because of a decrease in available Pb and thus highlighting a role F may play in Pb neurotoxicology.

In summary, the current F and/or Pb exposure model revealed significant decreases in the expression of CaMKII in the rat hippocampus. Further studies on the reduced level of CaMKII by F and Pb hold the possibility of contributing to a more complete understanding of how they adversely affect learning and memory.

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