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Expression of c-fos in Rat Brain as a Prelude Marker of Central Nervous System Injury in Response to Methylmercury-stimulation[1]

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Objective To probe into the prelude marker of central nervous system injury in response to methyl mercury chloride (MMC) stimulation and the signal transduction molecular mechanism of injury in rat brain induced by MMC. **Methods** The expression of c-fos mRNA in brain and the expression of c-FOS protein in cortex, hippocampus and ependyma were observed using reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemical methods. The control group was injected with physiological saline of 0.9%, while the concentrations for the exposure groups were 0.05 and 0.5, 5 mg/kg MMC respectively, and the sampling times points were 20, 60, 240, 1440 min. **Results** The expression of c-FOS protein in cortex and hippocampus increased significantly, the accumulation of mercury in the brain induced by 0.05 mg/Kg MMC for 20 min had no significant difference compared with the control group. The mean value was 0.0044 mg/Kg, while the protein c-FOS expression had significant difference compared with the control group (P<0.01). More sensitive expression occurred in hippocampus and cortex, but not in ependyma. **Conclusion** The expression of c-FOS protein in cortex and hippocampus can predict the neurotoxicity of MMC in the early time, and immediately early gene (IEG) c-fos participates in the process of brain injury induced by MMC.

Key words: Methyl mercury; c-fos; Central nervous system; Early prediction

INTRODUCTION

Methylmercury is a well-documented neurotoxicant, which may cause adverse effects on the developing brain. Moreover, this compound readily passes the placental and blood-brain barrier. Methylmercury is highly toxic, and the nervous system is its principal target. In adults, its earliest exposure produces non-specific symptoms such as paresthesia, malaise, and blurred vision. When exposure is prolonged, signs appear such as concentric constriction of the visual field, deafness, dysarthria, ataxia, and coma, leading to death. The c-fos proto-oncogene is a member of the IEGs. IEGs including c-fos and c-jun in neurons are easily induced by a variety of extra cellular stimuli. Immediate-early genes (IEGs) and their protein products act as transcription factors and therefore can influence the expression of other genes[1-4]. Because of this ability, activation of IEGs within a neuron indicates that a response has been elicited by a particular stimulus. The IEG c-fos can be expressed at very low levels in the unstimulated brain, and has been used to show the activation of brain region in response to different stimulants, including many pollutants[5-7]. The objective of this study was to probe into the methyl mercury chloride (MMC) neurotoxicity and the signal transduction molecular mechanism of MMC-induced injury in rat brain.

MATERIALS AND METHODS

Animals and Experimental Treatment

Sprague-Dawley rats weighing 195-205 g purchased from Shanghai Animal Experimental Center of Chinese Sciences were housed singly and maintained at 22℃ in a 12 h light/dark cycle with free access to food and water. Rats were raised for several days before experiment. They were divided into 16 groups and each group had 5 rats (3 males, 2 females), the control group was injected with

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physiological saline of 0.9%, and 0.05, 0.5, 5 mg/Kg body weight of MMC were given to the experimental groups. The sampling time points were 20, 60, 240, 1440 min (Table 1). In exposure time, rats were anaesthetized with 10% ketamine clorhydrate (0.5 mL/100 g body weight) before perfusion via the ascending aorta with 0.1 mol/L phosphate buffered saline. The brains were dissected quickly. One part of the tissue was post-fixed in 4% paraformaldehyde solution for 24 hours, then washed in water, dehydrated and embedded in paraffin. The tissue was cut into 5 μm thick sections. Five sections were used to examine the c-FOS protein expression. The other part of the tissue was rapidly removed and immediately frozen in liquid-nitrogen and stored at –70℃ until assay.

TABLE 1

Groups of Experiment Rats

Time \ Dose	0 (D1)	0.05 mg/kg (D2)	0.5 mg/kg (D3)	5 mg/kg (D4)
20 min (T1)	D1T1	D2T1	D3T1	D4T1
60 min (T2)	D1T2	D2T2	D3T2	D4T2
240 min (T3)	D1T3	D2T3	D3T3	D4T3
1440 min (T4)	D1T4	D2T4	D3T4	D4T4

Reverse Transcriptase Polymerase Chain Reaction

The tissue sections were placed into a suitably sized vessel for homogenization. Total RNA was isolated using the RNeasy protocol (Qiagen, Germany). The concentration of RNA in each sample was determined by photo spectroscopy. An aliquot of total RNA (0.5 μg) from each sample was used for cDNA synthesis. RT-PCR was performed using Qiagen onestep RT-PCR kit (Qiagen, Germany) on a Touchgene Gradient PCR system (Touchgene Gradien, England). PCR conditions included denaturing at 94℃ for 30 s, annealing at 60℃ for 50 s, and extension at 72℃ for 60 s, for 30 cycles. The sequences of primers (Qiagen, Germany) used for analysis are listed in Table 2. PCR products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide using a DNA molecular weight marker for comparison. After electrophoresis, the expression of c-fos and GADPH mRNA was indicated by measuring the density of the respective specific bands using the electrophoresis documentation and analysis system along with the Tanon (Shanghai, China) image analysis Software program (Ver. 3.61). The amount of mRNA expression was determined by dividing the densito metry value of the mRNA RT-PCR product by that of the GAPDH product and the control was set as 1.0[8-10].

TABLE 2

Nucleotide Sequence and Size of the Expected PCR Products for Oligonucleotide Primers Used for RT-PCR

Gene	Sequence	PCR Product (bp)
GAPDH	5′-ATGGAAGAAGAAATCGCCGC-3′ 5′-ACACGCAGCTCGTTGTAGAA-3′	287
c-fos	5′- ATGATGTTCTCGGGTTTCAA -3′ 5′- TGACATGGTCTTCACCACTC -3′	348

Immunocytochemistry

Five μm-thick paraffin sections were deparafinized and dehydrated, then subjected to an immunohistochemical staining procedure on microscopic slides. The results were visualized using the avidin-biotin-peroxidase method. In brief, the sections were incubated for 20 min in 1-3 drops of serum block, then the serum was aspirated from slides, and 1-3 drops of pre-diluted primary antibody (Santa Cruz Biotechnology, santa Cruz) was immediately added. After that, the sections were incubated for another 2 h, rinsed with PBS, then washed in PBS twice for 2 minutes each, excess liquid was aspirated from slides. The sections were incubated for 30 minutes in 1-3 drops of biotinylated secondary antibody (Santa Cruz Biotechnology, santa Cruz), and washed twice with PBS. The sections were incubated for another 30 minutes in 1-3 drops of HRP-streptavidin complex washed with PBS, and 1-3 drops HRP substrate mixture was added. The sections were developed for 30 seconds-10 minutes, or until the appearance of desired stain intensity, rinsed with demonized H_2O and transferred to a demonized H_2O for 2 minutes on a stir plate, counterstained, dehydrated and mounted on slides[11-13]. Control slides were tested for the specificity of the primary antibody. These slides were included in every step of the staining protocol and 10% Trition was applied. Analysis of these slides revealed virtually no staining. Labeled sections were examined by bright field microscopy throughout the rostra-caudal extent of the striatum from each animal. Digital bright-field images were obtained with a video camera attached

to an item microscope and analyzed with Tanon (Shanghai, China) image analysis software program (Ver. 3.61). The program was used to measure the number of labeled cells.

Statistical analysis[14-16] of data included the mean and SD. Statistical comparisons between the exposure and control groups were made with paired Student's *t*-test. ANOVA test was also applied for each group (five different treatments) in every location analyzed. $P<0.05$ was considered statistically significant.

RESULTS

Mercury Concentrations in Rat Brain

Total mercury (T-Hg) in brain of rats was detected with the AMA-254 liquid/solid mercury analyzer (Milestone, Italy). The mercury concentrations in brains are listed in Table 3. The concentrations of mercury in brain of rats exposed to 0.05 mg/Kg MMC for 20 min were not significantly different from those exposed to physiological saline, indicating that the mercury did not accumulate in the brain. But in the 0.5 mg/Kg and 5 mg/Kg groups, the content of mercury in the brain was significantly different from that in the control group ($P<0.01$), showing that mercury obviously accumulated in the brain and increased with increase of the dose. After exposure for 60 min, the content of mercury in the brain increased obviously with increase of the dose.

c-fos mRNA Expression

Fig. 1A shows that the expression of c-fos mRNA in the control group (D1 group) was so weak that the light strip could not be seen while that of the other groups (Figs. 1 B, C, and D) had different extents of light strip. The relative expression level of c-fos mRNA is shown in Fig. 2.

TABLE 3
Total Mercury Concentration in Rat Brain (n=5, ng/kg)

Time \ Dose	0 (D1)	0.05 mg/kg (D2)	0.5 mg/kg (D3)	5 mg/kg (D4)
20 min (T1)	4.4±0.2	4.4±0.9	17.3±2.3**	526.6±110.4**
60 min (T2)	4.1±0.5	6.4±1.8*	30.2±4.4**	472.5±111.5**
240 min (T3)	3.7±1.0	14.7±3.9**	119.6±28.6**	1284.0±140.0**
1440 min (T4)	4.4±0.9	10.0±1.0**	92.9±11.9**	1140.2±212.5**

Note. *$P>0.5$, **$P>0.01$. Compared with the control group.

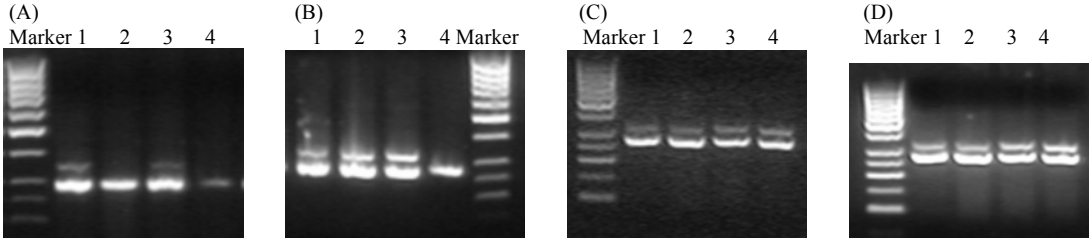

FIG. 1. RT-PCR products of c-fos and GAPDH on a 2.0% agarose gel stained with ethidium bromide. (A) Rats exposed to physiological saline for 20, 60, 240 and1440 min, (lanes 1, 2, 3, and 4). (B) Rats exposed to 0.05 mg/Kg MMC for 20, 60, 240, and 1440 min (lanes 1, 2, 3, 4). (C) Rats exposed to 0.5 mg/Kg MMC for 20, 60, 240, and 1440 min (lanes 1, 2, 3, and 4). (D) Rats exposed to 5 mg/Kg MMC for 20, 60, 240, and 1440 (lanes 1, 2, 3, and 4). DNA marker (Sangon, China) sizes were 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, and 750-bp from bottom to top.

Fig. 2A shows that the c-fos mRNA expression in brain exposed to 0.05 mg/Kg MMC was not significantly different from that in the control group ($P>0.05$). The other exposure groups had a significant difference ($P<0.05$ or $P<0.01$), and there were also significant difference between the neighboring exposure groups. Fig. 2B shows that the c-fos mRNA expression in brains exposed for 60 min increased at the beginning and decreased later with the increased concentration. The strongest c-fos mRNA expression was induced by 0.5 mg/Kg MMC. Compared with the control group, the exposure groups had a significant difference ($P<0.01$). There was no significant difference between the neighboring exposure groups. Fig. 2C shows that the c-fos mRNA expression in brains exposed for 240 min increased with the increased concentration. There was no significant difference between the neighboring exposure groups. Fig. 2D shows the c-fos mRNA expression in brains exposed to 0.05 mg/Kg MMC for 1440 min was not significantly different from that in the control group. There was a significant difference between the neighboring exposure groups ($P<0.01$).

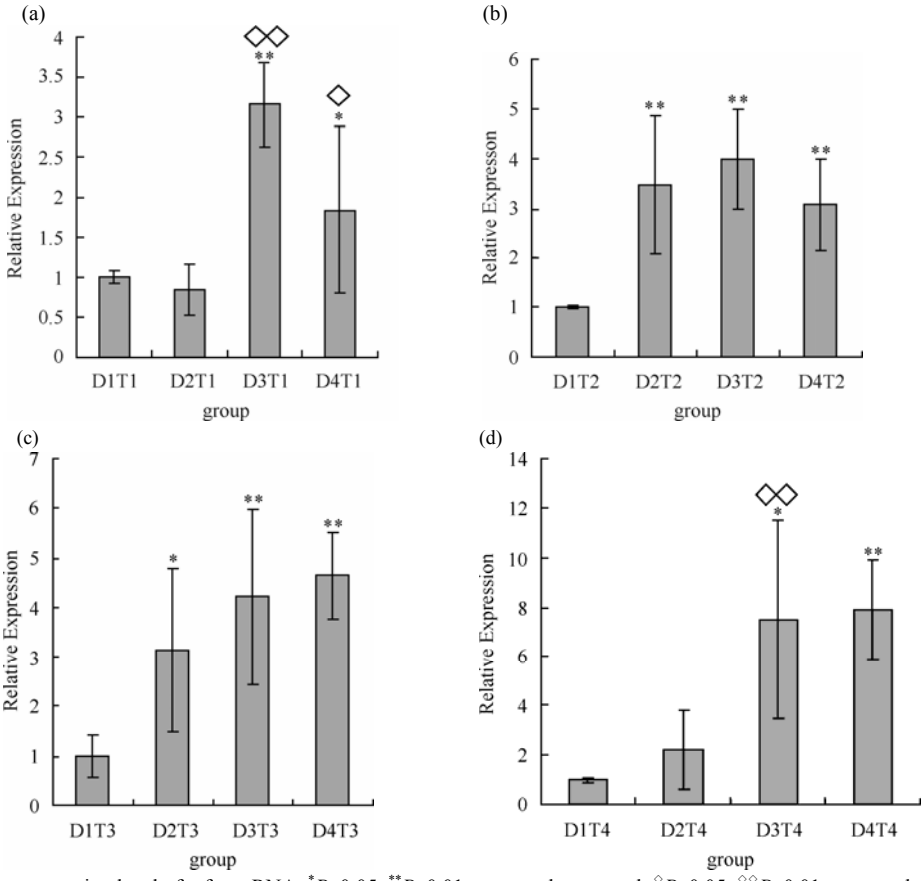

FIG. 2. Relative expression level of c-fos mRNA. $^{*}P<0.05$, $^{**}P<0.01$ compared to control. $^{\diamond}P<0.05$, $^{\diamond\diamond}P<0.01$ compared to exposure for 20, 60, 240, and 1440 min.

c-FOS Protein Expression in Cortex and Hippocampus

Fig. 3 shows the distribution of c-FOS positive cells in the cortex. Very few c-FOS positive cells could be detected in cortex of rats exposed to physiological saline (Fig. 3A), 0.5 mg/Kg MMC produced a significant number of c-FOS positive cells compared with others groups (Fig. 3C), c-FOS positive cells were observed in the cortex exposed to 0.05 mg/Kg and 5 mg/Kg MMC (Figs. 3B, D). The quantitative data are shown in Table 4. Some c-FOS positive cells were found in ependyma of the exposure groups and the number of positive cells in the ependyma was difficult to count. So we only calculated the number of labeled cells in hippocampus and cortex.

The c-FOS expression in the control group was very low. There was a significant difference between the exposures for 1440 min and 20 min ($P<0.01$). The expression decreased at 60 min and resumed after 1440 min. There was no significant difference between the neighboring groups. The expression of c-FOS in the exposure groups was significantly different from that in the control groups. Mercury induced significant c-FOS protein expression in hippocampus and cortex.

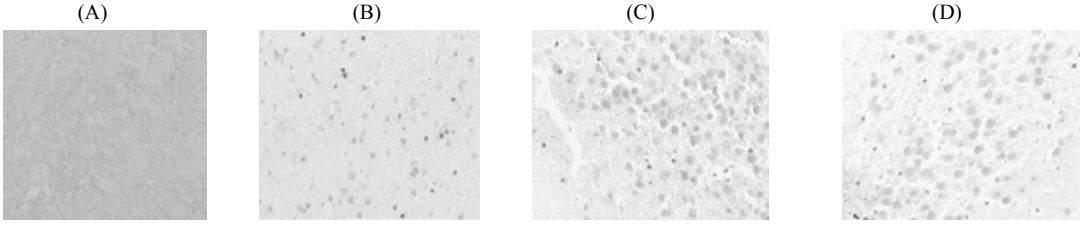

FIG. 3. Distribution of c-FOS positive cells in the cortex of rats. (A) Exposed to physiological saline for 20 min. (B) Exposed to 0.05 mg/Kg MMC for 20 min. (C) Exposed to 0.5 mg/Kg MMC for 20 min. (D) Exposed to 5 mg/Kg MMC for 20 min.

TABLE 4

Number of c-FOS-positive Cells in Rat Hippocampus and Cortex (n=5)

Time \ Dose	0 (D1)	0.05 mg/kg (D2)	0.5 mg/kg (D3)	5 mg/kg (D4)
20 min Cortex	9.8±2.4	41.4±11.3**	52±5.2**	49.4±12.1**
T1 Hippocampus	13.4±6.6	26.2±7.9*	56.8±8.1**	72.4±13.4**
60 min Cortex	10±5.5	46.4±10.5**	56.8±13.8**	54.2±14.7**
T2 Hippocampus	8±2.3	38.6±11.4**	63.2±15.8**	64±11.3**
240 min Cortex	6.4±2.5	58.8±4.3**	81±7.3**	73.6±7.1**
T3 Hippocampus	7.4±2.4	57±18.3**	67±15.6**	58.6±10.9**
1440 min Cortex	4.6±2.0	47.2±9.4**	57.4±19.4**	53.2±15.8**
T4 Hippocampus	8.8±3.8	34.2±11.1**	51.4±10.6**	30.4±9.7**

Note. *P<0.05 compared to control, **P<0.01 compared to control.

DISCUSSION

The content of mercury accumulated in the brain changed after injecting MMC (Table 3). Before exposure for 240 min, the content increased and reached the peak at 240 min. After exposure for 1440 min, the content in 0.05 mg/Kg group decreased obviously and the other two dose groups showed the tendency to decrease. This may be caused by the metabolites of MMC in the body. In the beginning, the speed of MMC accumulation in the brain was faster than that of MMC metabolism in the body and the content of mercury in the brain increased. Then, the speed of metabolism was faster than that of accumulation and the content of mercury in the brain decreased.

c-fos is an immediate-early gene that has been implicated in the conversion of short-term stimuli into long-term changes in the phenotype of cells by regulating gene expression, and is therefore used as a functional marker of neural activation[17-19]. In the normal condition, c-fos gene has low expression in the nerve cells and takes part in the cell growth, cell polarization and message transfer. The outer stimulants such as pollutants, cold or lack of blood in the brain could induce the express of c-fos. The immediately-early genes of the activator protein-1 (AP-1) family (c-fos, junB, c-jun) encode for transcription factors, activating promoters of target genes involved in the regulation of neuronal function, adaptive processes or apoptotic cell death[20-21]. Gene expression changes were studied for 20, 60, 240, and 1440 min of exposure to 0.05, 0.5, and 5 mg/kg MMC (Fig. 2, Table 4). Enhanced gene expression of c-fos was observed in response to methylmercury under the above-mentioned conditions. After 20 min of exposure, there was a concentration-dependent response in c-fos gene expression with no further increase in gene expression following 240 and 1440 min of exposure. Low concentration of methylmercury caused a modest increase at 60 min, whereas 0.5, 5 mg/kg methylmercury produced a significant increase at 20 min. The significance of the transient increase in c-fos shortly after exposure to low concentration of MeHg is unknown. The c-FOS expression in the control group was significantly different between the exposures for 1440 min and 20 min (P<0.01, Table 4), showing that the expression of c-FOS in cortex and hippocampus of the control group is stable until 1440 min. This might be due to the fact that the temporal stress effect induces the expression of c-FOS during the injection.

The expression of c-fos in brain exposed to 0.5 mg/Kg MMC for 20 min increased significantly compared with that in the control group (P<0.01), while its protein c-FOS exposed to 0.05 mg/Kg MMC for 20 min was significantly different from that in the control group (P<0.01). In the study of MMC accumulation, we could see that after exposed to 0.05 mg/Kg MMC for 20 min, the accumulation of mercury in the brain was not significantly different from that in the control group, and the mean was 0.0044 mg/Kg; while the c-FOS protein expression was significantly different from that in the control group (P<0.01). We also found that after exposure to MMC, the expression of protein c-FOS in the hippocampus and cortex reached the peak before exposure for 240 min while the accumulation of mercury reached the peak after exposure for 240 min (Fig. 2, Tables 3 and 4), suggesting that the expression of c-FOS is faster than the accumulation of mercury. c-FOS protein begins to express before the mercury arrives at the brain. The nerve toxicity of mercury is a message transfer process. So the expression of c-FOS protein may be a prelude to neurotoxicity of MMC, and c-fos induction takes place much earlier than apparent cell apoptosis caused by MMC. By comparing the expression intensity of c-FOS protein in different regions of hippocampus and cortex as well as ependyma, we found that more sensitive expression occurred in hippocampus and cortex, but not in ependyma, demonstrating that hippocampus and cortex are the selected regions for the early warning against mercury toxicity.

To understand the neurotoxic mechanism and the signal transduction mechanism of mercury, we studied the effects of mercury on some neurotransmitters and oxidative damage of rats. We found that some neurotransmitters were highly sensitive to the MMC. After exposed to low dose of MMC (<0.5 mg/Kg) for a short time (20 min), acetylcholine (Ach) and NO changed obviously. We also investigated the effect of MMC on the free radicals, and found that after exposed to MMC, the content of MDA was related with the dose of MMC. When exposed to low dose of MMC, the content of MMC did not increase obviously, the content of MMC increased at first and decreased thereafter when exposed to high does. The vigor of SOD and GSH-px in the liver and kidney decreased at the beginning and increased later, suggesting that the damnification of free radicals induced by MMC is a dynamic process. When exposed to a low dose of MMC, the damage could recover quickly. To screen the sensitive genes in the expression maps of the neuotoxicity by MMC, the differently expressed genes between the control and exposure groups were observed by the cDNA microarray. We found that gene chip could be used to analyze the gene expression patterns in the neuotoxicity of MMC, and to screen the sensitively expressed genes[22-23].

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