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Effect of lead on ERK activity and the protective function of bFGF in rat primary culture astroglia*

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Abstract: Objective: To observe the effects of lead on levels of phosphorylated extracellular signal regulated kinase (p-ERK) in the cytoplasm of primary cultures of rat astroglial cells and the possible protective effect of basic fibroblast growth factor (bFGF) on lead-induced effects. Methods: The primary astroglia cells from 1~6 d old Wistar rats were cultured. The cells pretreated with the MEK1 (mitogen-activated protein kinase kinase 1) inhibitor PD98059 and bFGF, respectively, were exposed to Pb acetate of different concentrations for different times. Western blotting and reverse transcription polymerase chain reaction (RT-PCR) methods were used to detect the protein and mRNA expressions of ERK. Results: mRNA expression for ERK peaked 15 min after initiation of lead exposure (P<0.05) and protein expression of p-ERK peaked at 30 min (P<0.05). ERK mRNA levels and p-ERK protein levels returned to baseline after 60 and 120 min of lead exposure, respectively (P>0.05). The increase in p-ERK levels in lead-treated cells could be inhibited by PD098059. Activation of ERK in the cells by lead was prevented by pretreatment with bFGF. Total ERK protein levels did not change under the same experimental conditions (P>0.05). Conclusion: Low-level lead exposure resulted in transient activation of ERK through the MEK pathway, which then returned to basal levels in the continued presence of lead. Exogenous bFGF protected ERK signaling components in astroglia from lead poisoning.

Key words: Lead, Astroglia, Extracellular signal regulated kinase (ERK), Basic fibroblast growth factor (bFGF)
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INTRODUCTION

Neurotoxic effects from exposure to low levels of Pb in the environment are a problem of significant magnitude in the whole world, especially in children and infants (Qian *et al*., 2000; Zhu *et al*., 2005; Suresh *et al*., 2006). Though numerous studies have shown that Pb could accumulate in the brain when its concentration in the blood is elevated (Bradbury and Deane, 1993), the mechanisms of Pb deposition in brain are not yet fully known. The cell type most responsible for Pb accumulation and storage in the central nervous system (CNS) is the astroglia (Sierra and Tiffany-Castiglioni, 1991). In brain, astroglia could accumulate 24 times more lead than mature neural cells (Lindahl *et al*., 1999) and in cell culture, astroglia has the capacity to concentrate Pb up to four times of magnitude higher than extracellular levels (Tiffany-Castiglioni, 1993). ERK1/2 (extracellular signal regulated kinase 1/2) is an important element on the forming process of long-term potentiation (LTP) (Adams and Sweatt, 2002). Pb could affect LTP by disturbing the expression of ERK and other signal molecules (Kanterewicz *et al*., 2000; Lin *et al*., 2003). Basic fibroblast growth factor (bFGF) is one of neurotrophic factors which could protect the neural cells from the damage of poison (Exkenstien, 1994; Neben *et al*., 2001). In order to investigate the possible protective effect of bFGF on the primarily cultured astroglia, we observed the mRNA and protein expression of ERK in the astroglia treated with bFGF and Pb acetate.

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MATERIALS AND METHODS

Materials

Healthy Wistar rats (1~6 d, provided by Animal Experimental Center, China Medical University, Shenyang, China), glial fibrillary acidic protein (GFAP) antibody and streptavi biotin-peroxidase complex (SABC) kit (Boster Biotechnology Co. Ltd., China), total ERK (t-ERK) and phosphorylated ERK (p-ERK) antibodies (Promega Biotechnology Co. Ltd., USA), bFGF (Beijing SL Pharmaceutical Co. Ltd., Beijing, China).

Methods

1. Cell culture and identification

The hippocampus and cortex of Wistar rats were taken out and digested in Ca^{2+}- and Mg^{2+}-free Hanks' balanced saline solution containing 0.125% trypsin. Cells were dissociated and plated into poly-L-lysine solution-coated plastic culture dishes and routinely cultured at 37 °C under 5% CO_2 with a mixture (1:1, v/v) of Dulbeccco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS). Thirty to forty-five minutes later, the unattached cells (i.e. astroglia cells) were transferred to another plastic dish with the same medium. When they grew to 80%~90% confluence, cells were incubated in 3 g/L H_2O_2 for 30 min, and then blocked with goat serum for 10 min. Then, cells were incubated with GFAP antibody, goat anti-rabbit IgG, and SABC reagent for 2 h, 20 min, and 20 min at 37 °C, respectively. Next, the cells were stained with diamino benzidine (DAB) for 20 min. PBS (0.01 mol/L) was used to fully rinse the cells for 5 min×3 times between steps. Hematoxylin was used to stain nuclei. Finally, the cells were routinely dehydrated, cleared and mounted on glass slides. The control sections were incubated with PBS to rule out false positive results.

2. Cell experimental treatments

Pb acetate was added to the same FBS-supplemented DMEM/F-12 medium at final concentrations of 0.2, 1.0 and 10 μmol/L. Cells were exposed to Pb acetate of different concentrations for 30 min or 1.0 μmol/L Pb acetate for 0, 5, 15, 30, 60 or 120 min. For inhibitor and bFGF groups, cells were pretreated with 100 μmol/L MAPK (mitogen-activated protein kinase) kinase 1 (MEK1) inhibitor PD98059 or 40 ng/L bFGF for 1 h respectively, and then exposed to 1.0 μmol/L Pb acetate for 0, 5, 15, 30, 60 or 120 min.

3. RT-PCR analysis

Total RNA was extracted with Trizol extraction liquid and reverse transcribed to synthesize cDNA (42 °C for 30 min, 99 °C for 5 min, 5 °C for 5 min). An ERK2 primer (sense: TGTTGTCCTCCCTCCTC; antisense: CGCCTTCTCCGATGTAC; product sizes: 343 bp) was incubated with the internal control β-actin primer (sense: AGACCTCTATGCCAACACAGTG CTG; antisense: TCATCGTACTCCTGCTTGCTG CTGA; product sizes: 218 bp) under the following conditions: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. After 30 cycles, desired DNA products were obtained and identified by 1.5% agarose gel electrophoresis with a PCR marker as a molecular weight comparison. The densities of the ERK2 band and the internal control β-actin band were determined by DNA scanner. The value of the density ratio (ERK2/β-actin) represented the level of each subunit mRNA.

4. Western blot analysis

The harvested cells were ruptured with a Sonifer Cell Disruptor and centrifuged. The supernate (i.e. total protein) was separated on a 12% acrylamide SDS-PAGE gel and electroblotted to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) free-fat milk and then incubated with a monoclonal antibody to p-ERK (rat origin, 1:400) and a polyclonal antibody to t-ERK (rabbit origin, 1:400) overnight at 4 °C, respectively, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Membranes were exposed to the electrochemiluminescence (ECL) reagents and then to the X-ray film. Chemiluminescent reagent 5500 and Scion image software were used to detect results for expressions of protein. The value of density ratio (p-ERK/β-actin) represented the level of each subunit protein.

Data analysis

Measurements were expressed as mean±*SD* and statistical analysis was performed by one-way ANOVA test with SPSS 13.0 software. $P<0.05$ was regarded as a significant difference. Each experiment was repeated three times, and the value of three samples averaged was compared among groups.

RESULTS

Qualitative analysis of the experimental astroglia

By immunohistochemical staining and observing under the microscope, more than 95% cells were stained positively for GFAP as indicated by the brown DAB reaction product in the cytoplasm, indicating that they were astroglia. The nuclei were stained blue (Fig.1).

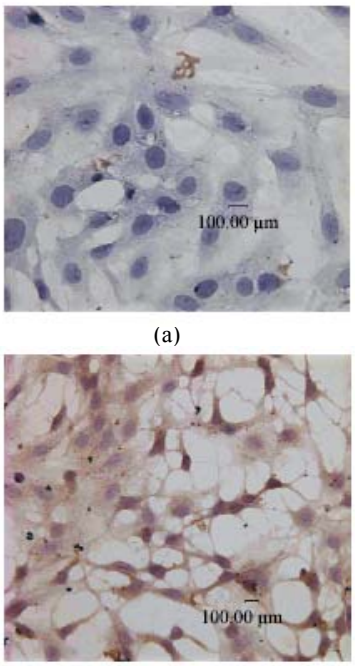

Fig.1 Immunohistochemical test for cell purity. (a) Negative control; (b) GFAP immunohistochemical stain
Cells were incubated with GFAP antibody (PBS was used as substitute of GFAP in negative control) and observed under the microscope. Almost all cells were stained with brown in the cytoplasm and blue in the karyon (cells were not stained with brown in the cytoplasm in negative control)

mRNA expression of ERK2

The result of the agrose gel electrophoresis is shown in Figs.2 and 3.

Compared with that of the control, the level of ERK2 mRNA expression began to increase after 5 min exposure to Pb and peaked at 15 min ($P<0.05$) (Table 1), and then gradually returned to the basal level. No statistically significant differences were found between the control and the groups pretreated with bFGF prior to Pb treatment ($P>0.05$) (Table 1).

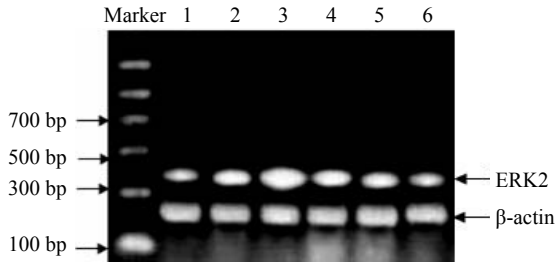

Fig.2 ERK2 and β-actin simultaneous gel electrophoresis of lead exposure group
1: Blank control; 2~6: 1.0 μmol/L Pb acetate treated for 5, 15, 30, 60 or 120 min. Compared with that of the control, the mRNA expression of ERK2 began to increase at beginning 5 min after the initiation of lead exposure, peaking at 15 min and returning to the basal level thereafter. The experiment was conducted 3 times and a representative gel is shown

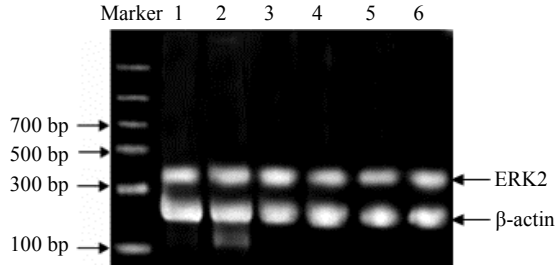

Fig.3 ERK2 and β-actin simultaneous gel electrophoresis of bFGF pretreated group
1~6: 1.0 μmol/L Pb acetate treated for 0, 5, 15, 30, 60 or 120 min after bFGF incubation for 1 h. Compared with the control, the level of ERK2 mRNA expression had no significant change. The experiment was conducted 3 times and a representative gel is shown

Table 1 Comparison of ERK2 mRNA expression in astroglial cultures exposed to 1.0 μmol/L lead, with and without bFGF pretreatment ($\bar{\chi} \pm s$, n=3)

Time (min)	ERK2/β-actin	
	Pb	bFGF+Pb▲
0 (control)	0.915±0.006	0.963±0.004
5	1.052±0.004*	0.964±0.004
15	1.108±0.006*	0.964±0.004
30	1.061±0.003*	0.964±0.003
60	0.940±0.007	0.963±0.007
120	0.926±0.007	0.964±0.003

▲ Cells were treated with 1.0 μmol/L Pb acetate for 0, 5, 15, 30, 60 or 120 min after bFGF incubation for 1 h; * Compared with the control in the respective groups, $P<0.05$

Protein expression of t-ERK and p-ERK

The results of the SDS-PAGE gel electrophoresis are shown in Figs.4~7.

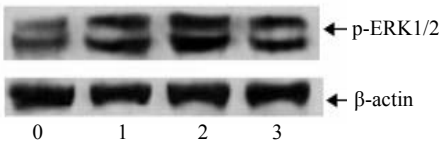

Fig.4 p-ERK and β-actin simultaneous SDS-PAGE gel electrophoresis of groups exposed to different concentrations of lead for 30 min

0: Blank control; 1~3: Concentrations of Pb acetate were 0.2, 1.0 and 10 μmol/L in order. Compared with that of the control, the results showed an inverted U shaped curve. The experiment was conducted 3 times and a representative gel is shown

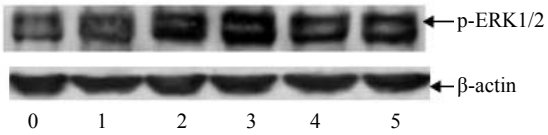

Fig.5 p-ERK and β-actin simultaneous SDS-PAGE gel electrophoresis of groups exposed to 1.0 μmol/L lead for various time

0: Blank control; 1~5: 1.0 μmol/L Pb acetate treated for 5, 15, 30, 60 or 120 min respectively. Compared with that of the control, the level of p-ERK protein expression began to increase from the 5 min point to the peak at 30 min, and then came back to the normal level. The experiment was conducted 3 times and a representative gel is shown

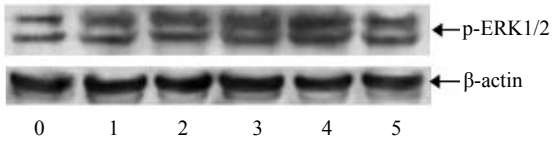

Fig.6 p-ERK and β-actin simultaneous SDS-PAGE gel electrophoresis for PD98059 pretreated group

0~5: 1.0 μmol/L Pb acetate treated for 0, 5, 15, 30, 60 or 120 min after 100 μmol/L PD98059 incubation for 1 h. PD98059 prevented the lead-induced increase of p-ERK protein. The experiment was conducted 3 times and a representative gel is shown

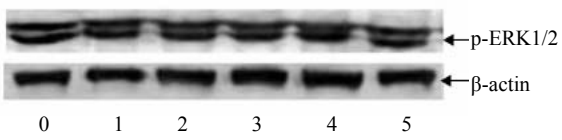

Fig.7 p-ERK and β-actin simultaneous SDS-PAGE gel electrophoresis for bFGF pretreated group

0~5: 1.0 μmol/L Pb acetate treated for 0, 5, 15, 30, 60 or 120 min after 40 ng/L bFGF incubation for 1 h. bFGF could restrain the increase of lead-induced p-ERK protein. The experiment was conducted 3 times and a representative gel is shown

After incubation with 0.2, 1.0 and 10 μmol/L Pb acetate for 30 min, the expression of p-ERK was higher than that of the control group ($P<0.05$) (Table 2). After 5, 15, 30, 60 and 120 min incubation in 1.0 μmol/L Pb acetate, the expression of p-ERK was gradually increased, peaking at 30 min ($P<0.05$) (Table 3). In the PD98059 pretreatment group, ERK activity was unchanged ($P>0.05$) (Table 3). In the bFGF pretreatment group, bFGF and Pb acetate increased the expression of p-ERK protein respectively, but bFGF prevented increase of lead-induced p-ERK protein. Compared with that of the blank control, the expression of p-ERK showed no change ($P>0.05$) (Table 3). The levels of t-ERK showed no significant changes under different experimental conditions ($P>0.05$).

Table 2 Comparison of p-ERK protein expression after exposure to different Pb acetate concentrations ($\bar{\chi} \pm s$, n=3)

Pb acetate (μmol/L)	p-ERK/β-actin
0 (control)	0.777±0.003
0.2	0.934±0.004*
1.0	1.324±0.003*
10	0.903±0.004*

* Compared with the control, $P<0.05$

Table 3 Comparison of p-ERK protein expression in 1.0 μmol/L lead-exposed groups after PD98059 or bFGF pretreatment ($\bar{\chi} \pm s$, n=3)

Time (min)	p-ERK/β-actin		
	Pb	PD98059+Pb▲	bFGF+Pb▲
0 (control)	0.776±0.003	0.717±0.004	0.779±0.001
5	0.780±0.002	0.765±0.005*	0.775±0.001
15	1.067±0.002*	0.765±0.004*	0.776±0.001
30	1.405±0.004*	0.766±0.003*	0.778±0.002
60	1.073±0.002*	0.763±0.004*	0.777±0.002
120	0.781±0.003	0.768±0.002*	0.776±0.002

▲ Cells were treated with 1.0 μmol/L Pb acetate for 0, 5, 15, 30, 60 or 120 min after PD98059 or bFGF incubation for 1 h; * Compared with the control in the respective groups, $P<0.05$

DISCUSSIONS AND CONCLUSION

Lead is a widespread environmental toxicant whose developmental neurotoxicity remains a major medical issue. There is growing evidence that lead can directly alter cellular physiology at multiple levels which include interference with ion channels and

activation of second messengers, in particular calcium-dependent messengers, which ultimately affect transcription factors and gene expression (Mir *et al*., 2000; Cui *et al*., 2005). In addition, numerous studies have shown that astroglia are the main site of Pb deposition in the CNS (Sierra and Tiffany-Castiglioni, 1991; Tiffany-Castiglioni, 1993). This report addresses the change of selected signal molecules in astroglia exposed to lead in primary culture.

ERK1/2 is an important element on the forming process of LTP which is regarded as the accepted mechanism for learning and memory function (Kanterewicz *et al*., 2000; Adams and Sweatt, 2002). Activation of mitogen-activated protein kinases (MAPKs) is regulated through a three-kinase complex composed of a MAPK, a MAPK kinase (MEK) and a MEK kinase (MEKK) (Kolch, 2000; Nebreda and Porras, 2000; Chang and Karin, 2001). These MAPK complexes are connected to the cell surface receptor and activated through interaction with a family of small GTPases and MEKK kinases. ERK1/2 exemplifies one class of MAPK that undergoes activation by a range of stimuli including growth factors, cytokines, cell adhesion, tumor-promoting phorbol esters and oncogenes (Whitmarsh and Davis, 2000). It is well known that ERK1/2 activation is necessary for cell growth because it phosphorylates and activates numerous substrates involved in nucleotide synthesis, gene transcription, protein synthesis and cell cycle progression (Dhandapani *et al*., 2007).

bFGF, a pleiotropic cytokine, is important for embryogenesis, angiogenesis, wound healing, and mesodermal development. Due to its stimulatory capacity on the formation of new blood vessels, bFGF is suggested to play a major role in growth and dissemination of tumors (Basilico and Moscatelli, 1992; Ensoli *et al*., 1994; Spence *et al*., 2007). Many studies have suggested that bFGF is one of neurotrophic factors which could protect neural cells from toxicant-induced damage.

The abnormal expression of p-ERK may affect LTP and then disturb the functions of learning and memory. PD98059 is the special inhibitor of MEK1, which is upstream kinase of ERK. Our results demonstrated PD98059 could prevent the activation of p-ERK induced by Pb which indicated that low-level lead exposure of primary rat astroglial cultures resulted in transient stimulation of ERK mRNA levels and p-ERK protein levels through the MEK pathway. This stimulation was followed by recovery to basal levels, even in the continued presence of lead. When cells were pretreated with 40 ng/L bFGF for 1 h and then exposed to 1.0 μmol/L Pb acetate for up to 2 h, the lead-induced increase of p-ERK levels was prevented. Thus bFGF protected astroglia culture from this effect of Pb on intracellular signaling. The maintenance of normal activity of ERK is essential and necessary for LTP and its function in learning and memory. Our results show that exogenous bFGF can protect ERK activity against the influence of lead. Therefore, bFGF may be a neurotrophic and protective factor modulated by MEK1/ERK signal pathway.

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