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Effects of Chronic Lead Exposure on Glutamate Release and Uptake in Cerebellar Cells of Rat Pups

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Changes in the release and uptake of glutamate in cerebellar granule and glial cells of offspring of lead-exposed mothers were determined. In cultured cerebellar granule cells exposed to lead for 5 days, glutamate release was less influenced upon N-methyl-D-aspartate (NMDA) stimulation than that in the control. Although the NMDA-stimulated release of glutamate in cerebellar granule cells prepared from lead-exposed first generation pups was not different from that of the control group, the S-nitroso-N-acetylpenicillamine (SNAP)-stimulated release of glutamate in cerebellar granule cells obtained from lead-treated pups was less elevated than that in the control. Furthermore, in cerebellar granule cells obtained from lead-exposed second generations pups, glutamate release did not respond to both NMDA and SNAP stimulation. In cerebellar glial cells exposed to lead, the basal glutamate uptake was not changed. However, the L-trans-pyrollidine-2,4-dicarboxylic acid (PDC)-blocking effects was significantly reduced. In glial cells obtained from lead-exposed pups, the glutamate uptake was also less blocked by PDC than that in the control. Further decreases in PDC-blocking effects were observed in cerebellar glial cells obtained from lead-treated second generation pups compared to those from the control group. These results indicate that lead exposure induces the changes in the sensitivities of the glutamate release and uptake transporter. In addition, these results suggest that lead exposure might affect the intracellular signalling pathway and transmission in glutamatergic nervous system.

Key words : Lead, Cerebellar granule cells, Glial cells, Glutamate release, Glutamate uptake

INTRODUCTION

Brain is known to be one of the major target organs for lead toxicity. Chronic lead exposure is known to produce adverse effects on the neurobehavioral activities, such as increased seizure susceptibility and disruptions in learning and memory (Alder *et al.*, 1977; Booze and Mactutus, 1990; Rice, 1993).

Lead is a divalent cation which is known to be a potent blocker of calcium channels and induces the disturbance of Ca^{2+} homeostasis (Busselberg *et al.*, 1993; Goldstein, 1993). It has been reported that lead may act on the MK-801 binding site, and on the Zn-binding site of N-methyl-D-aspartate (NMDA) receptor, thereby altering the receptor function (Alkondon *et al.*, 1990; Uteshev *et al.*, 1993). Lead-exposed rats have also been reported to show altered sensitivity to NMDA (Petit *et al.*, 1992) and changes in [^{3}H]MK-901 binding sites (Guilarte and Miceli, 1992; Guilarte *et al.*, 1993). Thus, these results imply that lead exposure affects the glutamatergic nervous activities in the brain.

It has been reported that granule cell packing in cerebellum is decreased by high dose postnatal lead exposure (Lorton *et al.*, 1986) and the morphology of neurons and astrocytes is affected by exposure to lead (Legare *et al.*, 1993). Furthermore, low concentration of lead inhibits nitric oxide production in brain endothelial cells (Blazka *et al.*, 1994). It is known that nitric oxide mediates enhancement of cGMP and induces the release of neurotransmitters (Bredt and Snyder, 1989; Lawence and Jarrott, 1993). Lead also inhibits the activities of glutamine synthetase in astrocytes (Tiffany-Castiglioni *et al.*, 1989; Tiffany-Castiglioni, 1993). Since the glutamate/glutamine cycle in brain plays an important role in maintaining the glutamatergic nervous activities, and glial cells, especially astrocytes, have a specific mechanism for glutamate uptake (Bloc *et al.*, 1995; Gilad *et al.*, 1990; Nicholls and Attwell, 1990), the balance between glutamate/glutamine becomes important in the glutamatergic nervous activities. However, the changes in the glutamate level after lead exposure is not well understood. To explore the mechanism of the lead toxicity, it is neces-

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sary to determine changes in glutamate release and uptake in neurons and glial cells after lead exposure.

Therefore, the present study is designed to investigate changes in glutamate release and uptake using cerebellar granule and glial cells prepared from pups exposed previously to lead. Changes of glutamate release and uptake are also determined in each cells after the *in vitro* lead exposure to compare with the effects of *in vivo* lead exposure.

MATERIALS AND METHODS

Animals and Materials

Eight-weeks old male and female Sprague-Dawley rats (Dae Han Lab., Taejon, Korea) were used. Animals were housed in 23±2°C, 12 hr light/12 hr dark cycled room and had free access to food and water. N-methyl-D-aspartate (NMDA), S-nitroso-N-acetylpenicillamine (SNAP) and L-trans-pyrollidine-2,4-dicarboxylic acid (PDC) were purchased from Research Biochemical Inc. (Natick, MA, USA). Fetal bovine serum and bovine calf serum were purchased from Gifco (Gaithersburg, MD, USA). Other basic chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animal treatment

Rats were treated with 0.25% lead acetate in drinking water, beginning gestation. Control rats were treated with 0.125% sodium acetate. During the growing period of rat pups, animals were continuously provided with 0.25% lead acetate through the maternal pathway and drinking water. After the rats were grown up, they bred again under the lead treatment and their offsprings (the second generation) were also used. According to Widmer *et al.* (1992), this dose of lead acetate does not affect the general health of pups and dams. For the in vitro lead-treatment of cerebellar granule and glial cells, appropriate concentrations of lead acetate were added during the change of the growth medium after 2 days of the stabilization. According to our preliminary study, the nontoxic concentrations of lead were used.

Cerebellar granule and glial cell culture

Cells were prepared by the method of McCaslin and Morgan with a slight modification (1987). Briefly, seven-day-old pups were decapitated, and the head were partially sterilized by dipping them in 95% ethanol. The cerebella were excised and placed in a culture medium which lacked serum and bicarbonate. Then, cells were mechanically dissociated. The growth medium (5 ml/60 mm dish) was Dulbecco's modified Eagle's medium (DMEM) supplemented with sodium pyruvate (1 mM), glutamine (4 mM), sodium bicarbonate (44 mM), glucose (25 mM), 6% fetal bovine serum and 6% bovine calf serum. After 2 days of stabilization, the growth medium was aspirated from the cultures and new growth medium was added. For preparing cerebellar granule cells, new growth medium containing 25 mM KCl was added with 5 μM cytosine arabinoside to prevent proliferation of nonneuronal cells (MaCaslin and Ho, 1994). For preparing cerebellar glial cells, the previous growth medium was used again. The viability and the developed neuron and glia prepared from lead-exposed pups were compared with those from control pups using the MTT assay (Cookson *et al.*, 1995).

Glutamate release from cerebellar granule cells

The release of glutamate was measured in cultures grown for 7 days after plating on slides. At the end of growing period, cells were placed in a physiological saline HEPES (PSH) buffer containing following chemicals; 5 mM HEPES, 135 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 10 mM glucose and 44 mM sodium bicarbonate (pH 7.4, 300 mOsm). After prewashing in the dark for 60 min, cells were incubated in the presence of either NMDA or SNAP at 37°C for the indicated period (15 min or 1 hr). Then, the amount of glutamate secreted into the buffer was analyzed by HPLC-ECD as described below. To determine changes of glutamate release in granule cells, the used glutamate releasers were NMDA and SNAP, which are a NMDA agonist and a nitric oxide generating agent, respectively (Southam and Gathwaite, 1991).

Glutamate uptake from cerebellar glial cells

The uptake of glutamate was measured using cultures grown for 10 days after plating on slides. At the end of growing period, growth medium was discarded from cells. After washing with PSH buffer for 1 hr, cells were incubated in the presence of both glutamate and various concentrations of PDC at 37°C for 30 min. The applied concentration of glutamate was 20 μM. Then, the amount remaining in the buffer was separated and quantified by HPLC-ECD as described below. To determine changes of glutamate uptake in glial cells, PDC was chosen as a competitive inhibitor of glutamate uptake (Swanson *et al.*, 1997)

Determination of glutamate

The level of glutamate was determined by the method of Roetger and Goldfinger (1991). The glutamate concentrations were quantified by HPLC with an EC detector after precolumn derivatization of small aliquots with *o*-phthaldialdehyde/2-mercaptoethanol reagent. Separations were achieved using a C18 reverse

type column and 0.1 M sodium phosphate buffer (pH 5.2) containing 37% methanol was used as mobile phase. The concentration of glutamate was determined by direct comparison of sample peak heights with those of an external standard containing the amino acid.

Statistics

The statistical significance of difference were determined using Student's t-tests.

RESULTS

The effect of NMDA on changes in glutamic acid in the incubation medium obtained from the *in vitro* lead-exposed granule cells for 5 days is summarized in Table I. The basal level of glutamic acid in the media was not affected by the exposure of the cells to 50 μM lead. However, the level of glutamic acid stimulated by 50 μM NMDA was significantly lower in the *in vitro* lead-exposed cerebellar granule cells than that in the control.

From the cerebellar granule cells obtained from the first generation of lead-treated pups, the effects of the addition of either NMDA or SNAP on concentrations of glutamic acid in the incubation media are shown in Table II. The levels of glutamic acid in the media were significantly increased by the addition of NMDA in both groups. However, while the level of glutamic acid in the media of the cerebellar granule cells prepared from the control pups was significantly increased by the addition of 50 μM SNAP, that from the cerebellar granule cells prepared from lead-treated pups was not changed by SNAP. From the cerebellar granule cells prepared from the second generation of lead-treated pups, the effects of the addition of either NMDA or SNAP on concentrations of glutamic acid in the incubation media are shown in Table III. The level of glutamic acid in the media of the cerebellar granule cells prepared from the control pups was significantly increased by the addition of 50 μM NMDA. However, that from the cerebellar granule cells prepared from lead-treated pups was not changed by the stimulation of NMDA. Also the significant increase in the level of glutamic acid from the cerebellar granule cells obtained from the control pups was observed only 15 min after the addition of SNAP.

Table I. Effects of NMDA stimulation on the release of glutamate in *in vitro* lead-exposed cerebellar granule cells

	Glutamate released (μM)	
	Control	Treated
Basal	0.350±0.032	0.357±0.019
NMDA	0.521±0.030** (1.49)	0.387±0.019## (1.08)

Cells were grown in media containing lead acetate (50 μM) from second to 7th day after plating. On the 7th day after plating, cells were stimulated with a designed compound (50 μM) for 15 min. The media were collected and analyzed by HPLC. Values are mean±S.E. for 4 or 5 determinations. Numbers in parentheses represent fold increases over basal values.

** Significantly different from the basal level of each group ($P<0.01$).

##Significantly different from the control group ($P<0.01$).

Table II. Effects of NMDA and SNAP on the release of glutamate in cerebellar granule cells obtained from the chronic lead-treated pups (the first generation)

	Glutamate released (μM)	
	Control	Treated
Basal	0.402±0.034	0.423±0.033
NMDA	0.868±0.103** (2.16)	0.846±0.037** (2.00)
SNAP	0.831±0.050** (2.07)	0.411±0.066## (0.97)

Cells were stimulated by a designed compound (50 μM) for 15 min. The media were collected and analyzed by HPLC. Values are mean ± S.E. for 4 or 5 determinations. Numbers in parentheses represent fold increases over basal values.

** Significantly different from the basal level of each group ($P<0.01$).

##Significantly different from the control group ($P<0.01$).

Table III. Effects of NMDA and SNAP on the release of glutamate in cerebellar granule cells obtained from the chronic lead-treated pups (the second generation).

		Glutamate released (μM)	
		Control	Treated
15 min.	Basal	0.250±0.024	0.281±0.032
	NMDA	0.392±0.031** (1.57)	0.330±0.038 (1.17)
	SNAP	0.470±0.058** (1.88)	0.284±0.038 (1.01)
60 min.	Basal	0.210±0.022	0.267±0.036
	NMDA	0.514±0.060** (2.45)	0.421±0.079 (1.58)
	SNAP	0.338±0.064 (1.61)	0.250±0.038 (0.94)

Cells were stimulated by a designed compound (50 μM) for either 15 min or 60 min. The media were collected and analyzed by HPLC. Values are mean±S.E. for 4 or 5 determinations. Numbers in parentheses represent fold increases over basal values.

**Significantly different from the basal level of each group ($P<0.01$).

##Significantly different from the control group ($P<0.01$).

Changes in the uptake of glutamic acid of the *in vitro* lead-exposed glial cells for 8 days are shown in Fig. 1. The basal uptakes of glutamic acid were not affected by exposure to either 1 μM or 5 μM lead for 8 days. However, the blocking effects of 50 μM PDC on the glutamate uptake in the *in vitro* lead-exposed glial cells were less effective (6~16%) than those in the control (40~60%).

From the cerebellar glial cells obtained from the the first generation of lead-treated pups, the effects of addition of PDC on the uptake of glutamic acid are shown in Fig. 2. The basal glutamate uptake in glial

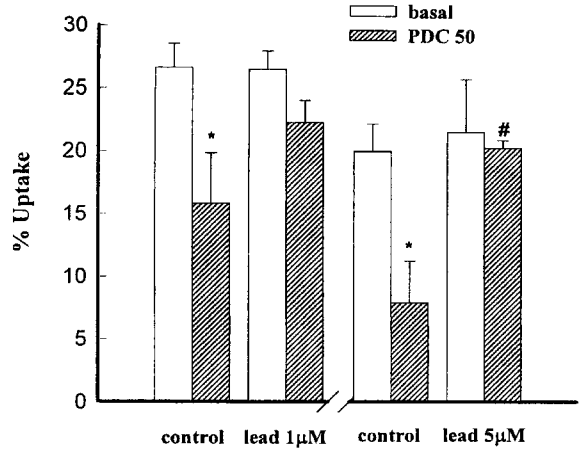

Fig. 1. Effects of *in vitro* lead exposure on glutamate uptake in cerebellar glial cells. Cerebellar glial cells obtained from 7 day-old rat pups were used. Cells were exposed to lead from second to 10th day after plating. On the 10th day after plating, cells were incubated with glutamate and designed compound for 30 min. The media were collected and analyzed by HPLC. Values are mean±S.E. for 4 or 5 determinations. *Significantly different from the basal level of each group (P<0.05). #Significantly different from the respective control group (P<0.05).

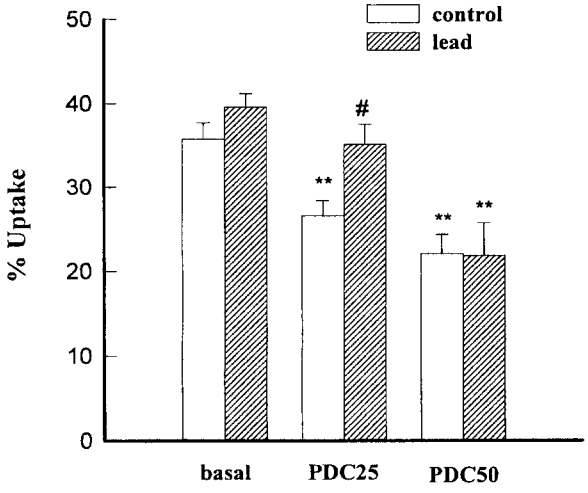

Fig. 2. Changes in glutamate uptake in cerebellar glial cells obtained from chronic lead-treated pups (the first generation). Cerebellar glial cells obtained from 7 day-old rat pups treated with 0.25% lead acetate (control was treated with 0.125% sodium acetate) through drinking water were used. On the 10th day after plating, cells were incubated with designed compound (μM) and glutamate for 30 min. The media were collected and analyzed by HPLC. Values are mean±S.E. for 7 or 8 determinations. **Significantly different from the basal level of each group (P<0.01). #Significantly different from the respective control group (P<0.05).

cells of lead-treated pups did not show any difference from that in the control. Although the uptakes of glutamic acid were similarly inhibited by the addition of 50 μM PDC in both groups (40~45%), the uptake of

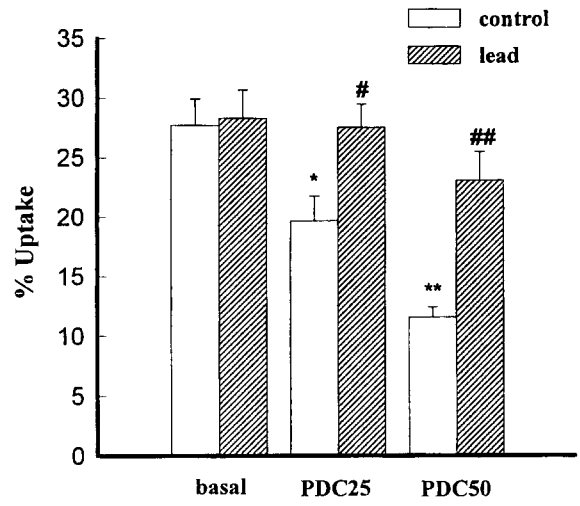

Fig. 3. Changes in glutamate uptake in cerebellar glial cells obtained from chronic lead-treated pups (the second generation). The experimental conditions were same as described in the legend to Fig. 2. *,**Significantly different from the basal level of each group (*P<0.05, **P<0.01). #,##Significantly different from the respective control group (#P<0.05, ##P<0.01).

glutamic acid in glial cells obtained from lead-treated pups (11%) was significantly less blocked by the addition of 25 μM PDC than that observed in the control (26%). For the cerebellar granule cells prepared from the second generation of lead-treated pups, the effects of addition of PDC on the uptake of glutamic acid are shown in Fig. 3. Changes in the uptake of glutamic acid in glial cells obtained from the second generation of lead-treated pups were similar to those of cells from the first generation. However, the decreased blocking effects of PDC on the uptake of glutamic acid were intensified. The uptakes of glutamic acid by the additions of either 25 or 50 μM PDC were significantly less inhibited in the glial cells obtained from the second generation of lead-treated pups (2.8 and 18.5%) than in the control groups (29 and 58%).

DISCUSSION

The present study demonstrates that the responses to the glutamatergic agents of both *in vitro* lead-exposed cells and cells obtained from lead-exposed pups are altererd. The release of glutamate in cerebellar granule cells obtained from lead-exposed pups was less responsive to NMDA and SNAP than that in cells from control pups. In addition, the glutamate uptakes in cerebellar glial cells from lead-treated pups were less blocked by PDC than that in cells from control pups. Furthermore, the release and uptake of glutamate after the *in vitro* lead-exposure were very similar to those after the *in vivo* lead-exposure.

It has been reported that after chronic prenatal lead-exposure, Ca^{++} mobilization in the cortex was reduced in response to inositol-1,4,5,-triphosphate (Singh, 1993). Guilarte *et al.* (1994) have reported that lead has a marked inhibitory effect on the hippocamphal NMDA receptor-ion channel complex. Also the decrease of [^{3}H]MK-801 binding sites in the cerebral cortex of neonatal rats was reported after chronic lead exposure (Guilarte and Miceli, 1992). It was reported recently that the increase in intracellular Ca^{++} concentration and release of glutamic acid in cerebellar granule cells from lead-exposed pups were less influenced by NMDA and kainic acid (Lim and Ho, 1998). Also in the present study, smaller responsiveness in NMDA-induced release of glutamate develops after the longer lead-exposed pups, which is clearly consistent with the previous report (Lim and Ho, 1998). Furthermore, the magnitude of the response to NMDA after the *in vitro* lead-exposure also decreased. Thus, the present results suggest that during development, lead might affect the presynaptic release of glutamate.

Nitric oxide is formed in neuronal cells and tissues upon NMDA or non-NMDA receptor activation (Garthwaite, 1991). The agonist-induced nitric oxide formation is associated with increased cyclic GMP (cGMP) (Bredt and Snyder, 1989; Dawson *et al.*, 1991). The increased cGMP as well as nitric oxide itself stimulates the release of glutamate (Bruhwyler *et al.*, 1993; Lawrence and Jarrott, 1993). Fox *et al.* (1994) reported that lead inhibited retinal cGMP-phosphodiesterase activities and the level of cGMP was increased. However, recently we reported that the normal kainic acid-induced increase in the level of cGMP did not occur in cerebellar granule cells prepared from lead-exposed pups (Lim and Ho, 1998). Since the release of glutamate by addition of SNAP was not stimulated in cerebellar granule cells prepared from lead-treated first and second generation pups, suggesting that nitric oxide-related neuronal activities are impaired after chronic lead-exposure. Because nitric oxide is a potent activator of guanylyl cyclase, and cGMP also stimulates the release of glutamate (Bredt and Snyder, 1989; Bruhwyler *et al.*, 1993), the decrease in release of glutamate by SNAP might be due to the defect in the second messenger system, especially cGMP, after chronic lead-exposure. Furthermore, the present results indicate that the impairment of the nitric oxide-related activities is earlier than that of NMDA receptor-related activities after chronic lead-exposure and suggest that lead exposure might change the receptor-mediated neurotransmission and intracellular signalling pathway in the glutamatergic nervous system.

Glutamate reuptake is known to play an important role in regulating synaptic and extracellular concentrations of glutamic acid. In the brain, the majority of glutamate uptakes are produced by astroglias, rather than neurons (Nicholls and Attwell, 1990; Rothstein *et al.*, 1996). Three subtypes of glutamate transporter are identified in the brain and each transporter differs in its kinetic property and distribution in the brain (Arriza *et al.*, 1994; Swanson *et al.*, 1997). However, it has been reported that glutamate uptake is regulated by the various factors, such as intracellular pH and xenobiotics (Aschner *et al.*, 1992; Judd *et al.*, 1996). Either the inhibition or the increase of glutamate uptake was reported by the exposure of either methylmercury or stressful stimui (Aschner *et al.*, 1992; Gilad *et al.*, 1990). The present results indicate that the blocking effects of PDC on the glutamate uptake were reduced in cerebellar glial cells after either *in vitro* or *in vivo* chronic lead-exposure. Also the longer (or higher) exposure to lead induced the less sensitive to PDC. It was reported recently that expression patterns of two astrocytic glutamate transporters vary during development and they have a different sensitivities to PDC (Sutherland *et al.*, 1996; Swanson *et al.*, 1997). After chronic lead-exposure, the changes in the sensitivity of glutamate uptake transporter as the present results imply that lead might affect the developmental patterns of glutamate glial transporters. Furthermore, the alterations in the glutamate uptake transporter during the development suggest that the glutamate/glutamine cycle in central glutamatergic nervous system might be affected after chronic lead-exposure.

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