The protective role of astroglia in the early period of experimental lead toxicity in the rat

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Abstract. Although the clinical manifestations of lead (Pb) neurotoxicity are documented, the subcellular mechanisms of its action are still an open question. The purpose of this study was to assess the function of nerve ending particles after acute lead exposure and to investigate whether it exerts a toxic effect on astroglial functions. The studies were performed using the rodent model of acute lead toxicity. Cellular fractions were used in biochemical measurements – synaptosomes and glial plasmalemmal vesicles (GPV). Since a procedure for the isolation of the fraction of astroglial origin has been developed, it becomes possible to investigate lead-astroglia interactions after *in vivo* exposure. It is of importance because most of the studies concerning lead toxicity were performed using astroglial culture systems. It was found that the uptake of glutamate (Glu) to the synaptosomes was lowered and KCl-dependent release was increased, suggesting the impairment of glutamatergic transmission leading to the elevation of extracellular amino acid concentration. In contrast, glutamate uptake to the GPV fraction was significantly elevated. The activity of the marker enzyme – glutamine synthetase (GS) was also significantly increased in the GPV fraction. The activation of glial functions suggest a regulatory role for these cells in the early period of acute lead toxicity.

INTRODUCTION

Lead (Pb) – a nonphysiological metal and environmental pollutant – is presented to most of the general human population below the level of toxic effect. However, its cumulative properties, which may lead to the exceeding of the "safe level", were demonstrated (Bercovitz and Laufer 1991, Mushak 1993). Despite a long history of scientific investigation, Pb toxicity remains a significant health problem of both industrial workers and those exposed environmentally (especially children). The collective data from *in vivo* experiments (Holtzman et al.1984) and from culture systems (Tiffany-Castiglioni et al. 1986, Holtzman 1987, Aschner and LoPachin 1993) suggest that astroglial cells posses the ability to sequester heavy metals, including lead. Holtzman (1984) proposed that astroglia serve as a "lead-sink" to protect neurons which are more sensitive to the toxic Pb effect. On the other hand, the glial Pb store may constitute a reservoir for its continuous release and thereby contribute to the toxicity of adjacent neurons (Holtzman 1987). Thus, it is of interest to assess to what extent astroglia can adapt to and tolerate the presence of intracellular lead.

The crucial role of astroglia in regulating the environment of adjacent neurons is beyond argument. One of most important roles is the regulation of glutamate (Glu) concentration. It is known that astrocytes are involved in the modulation of synaptic activity by the regulation of the extraneuronal level of released Glu (Vernadakis 1988). Glutamate, the predominant excitatory amino acid neurotransmitter in vertebrate brain, is taken up by glial cells after release from neurons and amidated to the non-neuroactive glutamine by the enzyme glutamine synthetase (GS), which is specific to astrocytes (Martinez--Hernandez et al. 1977, Norenberg, 1977). Thus, when that regulative mechanism cannot act properly, it may lead to excitotoxic damage of neurons. Taking into account that the ratio of astrocytes to neurons is 10:1 and approximately 30% of brain volume consists of these cells (Aschner and LoPachin 1993), it is probable that they may actively participate in brain injuries, also in that of toxic origin. So it becomes interesting, if not necessary, to assess the glia involvement in brain function after acute *in vivo* lead exposure.

Previous studies on glial toxicity by lead were mainly performed using cultured glial cells. In contrast to synaptosomes, which are a good model for nerve ending studies, there were no preparations available for the study of glial functions after *in vivo* exposure, until recently.

The present studies were undertaken to estimate the possible alterations in glial cells (as measured with the glia-derived fraction) that may occur in adult rat brain after acute lead exposure. Synaptosomal glutamate uptake and release, together with the response of the glial fraction, were investigated.

METHODS

Animal treatment

Male Wistar rats were used in the experiments of acute lead toxicity. Animals were injected intraperitoneally with lead acetate at a concentration of 25 mg/kg b.w. for 3 days. Controls were treated with distilled water. Pb doses and distilled water doses were administered at a constant small volume of 100 μl. During the time of experiments, animals had unrestricted access to the standard laboratory chow. In the group of lead-exposed rats we did not notice either incidents of death, or symptoms of severe lead toxicity like seizures.

Preparation of GPV fraction

A Percoll density gradient centrifugation technique by Daniels and Vickroy (1998), with slight modifications according to Nakamura and coworkers (Nakamura et al. 1993), was used to isolate glial plasmalemmal vesicles termed GPV. Briefly, forebrains from 2 rats were homogenized in 30 ml medium (0.32 M sucrose and 1 mM EDTA) and centrifuged at 1,000 g for 10 min using a fixed-angle rotor. The supernatant was diluted using a SEDH solution containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM dithiothreitol and 20 mM HEPES (pH 7.4) and centrifuged at 5,000 g for 15 min. Thereafter, the resultant supernatant was saved and the pellet resuspended in a new portion of SEDH solution and centrifuged at 1,000 g for 10 min. Both supernatants were combined into one and centrifuged at 33,500 g (20 min). Pellets, suspended in SEDH, were centrifuged in a three--step discontinuous Percoll gradient (20% : 10% : 6%) at 33,500 g for 10 min. The layer between 0% and 6% Percoll was collected and centrifuged 1,000 g (20 min) after dilution in SEDH. The supernatant was centrifuged at 33,500 g for 20 min to obtain the pellet – glial plasmalemmal vesicle fraction (GPV). The resultant pellet was suspended in SEDH solution and used for glutamate uptake measurements or for determining glutamine synthetase activity.

Preparation of synaptosomal fraction

The synaptosomes were prepared according to the Booth and Clark method (1978). Rats were decapitated and forebrains homogenized in sucrose buffer (0.32 M sucrose, 1 mM EDTA, 10 mM TRIS-HCl, pH 7.4). Samples were centrifuged at 1,300 g for 10 min and subsequently 17,000 g for 10 min. Then, the pellet was centrifuged in a discontinuous Ficoll gradient (7%/12%) 99,000 g for 30 min. Each fraction for further examination was obtained from one rat.

Glutamine synthetase assay

The enzyme activity in GPV samples was determined according to the method of Pishak and Philips (1979). A reaction mixture contained equal amounts (5 μl) of 500 mM imidazole-HCl, 150 mM $MgCl_2$, 100 mM ATP, 40 mM NH_4Cl, 10 mM dithioerithritol (DTE) and L-[G-^{3}H] glutamic acid (specific activity 1.06 mCi/mmol) mixed with unlabeled glutamic acid to a conc. of 75 mM. After the addition of 20 μl of GPV fraction, the reaction mixture was incubated for 30 min. at 37°C. The reaction was stopped with 1 ml of cold water and transferred to two stacked ion exchange columns (Dowex-1-H+ on top of Amberlite CG-50, H+) to separate the reaction product (^{3}H-glutamine) from other labeled compounds. Each column was eluted with 3 ml of cold water. The eluent was collected and radioactivity measured. Results are expressed as specific activity in nmoles glutamine per min per mg protein.

[^{3}H]-glutamate uptake

The GPV and synaptosomal fractions were used for glutamate uptake according to the filtration method described by Divac (Divac et al. 1977). For measurements of glutamate uptake, aliquots (50 μl) of fractions (0.8-1 mg protein/ml) were added to 400 μl of buffer (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 20 mM HEPES, pH 7,4). Isomolar concentrations of choline chloride were used instead of NaCl to measure sodium-independent uptake. Assay duplicates were preincubated in a shaking water bath at 30°C for 5 min. After the addition of 50 μl of radioactive glutamate (f.c. 5 μM, specific activity 45 Ci/mmol), the reaction was stopped by filtration under vacuum through a Whatman GF/B glass filter at the several time points. Tubes and filters were washed 3 times with 2 ml of cold normal or so-

dium-deficient buffer, respectively, and the activity counted after soaking the filters in 1 ml of 10% Triton X-100 for 10 min. Radioactivity trapped on the filters was measured by a liquid scintillation spectrometer. Sodium-dependent uptake was assessed as the difference between uptake in the two above mentioned buffers. The time course of glutamate uptake was done and the maximum was found to be in 4th min (synaptosomes) and 2nd min (GPV). In the case of synaptosomal fractions, the KCl-dependent release of taken up [^{3}H]-glutamate was measured after adding 50 mM KCl in the 4th min of the uptake process.

Measurement of lead content

The lead levels in blood and brain homogenate were measured by an atomic spectrometer with graphite furnace.

Protein measurement

The amount of protein in GPV and synaptosomal fractions was determined by the method of Lowry and co-workers (Lowry et al. 1951) using bovine serum albumin as a standard.

Data analysis

All values are expressed as a mean ± SEM from the number of experiments stated in legends to the figures. The number of experiments corresponds with either the number of animals (Table I) or the number of separately and freshly isolated fractions (Figs. 1-3). Single fraction used for one measurement was obtained either from 1 (in the case of synaptosomes) or from 2 (in the case of GPV) experimental animals and their respective controls. Statistical significance was determined using the Student's *t* test and accepted at a level of *P*<0.05.

RESULTS

The lead level

The lead dose received by the exposed rats produced blood levels of metal within the high concentration range, as observed in acute lead poisoning (Goyer and Chisolm 1972). Blood lead levels were much higher in lead-exposed rats than controls and reached 97.2 μg/dl. A significant penetration of lead from blood to brain was noticed. The amount of lead in the forebrain homogenates of exposed rats was 1.8 μg/g w.w. (Table I).

TABLE I

Lead levels in blood and brain homogenates of the control and lead-treated rats in the acute model of toxicity

Sample	Lead level	
	Control group	Lead-exposed group
Blood (µg/dl)	2.0 ± 0.2	97.2 ± 3.0
Brain homogenate (µg/g w.w.)	$<0.019*$	1.8 ± 0.4

Data expressed the means $\pm$ SD of 4 independent measurements performed on samples derived from 4 animals in each group. *Below the sensitivity of applied method.

Uptake and release of GLU in synaptosomes

Uptake of glutamate to the synaptosomes was impaired in a fraction obtained from Pb-treated rats. The decrease (calculated in maximum of uptake curve i.e.
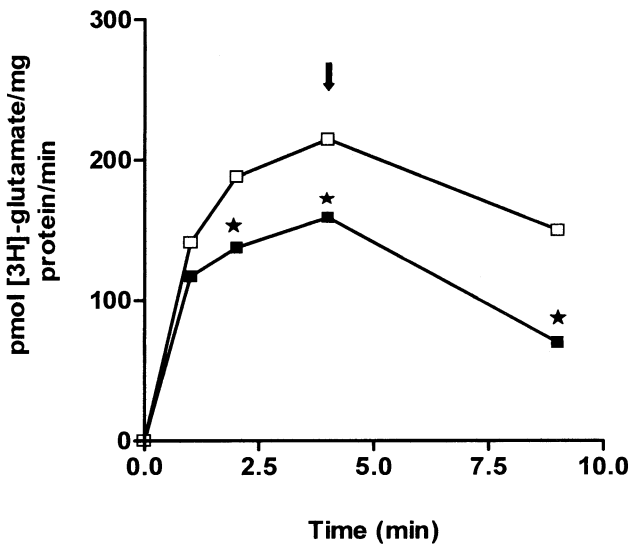
4 min.) reached 25%. The KCl-dependent release of glutamate from the synaptosomal fraction was found to be enhanced in Pb-treated rats by about 20% (Fig. 1).

GLU uptake to GPV

The major component of astrocytic glutamate uptake (high affinity transport system) is Na^+-dependent (Schousboe et al. 1977). It was determined as the difference between total and Na^+-independent uptake. Total glutamate uptake was determined in a buffer system with Na^+, Cl^- and Ca^{2+} ions, all of them identified to be necessary in that process (Flott and Seifert 1991). Na^+-independent uptake was determined with a buffer lacking sodium ions.

That astroglial high affinity glutamate uptake measured in the GPV fraction obtained from rats after Pb exposure, was found to be enhanced. The increase reached 45% in the maximum (2nd min) of uptake (Fig. 2).

Glutamine synthetase activity

Glutamine synthetase amidated glutamate released by neurons to nontoxic glutamine. A marked activation of specific activity of the enzyme was noticed as the result
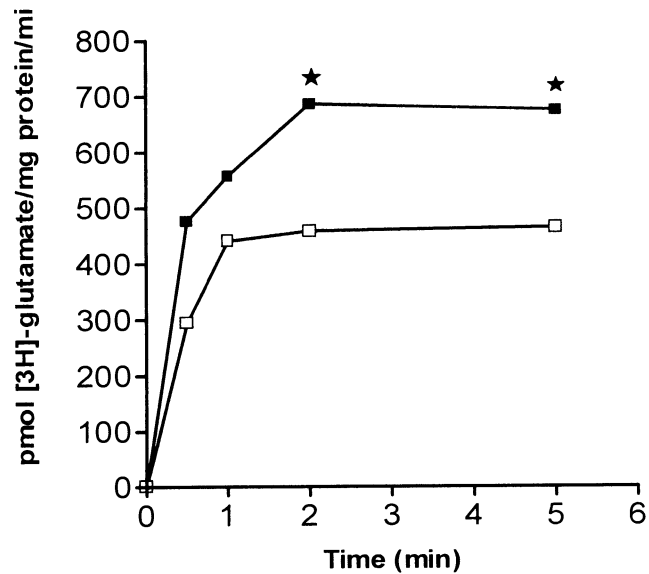

Fig. 1. Na^+-dependent uptake and release of [³H]-glutamate in synaptosomal fractions obtained from control (open square) and Pb-treated (filled square) rats. The release was measured after adding 50 mM KCl in the 4th min of the uptake curve (arrow). Points are means $\pm$ SEM of 6 independent experiments, each performed on 6 separately prepared fractions in both control and experimental group. SEM was less than 5%. *The values are significantly different at the level of $P<0.05$.

Fig. 2. Na^+-dependent uptake of [³H] glutamate to the GPV fraction obtained from control (open square) and Pb-treated (filled square) rats. Points are means $\pm$ SEM of 4 independent experiments each performed on separately prepared fraction. SEM was less than 5%. * The values differ significantly at the level of $P<0.05$.
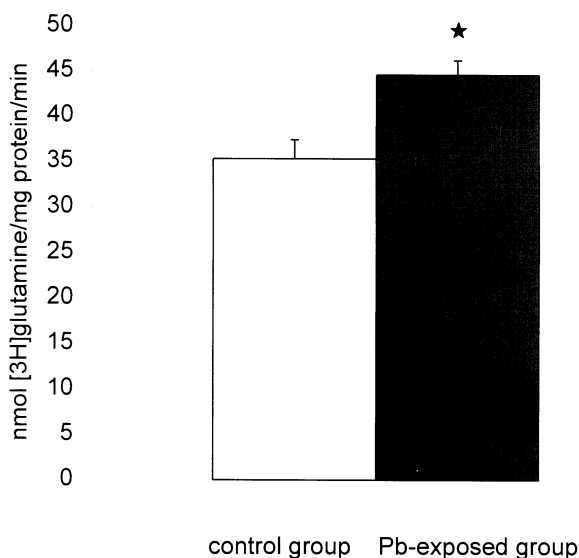
Fig. 3. The activity of glutamine synthetase in GPV fractions obtained from control and Pb-treated rats. The values represent the means ± SEM of 7 independent measurements in both groups, each performed on 7 separately prepared fractions. *P<0.05.

of acute lead exposure. The activity of GS in the glial fraction obtained from lead-treated rats was found to be significantly (P<0.05) higher than in controls (Fig. 3). The increase reached about 20%.

DISCUSSION

Lead - an ubiquitous environmental pollutant - may be dangerous not only for those who are occupationally or accidentally exposed. The greater population may also suffer from Pb toxicity due to its cumulative properties which may lead to the exceeding of the "safe level" and to various resulting consequences, including neurotoxicity. It is likely enough that the absorption of lead and its storage in the brain occurs at any level of blood lead, therefore man can not estimate the threshold (Niklowitz 1977). Unquestionably, neurotoxicity is one of the most serious toxicological events, since damage to even a small number of neurons can have consequences for the whole organism. Therefore, the necessity to study the subcellular mechanisms of neurotoxic action of lead exists.

Until recently, no biochemical method was available for the study of lead and glia interactions, so the attention was focused on cultured cells (Holtzman et al. 1987, Tiffany-Castiglioni 1993). Experimental procedures using culture cell systems may be vigorously standardized, the

toxic exposure is continuous and easily quantitated. However, the culturing process may alter some cellular properties and consequently develop an altered response to the tested agent that limits the range of applications for this method (Veronesi 1992). Additionally, it is known that pure astrocytic cultures do not respond in the same manner as cocultures (Veronesi 1992, Heidinger et al. 1999), so the question arises to what extent cultured cells reflect the functions of the cells in living tissue.

The GPV fraction obtained by the method of discontinuous density-gradient centrifugation by Daniels and Vickroy (1998) was verified by the authors for the structural integrity and analyzed for glial markers and functional specificity. The studies provide solid evidence for the highly-enriched glial nature of the GPV fraction.

Morphological examination performed for the present studies revealed a large number of membrane-encapsulated vesicles that could be classified as small spherical structures or large irregularly shaped structures (data not shown) (Strużyńska et al. 1999). The morphological characteristics of the GPV fraction is consistent with observations published in the original method.

Glutamine synthetase (GS) is an enzyme primarily localized to astroglia with a key role in glutamate and ammonia metabolism in the brain (Norenberg 1997). There are data about the dose- and time- dependent reduction of this enzyme activity, measured in cultured astroglial cells after lead exposure (Engle and Volpe 1990, Sierra and Tiffany-Castiglioni 1991). It is suggested that the reduced GS activity is a very sensitive indicator of Pb exposure in astroglia (Tiffany-Castiglioni 1993).

On the other hand, the induction of GS activity was observed in vivo in brain homogenates of lead-exposed rat pups (Cookman et al. 1988). The enhancement of this enzyme activity may be the expression of the intense activity of the astroglial cells (gliosis) as the result of neuronal damage of a different origin, including toxic conditions (Norenberg 1996). Glu-induced increases in GS activity were observed, showing this enzyme to be directly involved in neuroprotection (Heidinger et al. 1999). Interestingly, the results of the present experiments also show an increased GS activity. From our experiments it may be concluded that GS elevated activity signals a disruption of glutamate metabolism in neurons. The lowered uptake and enhanced potassium-evoked release of glutamate from the synaptosomal fraction after acute lead exposure suggest the impairment of glutamatergic transmission which may cause the excess of extracellular glutamate and in consequences lead to glutamate-induced neuronal injury.

The function of GS in the brain is related to the synaptic roles of glutamate and is a critical factor in the phenomenon of glutamate compartmentation. There is evidence that signaling from neurons influences glutamate uptake by astrocytes (Mennerick et al. 1996, Heidinger et al. 1999).

Data presented in the literature show that other astroglial functions, especially high affinity glutamate uptake, were impaired in astroglial primary cultures in the presence of lead (Rönnbäck and Hansson 1992). The results of the present studies are surprisingly different. We observed the enhancement of Na^+-dependent glutamate uptake in glia-derived fraction obtained from lead-treated rats. The data suggest that the astroglial response induced by Pb may differ in different developmental stages and especially in different models of studies. The animal model of studies presented herein is closer to the *in vivo* situation after lead exposure than experiments on cell cultures, especially one-line cell cultures. It is evident that in pure astroglial cultures the deprivation of signals derived from neurons may significantly change the astroglial response.

In these studies the enhanced glutamate uptake by the glia-derived fraction, together with an increased GS activity, may reflect the activation of the astroglial glutamate/glutamine system which is instrumental for the astroglial homeostatic function, i.e. controlling of glutamate concentration.

Since increases in GS levels are associated with the formation of reactive astrocytes (or with stimulated metabolism), the findings of lead-induced increases in that enzyme activity in the present experiments indicate that the glial compartment of the adult rat brain is actively responding to the effects of lead.

In conclusion, the present experiments demonstrate for the first time the induction of functional activity of the astroglia-derived fraction obtained from adult rat brain after acute lead exposure. All the results, taken together, may reflect the protective role of astroglia in the early period of lead toxicity in the rodent model of acute exposure. It does not exclude that a delayed response (in chronic, prolonged lead exposure) may be markedly different.

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