THE EFFECT OF LEAD ACETATE ON THE NITRIC OXIDE SYSTEM IN THE RAT HIPPOCAMPAL CELLS: AN IN-VITRO STUDY

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Abstract - The mechanism by which lead may cause a perturbation in the nervous system is through the nitric oxide synthase. In this study the effect of lead acetate on the hippocampal constitutive nitric oxide production was studied. The variation in the nitric oxide production may contribute to physiological lead neurotoxicity in vivo. Different concentrations of lead acetate (10^{-9} to 10^{-6} M), were added to cultures of hippocampal pyramidal cells obtained from one-day newborn rats. Sodium acetate was used as control. In this range of study viability of cells did not alter when it was compared to control ($P < 0.05$). In another part of study, cells were obtained from one day dams whose mothers were chronically intoxicated by lead acetate. The results showed that neither direct exposure of normal cells nor cells obtained from latter group could show any increase in the amount of nitric oxide. Administration of lead to the cells 10 minutes before measurement showed same results. These results showed that low concentration of lead acetate can not induce nitric oxide production in the pyramidal cells.
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Key Words: Lead, pyramidal cell, nitric oxide, culture, chronic toxicity, rat hippocampal cells

INTRODUCTION

Lead (Pb), the most ubiquitous toxic metal, is detectable in practically all phases of the inert environment and in all biologic systems. Children seem particularly vulnerable as a result of greater relative exposure to Pb and because of the enhanced sensitivity of the developing nervous system (1). In fact, prospective cross-sectional and longitudinal epidemiological studies in pediatric populations find low-level Pb exposure to be associated with subtle behavioral changes, such as deficits in IQ scores, impaired psychomotor development, and poor classroom performance (2,3). While higher level exposures have long been known to produce overt nervous system toxicity as manifested in seizures, coma and even death (4). The hippocampus has been suggested as a site of lead's toxic action (5) either as storage for Pb (6) or existence of more sensitive cellular targets to Pb in hippocampus (7). Nitric oxide (NO) is a highly unstable compound that acts as a messenger molecule in immune and endothelial cells (8) and as a neurotransmitter in the brain (9). Nitric oxide is the main product of a reaction in which nitric oxide synthase (NOS) converts arginine to citrulline (10). There are several isoforms of NOS that fall into two major classes : 1) inducible NOS (iNOS) present in macrophages and 2) constitutive calcium (Ca^{2+}) and calmodulin regulated NOS (cNOS), which includes two isoforms, endothelial (eNOS) and neuronal (nNOS) (11). Both eNOS and nNOS activities are found in the hippocampus (12).

It has been shown that Pb, a divalent metal, is capable of displacing Ca^{2+} from various Ca^{2+}-binding proteins such as calmodulin (13). Furthermore, Pb can inhibit membrane Ca^{2+}-dependent processes (14) and Ca^{2+}-dependent enzymes (15). Furthermore Pb can interfere with NMDA receptors at concentration about 5-20 μM (16). The glutamate/NO/soluble guanylyl cyclase system is of primary importance in a variety of physiological and pathological processes of the hippocampus (17).

In the present study we examined the effect of low concentrations of Pb on levels of NO production by hippocampal pyramidal cells, in the presence and absence of low level Pb in vitro. Furthermore the effect of chronic exposure of newborn rats from mothers that had received Pb by drinking water and lead levels in their blood had reached the steady state was examined.

MATERIALS AND METHODS

1. Cell Culture of hippocampus cells

Pregnant Sprague-Dawley rats (300-400 g) were housed in a room controlled at 23 ± 2 ° C with controlled lighting conditions (12 hrs light/12 hrs dark). Food and water were provided ad libitum. The hippocampus tissue of one-day-old pups removed aseptically (10 pups in each experiment in three

separate occasions). The tissue was then incubated in dissociation medium (90mM Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 0.25 mM $CaCl_2$ and 1 mM HEPES with the pH adjusted to 7.4) containing Trypsin (1X, Sigma) for 20 minutes. Cells were then filtered through 50 μm nylon filter. The cells were washed in growth medium (Dulbecco Modified Eagle Medium, DMEM) containing 5% fetal bovine serum (FBS), 5% horse serum (HS), 400 μg L-glutamine, and 17 mM D-glucose (18). The dissociated cells were plated at a density of aproximately 5.6×10^5 ml^{-1} in 35 mm poly-D-Lysine coated plates (Nunc, Denmark). Non-neuronal cells were omitted by 24 hrs cytosine arabinoside incubation (18). In a separate experiment the pregnant animals were chronically (one months prior to pregnancy) exposed to lead acetate and after delivery the pups were used in the same manner for the effect of lead in the NO production of cultured cells.

2. Nitric Oxide measurement in culture

Into a prewarmed (37°C) cuvette containing the buffer (e.g. 0.1 M Hepes. pH 7.0) add the NOS substrates as follows: 1mM L-arginine (100mM stock solution prepared in the buffer, stored at -20°C), 1 mM $CaCl_2$ (100 mM stock solution prepared in H_2O, stored at -20°C), 0.2 mM NADPH (20 mM stock solution prepared daily in the buffer, pH = 7.0), 0.5 μM flavine mono nucleotide (FMN) (0.5 mM stock solution prepared daily in the buffer, pH 7.0) and 10 μM tetrahydrobiopterin (BH4) (2 mM stock solution prepared in 10 mM HCl prepared every 5-6 hrs); it is highly unstable in dilute solution due to autoxidation.

To measure NO, add 4 μM HbO_2 (oxyhemmoglobin) (prepared according to Di Iorio) (19) to the cocktail, mix gently; record the absorbance at 401 nm. This cocktail is added to the cells, which are washed three times by PBS and incubated in 37°C for 20 minutes. MetHb (methemoglobin) formation is shown by increasing in the absorbance at 401 nm. It is quantified using a $\varepsilon_{401\ (metHb\text{-}HbO2)}$ of 49 mM cm^{-1}(20).

3. Lead administration to the culture

Different concentrations of lead acetate (10^{-9}, 5×10^{-9}, 10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7} and 10^{-6} M) were administered to 2 days culture of the hippocampus cells after pyramidal purification and let lead be exposed for 7 days in the medium. In separate experiment, the same concentrations of lead acetate were added to culture medium just 10 minutes before NO assessment.

4. Viability test

A. Trypan blue exclusion test: 0.4% v/v trypan blue was added to the cell suspension (cells were prepared by trypsinization) and those cells not stained by the dye were counted by light microscopy.

B. Determination of mitochondrial dehydrogenase activity (MTT)

100 μl solution of 3-[4,5-dimethyl thiazol-2y1] -2,5-diphenyl tetrazolium bromide (MTT) was added to each well. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of the yellow MTT to yield purple formazan crystals, which are insoluble in aqueous solutions. The crystals were dissolved in 300 μl acidified isopropanol and the UV absorbance of the resulting purple solution determined at 570 nm against 690 nm for blank solution. The amount of produced formazan is directly proportional to the number of viable cells.

5. Statistical methods

The data in each group were examined by paired student t-test and probability less than 0.05 ($P<0.05$) was assumed significant.

RESULTS

Figures 1 & 2 show the viability of cells when they were exposed to the lead acetate and sodium acetate (0, 10^{-8}, 10^{-7} and 10^{-6} M) using trypan blue dye exclusion test and MTT assay, respectively. As it is shown in these figures the viability of cells remain unchanged in the range of lead administration. Figure 3 shows time course study of NO production in normal culture of hippocampus cells. The optimum time for measurement of NO as it is shown in figure 3 is about 20 minutes after adding substrate for NOS. Figure 4 shows the effect of different concentrations of lead acetate in the NO production of cells in vitro. As it is shown in this figure there is no difference in the NO production of cells after exposure to lead acetate. In all experiments different concentrations of sodium acetate were studied as control for the effect of acetate ion in the NO production. The same study was conducted for the pups obtained from pregnant rat, which were chronically treated by lead acetate except in the following experiment lead acetate was not added directly to the medium. Figure 5 shows the plasma lead concentrations of animals that were administered lead acetate chronically. Figure 6 shows that the administration of lead chronically to the animals in vivo has no effect in the NO production when it is compared to the sodium acetate group. Figure 7 shows that the administration of lead acetate (the same concentrations in figure 4) for 10 minutes has no effect in the NO production when it is compared to the sodium acetate group.

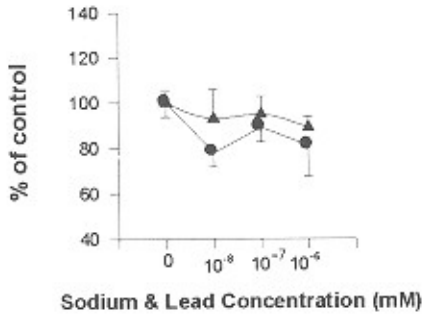

Fig. 1. The effect of different concentrations of lead acetate and sodium acetate on the viability of hippocampal pyramidal cells in culture by using trypan blue counting assay. The results were presented as percent of viability of control, (±SE, n=3).

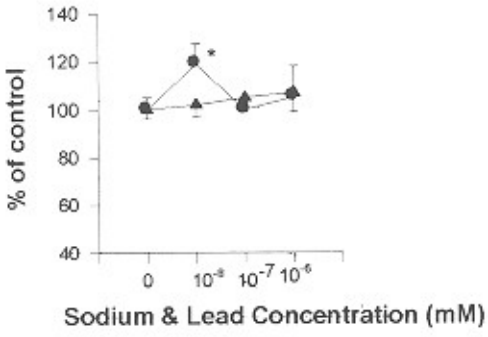

Fig. 2. The effect of different concentrations of lead acetate and sodium acetate on the viability of hippocampal pyramidal cells in culture by using MTT assay. The results were presented as percent of viability of control, (±SE, n = 3).

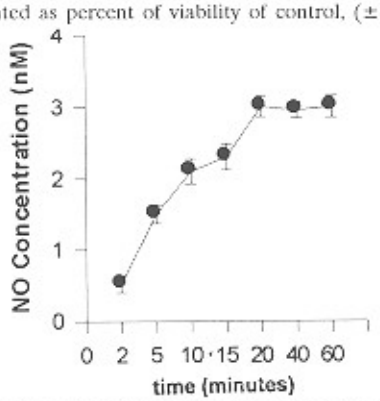

Fig. 3. The effect of time on nitric oxide production in normal hippocampus cell cultures (±SE, n=6).

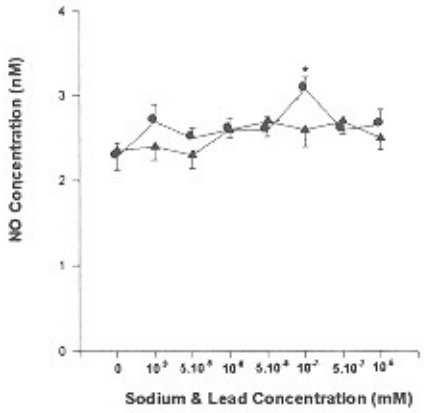

Fig. 4. The effect of different concentrations of lead acetate and sodium acetate on NO production in hippocampal pyramidal cells (±SE, n=9).

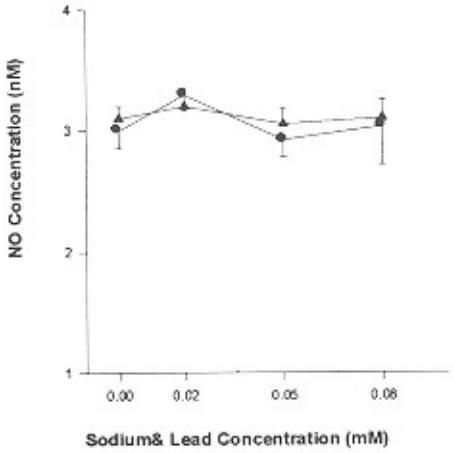

Fig. 5. Dose relationship with plasma level when the lead acetate was administered orally (±SE, n=5).

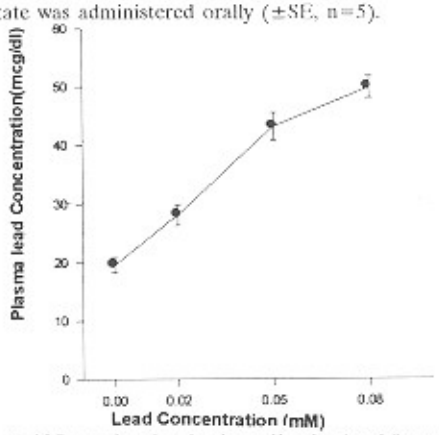

Fig. 6. NO production in the cells obtained from pups that their mothers were chronically treated by lead acetate compare to groups which were treated by sodium acetate (±SE, n=9).

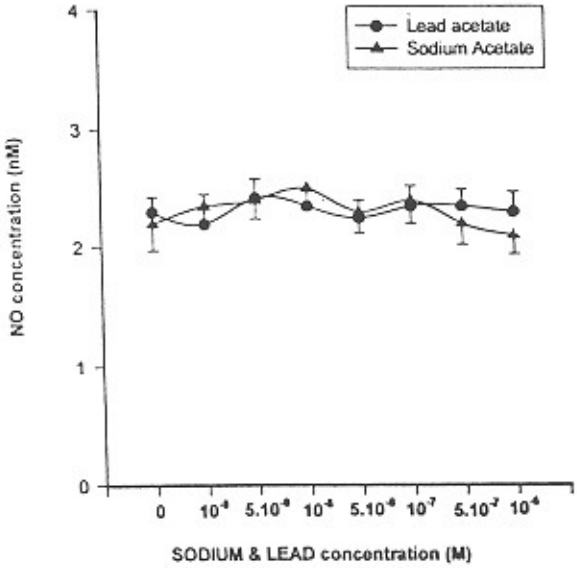

Fig. 7. The effect of different concentrations of lead acetate and sodium acetate with 10 minutes preincubation time on NO production in hippocampal pyramidal cells (±SE, n = 9).

DISCUSSION

NO is a biological messenger molecule that behaves as a neurotransmitter in the brain (9). Because of similarity between clinical presentation of lead neurotoxicity and NO production effect on the learning and memory in brain (2,3,11), the question arises whether there is a relation between these phenomena.

Pyramidal cells contain nNOS (21). cNOS is calcium and calmodulin dependent and plays a role in localized transcellular communication (22), relaxation of vascular smooth muscle (23,24), inhibition of platelet aggregation and adhesion (25) and neurotransmission (9,26). It has been shown that Pb is capable of inhibiting cNOS at concentrations that alters the activities of other Ca^{2+}-dependent enzymes, such as pyruvate kinase (27) and protein kinase C (15). Increasing the concentration of Ca^{2+} reversed the inhibitory effects of Pb suggesting that the effect of Pb on cNOS is in part due to its ability to mimic Ca^{2+}; this is in agreement with Habermann et al (1988) who have shown that Pb can displace Ca^{2+} from calmodulin (13). The hippocampus has been suggested as a site of Pb's toxic action (5). Moreover, It has been shown that Pb can be accumulated in the hippocampus (6). However, it has been claimed that the basis for any suggestion about selective vulnerability of hippocampus is not due to a preferential Pb accumulation. Instead, Pb may interact with cellular targets and alter biochemical or cellular processes that are uniquely associated with, or greatly enhanced in a particular region (7). 85-90% of hippocampus cells consisted of pyramidal cells (28). In this study we decided to evaluate cNOS function in relation to Pb toxicity. The inability of sodium acetate to affect the production of nitrite by cNOS suggests that the decrease in nitrite production can be attributed to Pb rather than to the acetate (29). Furthermore, the inhibition of nitrite production by cNOS occurred at concentrations of Pb that did not alter pyramidal cell morphology, induce cell membrane leakage, or alter the rate of ATP production (Fig. 1&2, respectively).

Earlier studies have shown that catalytic conversion of L-arginine to nitric oxide requires Ca^{2+} ions. It has been reported that activity of brain cNOS underwent time-dependent diminution when cytosolic preparations were preincubated with calcium ions (7). Pb and other heavy metals can interfere with cNOS activity through Ca^{2+} ion. However, this interference usually occurs in a concentration higher than approached in chronic toxicity (100 μM compared to 1 μM in whole brain respectively) (30). nNOS is permanently decreased in the cerebellum of rats subjected to chronic neonatal blockade of NMDA (31). However, in hippocampus, NMDA receptor seems not to be involved in the maintenance of basal concentration of cGMP (17). It has also been shown that Pb can interfere with NMDA receptors at concentration about 5-20 μM (16) which is near to the concentration achieved in chronic lead toxicity (7). Pb interacts with the NMDA receptor complex and inhibits receptor activation (32,33). It has been suggested that the effects of Pb on the NMDA receptor complex may be responsible, at least in parts, for some of the learning deficits which have been identified in experimental animals and human beings exposed to Pb during early development (32,33). This effect of Pb was very similar to the one observed for the divalent cation Zn , an allosteric modulator of the NMDA receptor. In fact Pb may inhibit NMDA receptor activation via an interaction at a Zn^{2+} allosteric site (16).

As shown in figure 4, different concentrations of lead acetate with 7 days preincubation in culture, have not altered NO production in hippocampus pyramidal cells. It may due to effect of Pb on NMDA receptor that inhibits stimulated NO production but does not affect basal NO production. Data presented in figure 6 shows that NO production was not altered in hippocampal pyramidal cells in cultures from neonates whose mothers had taken Pb in drinking water and Pb levels had reached the steady state figure 5 . Furthermore, figure 6 shows that Pb can not interfere with fetus NOS and/or after birth the NOS activity will be resumed. Data presented in figure 7 shows that incubation of different concentrations of lead acetate for 10 minutes could not alter NO production in hippocampal pyramidal cells in culture, which is in

agreement with that of Mittal et al (1995) (30) (similar to Fig. 4).

The data presented in this study makes two hypotheses. First in agreement with Guilarte et al (1997) (16) which shows that low concentration of Pb can bind to the NMDA receptors in day 14th postnatal rats in hippocampus region at concentration of 7.8 μM compared to cortex at concentration of 34 μM and so resulted inhibition of NO production but can interact in learning and memory as mentioned before. In this hypothesis Pb behaves as divalent cation competing with Zn at this receptor. Another possibility is interference of Pb with eNOS in endothelial cells in hippocampus blood vessels (29) which results in interruption of normal blood flow in the region and may cause damage in pyramidal cells. Latter concept means that if animals were treated with Pb chronically, they should present some pathological problem in the hippocampal or, at least, brain vessels.

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