LEAD EXPOSURE IMPAIRS NMDA AGONIST-INDUCED NO PRODUCTION IN PYRAMIDAL HIPPOCAMPAL CELLS

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ABSTRACT
Chronic exposure to Lead (Pb) affects neural functions in central nervous system (CNS) particularly the learning and memory. On the other hand, alteration of calcium level in the CNS results in activation of NOS where it is expected to increase nitric oxide level in hippocampus. In this study the role of Lead exposure in NMDA induced NO production in pyramidal hippocampal cells (CA1HP) was investigated. The NO level was determined by measurement of concentration of nitrite and nitrate as NO products using the metHb production at 401 nm. The ACBD (NMDA agonist)-induced NO level was almost reduced to the control level (2.5 nM) in the presence of 10 and 100 nM of Lead acetate. Lead acetate at concentrations which normally results in chronic toxicity did not increase the nitric oxide (NO) production by CA1HP. One reason for this finding could be the interaction of Lead with NMDA receptors due to similarity of Pb2+ to Zn2+ ion. Another reason may be related to direct interaction of Lead with NMDA receptors that inhibit the stimulated NO production.

Keyword: Lead acetate, ACBD, NMDA agonist, Pyramidal cell, Nitric oxide, Culture

INTRODUCTION
Lead is a heavy metal environmental toxicant that possesses a significant health threat, particularly to the developing CNS of infants and children (1-3). The neurological effects of low level of Lead exposure range from impaired cognitive performance to altered brain development (4, 5). Although the mechanisms involved in these neurological aberrations are not clearly understood, much attention has focused on Lead interactions with calcium mediated cellular events (6-9). Lead blocks long-term potentiation (LTP) in rat brain slice of hippocampus (10, 11) through mechanisms which may (12) or may not (13) involve interference with the NMDA receptors. NMDA receptors are densely distributed in the mammalian CNS and participate in several forms of synaptic Plasticity (14, 15, and 16). Activation of NMDA receptors is critical for the induction of LTP (17, 18). The influx of calcium through NMDA receptors channels activates a cascade of events that lead to persistent changes in synaptic efficacy (19, 20). Despite of the clear role of NMDA receptors in LTP, previous studies have shown that untimely activation of NMDA receptors prior to delivery of an LTP-inducing stimulus impairs the ability of generate LTP without persistent alteration baseline synaptic responses (21). The constitutive form of brain nitric oxide synthase (NOS) is Ca2+-calmodulin dependent (22, 23) and NOS activity may be regulated by phosphorylation (22, 24, 25). NOS catalyze formation of nitric oxide (NO) and citrulline from L-arginine in a reaction requiring molecular oxygen and NADPH. Recent evidences support NO as a retrograde messenger mediating LTP in the hippocampus (26-29) and a similar process in the cerebellum which is called long-term synaptic depression (30). It has been shown that NO is critical for the normal physiological regulation of the nervous system (31). Nitric oxide may also play a role in other events of neuronal plasticity that are involved in the early brain development (32-35). Nerve cells may employ several mechanisms of protection in response to Lead exposure including modification in NO production. NO plays a key role in morphogenesis, synaptic plasticity, and regulates release of neurotransmitter (36). Any alterations in the amount of NO may affects the neural functions. On the other hand, chronic exposure to Lead (Pb) affects neural functions in central nervous system (CNS) particularly the learning and memory by blocking voltage dependent calcium channels in CNS. While from the theoretical point of view, alteration of calcium level in the CNS results in activation of NOS where it is expected to increase nitric oxide level in hippocampus; our previous results did not support this idea. Therefore, it is possible that Lead inhibits elevation of NO through another mechanism such as the decrease in the effect of NMDA receptor on NO production. In this study the effect of Lead on NO production by CA1HP cultured cells following

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exposure to NMDA receptor agonist, ACBD was investigated.

MATERIALS AND METHODS

Materials
Trypsin 0.025% (Gibco, UK), 50 µm nylon filter (Portex, UK), Dulbecco modified eagle medium (DMEM; Gibco, UK), fetal calf serum (FCS; Gibco, UK), horse serum (HS; Gibco, UK), L-glutamine (Sigma, UK), Poly-D-Lysine (Sigma, UK), cell culture plasticware (Nunc, Denmark), tetrahydrobiopterin (BH4; Sigma, UK), ACBD (Tocris, UK), 3-[4,5-dimethyl thiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT; Sigma, UK), Anti-MAP2 antibody (Calbiochem, USA), FITC-immunoflurescent antimouse IgG (Sigma, UK) and other reagents were purchased from local distributor from high quality companies.

Methods

Preparation of CA1HP cells
Pregnant Sprague-Dawley rats (300-400 g) were purchased from Iranian Pasteur Institute and housed in a room controlled at 23 ± 2°C with controlled lighting conditions (12 hrs light and dark cycles). Food and water were provided ad libitum. The hippocampus of one-day-old pups were removed aseptically (10 pups in each experiment in three separate occasions). The tissue was then incubated in dissociation medium (90 mM Na2SO4, 30 mM K2SO4, 5.8 mM MgCl2, 0.25 mM CaCl2 and 10 mM HEPES with the pH adjusted to 7.4) containing 0.025% trypsin for 20 minutes. Cells were then filtered through 50 µm nylon filter. Followed by washing in Dulbecco Modified Eagle culture medium (DMEM) containing 5% FBS, 5% HS, 400 µM L-glutamine and 17 mM D-glucose (37). The dissociated cells were plated at a density of approximately 5.6 x 10^5 cells/ml in 35 mm poly-D-Lysine coated plates. Non-neural cells were omitted by 24 hrs exposure to cytosine arabinoside (37).

Nitric oxide measurement
NO was measured according to the method of Richter et al (39). Briefly, in a prewarmed (37°C) cuvette containing the buffer (0.1 M HEPES, pH 7.0), the NOS substrates and reagents were added as follows: 1mM L-Arginine, 1 mM CaCl2, 0.2 mM NADPH, 0.5 µM flavine mono nucleotide (FMN) and 10 µM tetrahydrobiopterin (BH4). This mixture is highly unstable in dilute solution due to auto-oxidation. To measure NO, 4 µM HbO2 (oxyhaemoglobin) which was prepared according to Di lorio (38) was added to the cocktail, mixed gently and absorbance was recorded at 401 nm. This cocktail was then added to the cells and incubated in 37°C for 20 minutes, and MetHb (methemoglobin) formation was measured by increasing in the absorbance at 401 nm (39).

Lead administration to the cultured cells
The CA1HP cells were purified as described above and at the second day were exposed to different concentrations of Lead acetate (10^-6 M) for 7 days. Then ACBD at concentration of 40 µM was added to the culture medium of the test group 15 minutes before NO measurement. NO was measured as explained in section 2.2.

Cell viability tests
a) Trypan blue dye exclusion test: 0.4% v/v trypan blue was added to the cell suspension (cells were prepared by trypsinization) and those cells which were not stained by the dye were counted by light microscopy.
b) Determination of mitochondrial dehydrogenase activity (MTT)
MTT (3-[4, 5-dimethyl thiazol- 2-yl]-2, 5-diphenyl tetrazolium bromide) was added (100 µl) 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 (55). The crystals were dissolved in 300 µl of the acidified isopropanol and the absorbance of the resulting purple solution was measured at 570 nm against 690 nm for blank solution. The amount of produced formazan is directly proportional to the number of viable cells.

Immunocytochemistry
Cultured neurons were stained with monoclonal anti-MAP2 antibody that recognizes phosphate independent epitope of the 280 KDa cytoskeletal MAP2 protein. Briefly, cells were fixed in 4% paraformaldehyde at room temperature for 4 minutes followed by washing with PBS and incubation in blocking reagent for 30 min. Then, cells were incubated with the anti-MAP2 antibody (1:100) in blocking reagent for 3 hrs at room temperature. Visualization was carried out using the FITC-immunofluorescent anti-mouse IgG. The numbers of the immunoreactive neurons were determined under the microscope (Olympus B201, Japan).

Statistical analysis
The data in each group were examined by paired student t-test (figures 1, 2 and 4) and one way ANOVA with Tukey post test (figure 5). Probability less than 0.05 (p<0.05) was assumed significant.

RESULTS
Figures 1 and 2 show the viability of cells exposed to the Lead acetate and sodium acetate using trypan blue dye exclusion and MTT assays. The viability of cells was remained unchanged at all
concentrations of Lead which were added to the culture medium. Figure 3 shows time course study of NO production in untreated hippocampus cells. The optimum time for measurement of NO (figure 3) was about 20 minutes after addition of the substrate for nitric oxide synthase. Figure 4 shows the effect of different concentrations of Lead acetate on in vitro production of NO by cells. Except one concentration (100 nM), there was no difference in the NO production by cells after exposure to different concentrations of Lead acetate. In all experiments different concentrations of sodium acetate were also studied as control for the effect of acetate ion in the NO production. On the sixth day after cell plating without treatment by cytosine arabinoside (Ara-C), it was impossible to distinguish between glial and neural cells under the phase-contrast microscope. The development of axons and dendrites were observed equally. More than 95% of cells were neuron cells when treated by Ara-C. The number of neurons, which were immunoreactive for MAP2 in six different 1-mm² areas in each well were measured. Picture 1 show neural cells which were stained with MAP2 antibody and FITC secondary staining system.

**Picture 1.** The neuronal cells from CA1 area of hippocampus in culture was stained by Antibody against MAP2a, b and conjugated to the FITC secondary antibody. The positive neuron has been detected by green light under fluorescent microscope. (×100)

Figure 5 shows the effect of concurrent exposure of cells to 40 μM ACBD and Lead (100 and 10 nM) on NO production. No marked difference was observed between the amounts of NO production in control group and the groups that were exposed to Lead acetate (100 and 10 nM). The NO increased significantly in the group that were exposed to 40 μM ACBD. In the group that was given 100 nM Lead acetate and 40 μM ACBD concurrently, no significant difference was observed in comparison to control group. However it was significantly different from the group that was treated with 10^{-8} M Lead acetate and 40μM ACBD concurrently. This difference was less than group that was exposed to ACBD alone [F (5, 42) = 33.218, p<0.05].

**DISCUSSION**

In the present study it is shown that chronic Lead exposure impairs elevated NO production by stimulation of NMDA agonist in hippocampal neurons. In neurons, NO synthesis is stimulated by Ca²⁺-influx which is induced by activation of glutamate receptors, preferentially NMDA receptors (40, 41, and 42). At least in some areas of the brain, there is a basal NO production, which causes synthesis of cGMP (43, 44, 17 and 18). The outflow of cGMP is greatly increased by activation of kainate/AMPA and NMDA receptors, as well as by electrical stimulation of pathways related to the excitatory amino acid utilizing neurons. Increase in the concentration of Ca²⁺ reverses the inhibitory effects of Pb²⁺ suggesting that the effect of Pb²⁺ on nNOS is in part due to its ability to compete with Ca²⁺. This is in agreement with the report which has shown that Pb²⁺ can displace Ca²⁺ from calmodulin (7). The hippocampus has been suggested as a site of Lead toxicity (46). Moreover, it has been shown that Pb²⁺ can be accumulated in the hippocampus (47). However, it has been claimed that the basis for any suggestion about selective vulnerability of hippocampus is not due to a preferential Pb²⁺ accumulation. Instead, Pb²⁺ may interact with cellular targets and alter biochemical or cellular processes that are uniquely associated with, or greatly enhanced by Pb²⁺ in a particular region (48). Since the majority (85-90%) of hippocampus cells consists of pyramidal cells (49), the function of nNOS in these cells in relation to Pb²⁺ toxicity were investigated. The inability of sodium acetate to affect production of nitrite by nNOS suggests that the decrease in nitrite production may be attributed to Pb²⁺ rather than to the acetate (50). Furthermore, it was observed that the inhibition of nitrite production by nNOS occurred at concentrations of Pb²⁺ that did not alter; pyramidal cell morphology, cell membrane leakage, or the rate of ATP production (Fig. 1 & 2). It has been shown that Pb²⁺ and other heavy metal ions can interfere with nNOS activity through Ca²⁺ ion. However, this interaction usually occurs in a concentration higher than those in chronic toxicity (100 μM compared to 1 μM in whole brain respectively) (51). It has also been shown that Pb²⁺ can interfere with NMDA receptors at concentration of 5-20 μM (54) which is almost the same concentration which is achieved in chronic Lead toxicity (48). Pb²⁺ interacts with the NMDA receptor complex and inhibits receptor activation (52, 10). It has been suggested that the effects of Pb²⁺ on the NMDA receptor complex
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**Figure 1.** The measurement of viability for pyramidal cells in presence of lead and sodium acetate by using trypan blue method as described in method section (Mean ± SD, n=8)

**Figure 2.** The measurement of viability for pyramidal cells in presence of lead and sodium acetate by using MTT assay as described in method section (Mean ± SE, n=8)

**Figure 3.** The time of incubation for the enzyme substrate reaction in the culture of hippocampal pyramidal cell culture. Note the minimum time for the best result is 20 minutes (Mean ± SD, n=6)

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

**Figure 5.** The effect of concurrent exposure of pyramidal cells to 40 µM ACBD with $10^{-7}$ and $10^{-8}$ M lead on the NO production (Mean SD, n=8)
may be, at least in parts responsible, for learning deficits. This effect of Pb$^{2+}$ has been shown in experimental animals and human beings which were exposed to Pb$^{2+}$ during the early stages of development (52, 12). This effect of Pb$^{2+}$ was very similar to that which was observed for the divalent cation Zn$^{2+}$, an allosteric modulator of the NMDA receptor. In fact Pb$^{2+}$ may inhibit NMDA receptor activation via an interaction at a Zn$^{2+}$ allosteric site (53). In present study, exposure to the Pb$^{2+}$ did not alter the basal amount of NO indicating that Pb$^{2+}$ did not change the basal release of NO in these cells (Figure 4). Our results showed that following addition of NMDA agonist (ACBD) to culture medium of cells, the level of NO was significantly increased (P<0.05). Concurrent treatment of cells with Pb$^{2+}$ (10 and 100 nM) and ACBD decreased the NO production compared to ACBD alone which indicates that NO production through this pathway is influenced by Pb$^{2+}$ exposure. Although Lead by itself did not change production of NO of pyramidal cells, it may affects learning and memory due to negative interference in NO production induced by NMDA agonist. However this mechanism requires further studies to be established.

REFERENCES

7. Habermann E, Crowell K and Janicki P. Lead and other metals can substitute for Ca$^{2+}$ in calmodulin, Arch. Toxicol., 1983; 54: 61-70
18. Dudek SM. and Bear MF. Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. Proc Nat Acad Sci USA 1992; 89: 4363-4367
28. Schuman EM and Madison DV. Locally distributed synaptic potentiation in the hippocampus. Science 1994; 263: 532-536
35. Schilling K, Schmidt HHHW. and Baader SL. Nitric oxide synthase expression reveals compartment of cerebellar granule cells and suggests a role for mossy fibers in their development. Neurosci 1994; 59: 893-903
44. Vallebuona F, Raiteri M. Extracellular cGMP in the hippocampus of freely moving rats as an index of nitric oxide synthase activity. J Neurosci 1994; 14: 134-139
52. Alkondon M, Costa ACS, Radhakrishnan V, Aronstam RS. and Albuquerq EX. Selective blockade of NMDA activated channel currents may be implicated in learning deficits caused by Lead. FEBS Let 1990; 261: 124-130