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Original Article

Lead exposure impairs the NMDA agonist-induced NOS expression 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. It has been shown that lead enters the neurons through calcium channels and displaces Ca^{2+} from calcium binding proteins such as calmodulin and troponin C thereby affecting calcium-mediated processes.

Our recently data showed that no prodaction due to NMDA receptor simulation in cultured CA1 pyramidal cells has been diminished in the presence of 10 nM of Lead acetate. Therefore, it is possible that Lead can inhibit the elevation of NO through blockade of NMDA receptor and interference of LTP through this mechanism. This finding may attribute to the effect of lead on the NOS activity or expression as key enzyme producing NO. In this study we have examined the effect of lead acetate on the NOS expression in the presence of NMDA agonist using immunocytochemical analysis. Expression of nNOS were examined in the CA1 pyramidal cells exposed to 10 and 100 nM lead acetate and 40 μM ACBD (NMDA agonist). The result of this experiment showed that the enhanced nNOS expression induced by ACBD significantly diminished by lead acetate. The trend of this inhibition is similar to amount of NO production indicating that the decrease of expression may major reason of decrease in NO production.

Keywords: Lead acetate; ACBD; NMDA agonist; Pyramidal cell; nNOS; Expression.

Introduction

Lead (Pb) is a heavy metal environmental toxicant that possesses a significant health threat, particularly to the development of CNS in infants and children (1-3). Furthermore, Pb is known to be a potent neurotoxin, inducing neuronal damage and behavioral disruptions (4, 5). The neurological effects of low level of Pb exposure range from impaired cognitive performance to altered brain development (6, 7). During brain development, chronic exposure to environmental levels of Pb results in accumulation of this metal at its highest level in the hippocampus (8). This has been the main hypothesis to explain why learning and memory are affected by chronic exposure to Pb (9). In this regards, Altmann et al. have reported that acute lead perfusion of hippocampal slices as well as chronic lead exposure impaired long-term potentiation (LTP) in CA_1 area (10, 11). It is known that activation of NMDA receptors which are densely distributed in the mammalian CNS and participate in several forms of synaptic Plasticity (12- 14), is critical for the induction

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of LTP (15, 16). However, the role of NMDA receptors in the Pb neurotoxicity has not been well defined. It has been reported that Pb blocks LTP in rat brain slice of hippocampus (17, 18) through mechanisms which may (19) or may not (20) involve interference with the NMDA receptors. The influx of calcium through NMDA receptor channels activates a cascade of events that lead to persistent changes in synaptic efficacy (21, 22). Despite 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 LTP generation without persistently altering baseline synaptic responses (23).

Other neurotransmitters have been proposed in the mechanisms of memory and LTP. Recent evidence supports nitric oxide (NO) as a retrograde messenger mediating LTP in the hippocampus (24-27) and in a similar process in the cerebellum called long-term synaptic depression (28). NO is produced by nitric oxide synthase (NOS) from L-arginine in an oxygen and NADPH requiring reaction. The constitutive form of brain NOS is Ca^{2+} -calmodulin dependent (29, 30) and NOS activity may be regulated by phosphorylation and affected by Ca^{2+} (29, 31, 32). It has been shown that NO plays a key role in morphogenesis and neuronal plasticity in the early brain development (33- 36) as well as synaptic plasticity and normal physiological regulation of the nervous system (37, 38). Therefore, changes in NO production could affect its regulatory role in CNS. It is known that chronic exposure to Pb affects neural functions in CNS particularly the learning and memory by blocking voltage dependent calcium channels. Since NO production in neuronal cell is Ca^{2+} dependent, alteration in calcium level in neuron could result in lower NO production in hippocampus. Our recent data showed that stimulation of NMDA receptor enhances NO production in cultured CA1 pyramidal cells diminished in the presence of 10 nM of lead acetate . Therefore, it is possible that lead can inhibit the elevation of NO through blockade of NMDA receptor and therefore can interfere with LTP through this mechanism (39).This finding may attribute to the effect of lead on the NOS activity or expression as key enzyme producing NO. In this study we have examined the effect of lead acetate on the NOS expression in the presence of NMDA agonist using immunocytochemical analysis.

Experimental

Preparation of CA1 hippocampal (CA1HP) cells

Pregnant Sprague-Dawley rats (300- 400 gr) were purchased from Iran Pasteur Institute and housed in a room controlled at 23 ± 2°C with controlled lighting conditions (12/12 hrs light /dark cycles) with food and water 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 Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 0.25 mM $CaCl_2$ and 10 mM HEPES with the pH adjusted to 7.4) containing 0.025% trypsin (Gibco, UK) for 20 minutes. Cells were then filtered through 50 μm nylon filter and washed in Dulbecco Modified Eagle culture medium (DMEM, Gibco, UK) containing 5% fetal bovine serum (FBS, Gibco, UK), 5% horse serum (HS, Gibco, UK), 400 μg L-glutamine and 17 mM D-glucose (40). 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 (Nunc, Denmark). Non-neural cells were omitted by 24 hrs exposure to cytosine arabinoside (Sigma, UK) (40).

Immunocytochemistry

Determination of MAP2 antigen

Cultured neurons were stained with monoclonal anti-MAP2 antibody that recognizes phosphate independent epitope of the 280 KD a cytoskeletal MAP2 protein (Calbiochem, USA). Briefly, cells were fixed in 4% paraformaldehyde at room temperature for 4 min, followed by washing in 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-conjugated anti-mouse IgG (Sigma, UK). The number of the immunoreactive neurons was determined under the fluorescent microscope

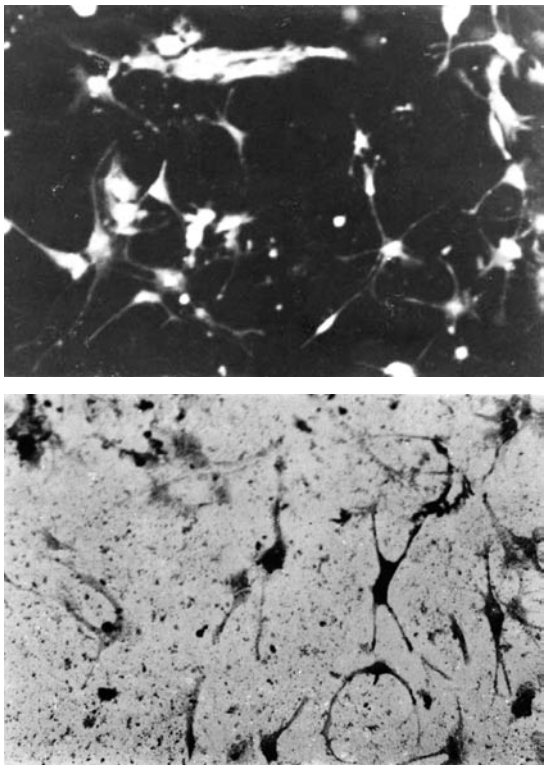


Figure 1. (Top) shows the immunofluorescence of anti-MAP2 antigen on the surface of pyramidal cells in culture (Bottom) The cell were stained with Meyer Haematoxylene (X100).

(Olympus B201, Japan).

Determination of NOS expression

Immunocytochemistry for nNOS was done as previously described (55). The anti-nNOS antibody (which corresponded to the N- terminal region of nNOS, Santa Cruz USA) was used at a 1:40 dilution of purified antibody (nNOS; 200 µg/ml to 5 µg/ml; Satna Cruz USA) have been used for specific immunoreactivity on cultured pyramidal cells, followed by incubation with secondary Streptavidin-HRP conjugated antibody. Briefly, the cells were fixed in methanol-acetone (1:1 v/v) for 10 minutes at 4°C and were washed twice with 3% hydrogen peroxide in order to remove endogenous peroxidase. The cells were then incubated in bovine serum albumin (BSA) 1% w/v for 1 hour to block nonspecific proteins and then washed twice with tris buffer (0.05 M). The cells were then incubated in humidified chamber with primary mouse antibody against NOS for 24 hours in 4°C (Optimum concentration of primary antibody 1:50) and were then washed

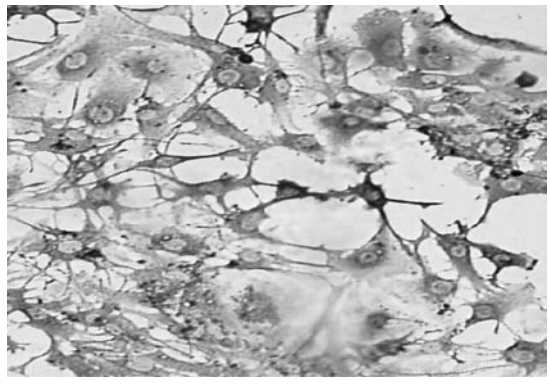


Figure 2. The CA1 pyramidal cells in culture after 8 days. Connection between cells is noted. Stained with nNOS antibody and secondary staining system with DAB chromogen (X200).

twice with Tris buffer (pH. 7.2). The cells were incubated for 2 hours in 1–3 drops of biotinylated secondary antibody. Rinse with PBS, and then wash in PBS twice for 2 minutes each on stir plate and stained as mentioned in the Santa Cruz kit (LSAB2 kit). Positive cells were counted in 1000 cells in 4 different slides. Specificity of nNOS antibody was examined by omission of the primary antibody. The results were reported as percent of control and statistically analyzed using one way ANOVA followed by Tukey multiple comparison post test and $p<0.05$ were considered significance.

Lead administration to the cultured cells

The CA1HP cells were exposed at day second of culture to different concentrations of lead acetate (10^{-9}-10^{-6} M) for 7 days. The ACBD (NMDA agonist) at concentration of 40 µM was added to the culture medium at the beginning of culture of hippocampal cells. At day seven the cells were fixed by methanol: acetone for 5 minutes in the refrigerator followed immunocytochemistry as explained previously.

Results

Figure 1 shows the immunofluorescence of anti-MAP2 antigen on the surface of pyramidal cells demonstrating the purity of pyramidal cells in culture which has been calculated more than 98% with the mentioned method. The pyramidal cells obtained from CA1 region of one day old of neonate rat were successfully grown in the culture in vitro. After 8 days they showed

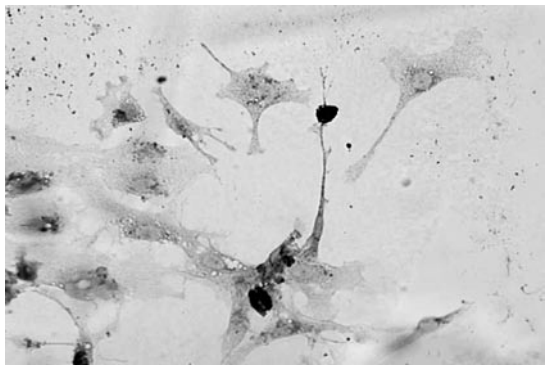


Figure 3. The CA1 pyramidal cells with stained with LSAB2 system without primary antibody against nNOS but stained with Meyer Haematoxylene. Note that no stained with DAB chromogen (X400).

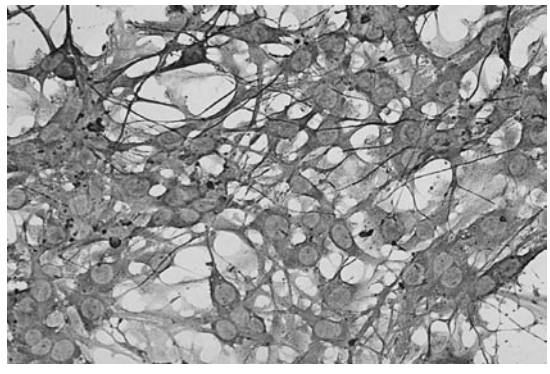


Figure 4. The CA1 pyramidal cells in culture treated with 40 μM ACBD (NMDA agonist). Note the expression of nNOS increased with this treatment. The cells stained with nNOS primary antibody with secondary LSAB2 kit with DAB chromogen (X200).

cell- cell connection properly and expressed constitutive nNOS (Figure 2). Elimination of primary antibody resulted in disappearance of nNOS expression indicating specificity of method for nNOS as antigen (Figure 3). Cells were all counterstained by Meyer Haematoxylene. ACBD at 40 μM concentration induced the expression of nNOS in these cells (Figure 4). On the other hand 100 nM of lead acetate did not alter the pattern of nNOS expression (Figure 5) comparing to control; however, it significantly reduced the ACBD-induced nNOS expression (Figure 6, $p<0.01$). Figure 7 shows the semiquantitative measurement of nNOS expression using digitized imaging system Olysia Software (Olympus DP70, Japan). As indicated in figure 7, ACBD as an NMDA agonistsignificantly increased NOS expression comparing to control group ($p<0.01$). The NOS expression induction significantly reduced in the presence of 10 and 100 nM of lead acetate (Figure 7).

Discussion

Nitric oxide (NO) is a lipophilic and chemically unstable free radical. NO also serves as a neuronal messenger since cerebellar neurons release an NO-like muscle relaxing factor (41) which is not stored in the vesicles but is produced on demand from L-arginine by the constitutive form of NOS (42). Recent research reports have confirmed that the distributed NOS in various regions of the brain produce NO (43). The NO may possess both neurodestructive and neuroprotective properties (44, 45). Neuronal nitric oxide synthase (nNOS) is a calcium dependent enzyme, and have been reported

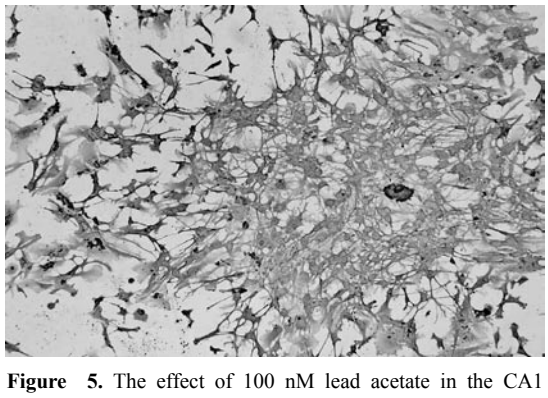


Figure 5. The effect of 100 nM lead acetate in the CA1 pyramidal cells in culture. The cells stained with nNOS primary antibody following secondary DAB immunostaining system. Note no apparent different expression of nNOS compare with basal expression has been observed (X100).

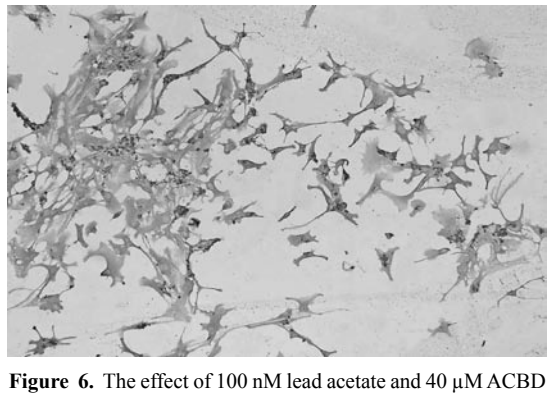
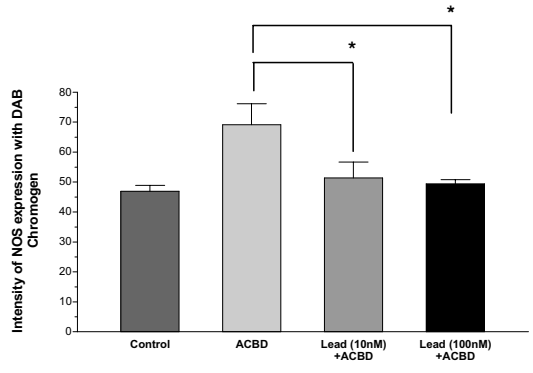


Figure 6. The effect of 100 nM lead acetate and 40 μM ACBD concurrently administered to CA1 pyramidal cells in vitro. Note that the induce effect of ACBD on nNOS expression has been diminished. The cells stained with nNOS primary antibody following DAB-HRP immunostained (X100).



The effect of concurrent exposure of pyramidal cells to 40 µM ACBD with 10 and 100 nM lead on the NO production.

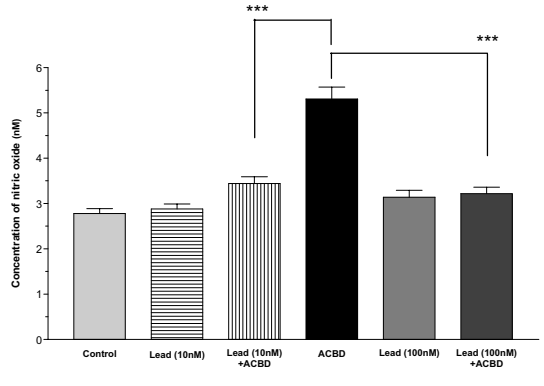


Figure 7. The effect of lead acetate and ACBD at NOS expression in CA1 pyramidal cells (n=4).

to be highly expressed in the cerebellum and the hippocampus (46, 47). Lead is known to exhibit a high affinity for calcium binding sites (48, 49). This could prevent accessibility of calcium to NOS, leading to a decreased activity of nNOS and reduced production of NO. This idea was supported by the results obtained from an in vitro model where lead inhibited the Ca^{2+} - calmodulin dependent NOS prepared from rat cerebellum (50), and from the whole brain cytosolic fractions (51, 52). However our previous results showed that the basal nNOS expression did not alter with 10 and 100 nM of lead acetate of hippocampal neurons, the concentration that usually achieved during chronic exposure. In the present study, our results showed that in hippocampal pyramidal cells, lead exposure impairs NOS expression induced by NMDA agonist. Neurons treated with lead (10 and 100nM) did not show any alterations in nNOS expression. However, the ACBD induced NOS expression was completely blocked to control level (Figure1), suggesting the role of lead on the excited neuron. Our previous study revealed that NMDA agonist can enhance net nitric oxide production in pyramidal cells (53). Howeverin the present study, lead acetate diminished the ACBD induced NO production. This effect was observed only when NMDA receptors on neurons were activated therefor lead acetate solely could not change the amount of nitric oxide production. One possible explanation for this phenomenon is the involvement of lead in changing expression of NOS as key enzyme for nitric oxide production through blockade of NMDA receptor. Our results confirmed that trend of NOS expression is in accord with NO production observed in our previous study. Furthermore, we observed that the inhibition of NO production by nNOS occurred at concentrations of Pb^{2+} that did not alter pyramidal cell morphology, induce cell membrane leakage or alter the rate of ATP production. This result may attribute to the alteration of NO as a result of alteration of NOS expression by lead in pyramidal cells. In vivo exposure to low level of Pb^{2+} during development impairs spatial learning and LTP and alters gene and protein expression of NMDA receptor in the hippocampus (54). Other reports were also confirmed the result, of our studies indicatins that lead affect, the NO production through NMDA receptor. Furthermore, this decrease in NO level may happens through decrease of NOS expression in the pyramidal cell.

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