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Lead increases Nitric Oxide Production in Immunostimulated Glial Cells

Min Sik Choi[1], Chan Young Shin[1], Jae Ryun Ryu[1], Woo Jong Lee[1], Jae Hoon Cheong[2], Chang-Rak Choi[3], Won-Ki Kim[4], and Kwang Ho Ko[1,*]

[1]*Department of Pharmacology, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul*
[2]*College of Pharmacy, Sahmyook University, Seoul,*
[3]*Department of Neurosurgery, St.Marys Hospital, The Catholic University of Korea, Seoul,*
[4]*Department of Pharmacology, Ewha Institute of Neuroscience, Ewha Medical School, Seoul, Republic of Korea*

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Abstract – Lead has long been considered as a toxic environmental pollutant that severely damages the central nervous system. In various neurodegenerative diseases, astrocytes become activated by proinflammatory cytokines. In the present study, we investigated whether lead (Pb^{2+}) affects inducible nitric oxide synthase (iNOS) expression in activated glial cells. Rat primary glial cells were stimulated with lipopolysaccharide (LPS, 1 μg/ml) plus IFNγ (100 U/ml). Pre-treatment of Pb^{2+} increased nitric oxide (NO) production in LPS/IFNγ-stimulated glial cells. Lead itself, however, suppressed the basal production of NO in control glial cells. Addition of the iNOS inhibitors L-NAME (1 mM) and L-NNA (800 μM) prevented the Pb^{2+}-induced increase in NO production. Western blot analysis showed that pre-treatment of Pb^{2+} augmented LPS/IFNγ-induced increase in iNOS immunoreactivity, which was well correlated with the increased NO production. In addition, pre-treatment of Pb^{2+} synergistically increased the iNOS mRNA expression induced by LPS and IFNγ. The present results indicate that lead intoxication adversely affect brain function by potentiating iNOS expression and NO production in activated glial cells observed in various neurodegenerative diseases.

Keywords □ Lead (Pb^{2+}), primary glial cells, lipopolysaccharide, nitric oxide, iNOS

Lead has long been considered as a heavy metal environmental toxicant that causes serious health problems, especially to the developing central nervous system (CNS) of infants and children (McMichael *et al.*, 1988; Mushak *et al.*, 1989; Petit, 1986). A long-term intoxication to low levels of lead causes significant impairment of cognitive function and delays in behavioral development (Needleman *et al.*, 1990; Bellinger and Dietrich, 1994).

In many neurological diseases such as ischemia, stroke, trauma, Alzheimer's disease, multiple sclerosis and AIDS dementia, the activation of glial cells by cytokines is commonly observed (Shrikant *et al.*, 1996). The activated glial cells produce NO to ultimately determine the pattern and degree of functional recovery of the CNS (Bruhwyler *et al.*, 1993; Nakashima *et al.*, 1995). Due to its cytotoxic activity, nitric oxide (NO) has been implicated in a large number of pathologies (Bruhwyler *et al.*, 1993). The cytotoxicity of NO is increased by reaction with superoxide anion ($O_2^{\cdot-}$) to form the highly reactive $ONOO^-$ (Beckman et al., 1990). Several researchers including us have reported that NO from cytokine-stimulated microglia or astrocytes potentiated NMDA-mediated or glucose deprivation-induced neural/glial toxicity (Hewett *et al.*, 1994, 1996; Choi and Kim, 1998; Kim and Ko, 1998; Kim *et al.* 1999a, b).

Certain pathophysiological effects of Pb^{2+} may be mediated by modulation of the production of NO or its congeners. However, the modulating effect of Pb^{2+} on iNOS gene expression and NO production seems to depend on the cell types and stimuli. Previously, Pb^{2+} was reported to increase iNOS expression and NO production in a pancreatic β-cell line RINm5F stimulated with IL-1β (Eckhardt *et al.*, 1999). In freshly isolated splenic macrophages, however, Pb^{2+} decreased iNOS activity without affecting the induction of iNOS expression (Tian and Lawrence, 1995, 1996). In murine brain endothelial cell culture, Pb^{2+} did not alter iNOS activity but inhibited eNOS activ-

*Corresponding author
Tel: 82-2-880-7848, Fax: 82-2-885-8211
E-mail: khk123@snu.ac.kr

ity by 50% (Blazka *et al.,* 1994).

At present, however, there is no clear demonstration of the interactive effect of Pb^{2+} on NO production in activated primary glial cells. In the present study, therefore, we investigated the effect of Pb^{2+} on NO production in rat primary glial cells stimulated with IFN-γ and lipopolysaccharide (LPS).

MATERIALS AND METHODS

Materials

LPS and $PbCl_2$ were purchased from Sigma (St. Louis, MO). Recombinant rat IFN-γ, DMEM/F12, glucose-free DMEM and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY). All other reagents were obtained from Sigma (St. Louis, MO).

Primary rat glial cell culture

Rat primary glial cells were cultured from the frontal cortices of 2- to 4-day-old Sprague-Dawley rat pups, as previously described (Kim *et al.,* 1999b). Briefly, cortices were freed of meninges and digested with trypsin for 10 min. Cells were carefully triturated and washed by centrifugation. After resuspension in normal growth media (DMEM/F12 supplemented with 10% FBS), cells were cultured in a T-75 culture flask. Upon confluence, cells were harvested with trypsin-EDTA and replated on 24-well culture plates. In this study, 80% confluent cells were used for experiments.

Determination of NO

NO production from immunostimulated cells was determined by measuring nitrite, a stable oxidation product of NO, as described previously (Green *et al.*, 1990). In brief, nitrite levels were determined by adding the Greiss reagent (mixing equal volumes of 0.1% napthylethylenediamine dihydrochlroride and 1% sulfanilamide in 5% phosphoric acid). After 10 min, the absorbance at 550 nm was determined using a UV spectrophotometer (Beckman DU-650, Fullerton, CA).

Measurement of DCF fluorescence

Glial cells were loaded with 2,7-dihydrodichlorofluorescein diacetate (DCF-DA, 30 μM) in PBS for 10 min and then rinsed with the same solution. After a 10-min incubation at room temperature, the fluorescence of DCF was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm (slit widths 20 and 25 nm, respectively) using a fluorescence microplate reader (TECAN, Austria). DCF-DA diffuses through cell membranes and is subsequently enzymatically deacetylated by intracellular esterases to the non-fluorescent DCF-H. Oxidants such as $ONOO^-$ effectively convert DCF-H to the highly fluorescent DCF (Possel *et al.*, 1997). Fluorescence intensities were corrected for autofluorescence (i.e., fluorescence of cells not loaded with DCF-DA).

Western blot

Total cellular proteins were isolated by incubating the cells with 100 μl of lysis buffer, which containing 1% Triton X-100. The samples were mixed with an equal amount of 2 x SDS-sample buffer and treated at 100°C for 3 min prior to electrophoresis on 8% SDS polyacrylamide gel. The resolved band was electrotransferred onto nitrocellulose (NC) membrane. The NC membrane was blocked with Blotto (5% non-fat dried milk in PBS/0.2% Tween-20) and then incubated at room temperature for 2 h with antibodies against rat iNOS which were diluted appropriately in Blotto, as suggested by the manufacturer (Transduction Laboratories, Lexington, KY). After three 10 min washes with PBS/Tween-20, the NC membranes were incubated with peroxidase labeled goat anti-mouse IgG at room temperature for 2 h. After extensive washing with PBS-Tween, the membranes were developed by enhanced chemiluminescence (ECL, Amersham, Buckinghampshir, UK).

RT-PCR

Total RNA was obtained from primary glial cells by Trizol method (GibcoBRL, Grand Island, NY). Reverse transcription was performed for 1h at 42°C with 5 μg of total RNA using 1 unit/μl of superscript II reverse transcriptase (GibcoBRL, Grand Island, NY) according to the manufacturer's instruction. Oligo $(dT)_{18}$ was used as a primer for this reaction. The samples were then heated at 94°C for 5 min to terminate the reaction. The cDNA obtained from 1.2 μg total RNA was used as a template for PCR amplification. Oligonucleotide primers were designed based on Genebank entries for the rat iNOS (accession number U03699). The following primers were used for the amplification reaction:

forward primer : 5'-CAAGAGTTTGACCAGAGGACC-3'
reverse primer : 5'-TGGAACCACTCGTACTTGGGA-3'

PCR mixes contained 10 μl of 10 X PCR buffer, 1.25 mM of each dNTP, 100 pmol of each forward and reverse primer and 2.5 units of Tag polymerase (Takara, Shiga, Japan). For amplification, the following PCR incubation times were used: 94 for

5 min followed by 30 cycles of 94°C for 30 s, 55 for 30 s, 72 for 1 min and continued by 72°C for 10 min. The amplified PCR products were electrophoresed and analyzed on 1.5% agarose-gel. For comparison, RT-PCR reaction was also performed for the housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The expected size of the amplified DNA fragments was 654bp for iNOS and 308bp for GAPDH.

Statistical analysis

Data are expressed as the mean ± standard error of mean (S.E.M) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Newman-Keuls test as a *post hoc* test and a p value <0.05 was considered significant.

RESULTS

At concentrations (1-20 μM) used in this study, Pb^{2+} itself suppressed the basal NO production in glial cells. Thus, a 48-h treatment of 1 μM Pb^{2+} decreased basal NO production in glial cells by 75% (data not shown, n=6. $p<0.01$). Immunostimulation of glial cells with LPS (1 μg/ml) plus IFNγ (100 U/ml) for 48 h significantly increased NO production (data not shown). Pretreatment with Pb^{2+} for 48 h further increased the immunostimulation-induced NO production (Fig. 1A). The potentiating effect was observed only after 12 h or longer pre-incubation with Pb^{2+} (Fig. 1B). Other divalent cations such as Cu^{2+} and Zn^{2+} (1~20 μM) did not alter the immunostimulation-induced NO production in primary glial cells (data not shown). Pretreatment of Pb^{2+} did not alter cell viability or the proliferation of immunostimulated rat primary glial cells, as evidenced by MTT reduction analysis and manual cell counting after trypsin/EDTA digestion (data not shown). Interestingly, the generation of reactive oxygen species (ROS) evoked by LPS (1 μg/ml) plus IFNγ (100 U/ml) was not altered after 48-h treatment with Pb^{2+} (Fig. 2).

To investigate whether the increased NO production was mediated by iNOS activation, we treated glial cells with NOS inhibitors N^G-nitro-L-arginine (NNA, 800 mM) or Nw-nitro-L-arginine methyl ester (L-NAME, 1 mM) during the immunostimulation period. Both inhibitors, NNA and L-NAME, markedly inhibited NO production in immunostimulated glial cells pre-treated or not pre-treated with Pb^{2+}. In the control group, NO production was increased in Pb^{2+} pre-treated immunostimulated glial cells by 162% compared with immunostimulated glial cells, and this increase by Pb^{2+} pre-treatment was completely blocked by NNA and partially blocked by L-NAME (Fig. 3). Pretreatment with Pb^{2+} concentration-dependently increased iNOS mRNA levels and immunoreactivity in immunostimulated rat primary glial cells (Fig. 4). Pb^{2+} did not itself change the basal iNOS mRNA levels and immunoreactivity in control rat glial cells (data not shown).

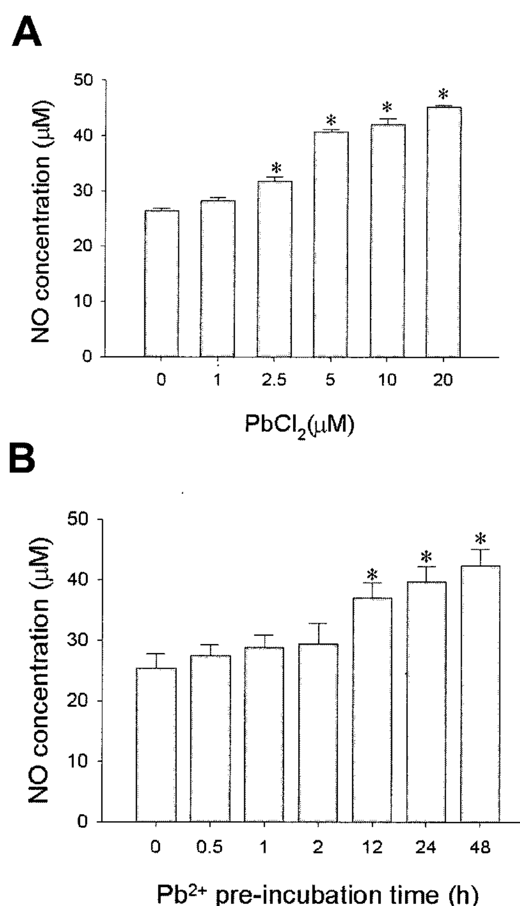


Fig. 1. Pre-treatment with $PbCl_2$ increases NO production in primary rat cortical astrocytes. A) Concentration-response relationship. Cells were stimulated for 48 h with various concentrations of $PbCl_2$. After thorough wash, the cells were stimulated with LPS (1 μg/ml) plus IFNγ (100 U/ml) for additional 48 h. B) Time dependency. Cells were pretreated with 5 μM $PbCl_2$ for the indicated times and then stimulated with LPS (1mg/ml) plus IFNγ (100 U/ml) for additional 48 h. NO levels, as assessed by measuring the formation of nitrite, were determined by using Griess reaction. All Data are expressed as a net increase of NO production and are plotted as the mean S.E.M. (n=4). *$p<0.05$, significantly different from $PbCl_2$-untreated control.

DISCUSSION

The present study demonstrated that at low micromolar concentrations Pb^{2+} increased NO production in activated glial cells by increasing the expression level of iNOS. The increased

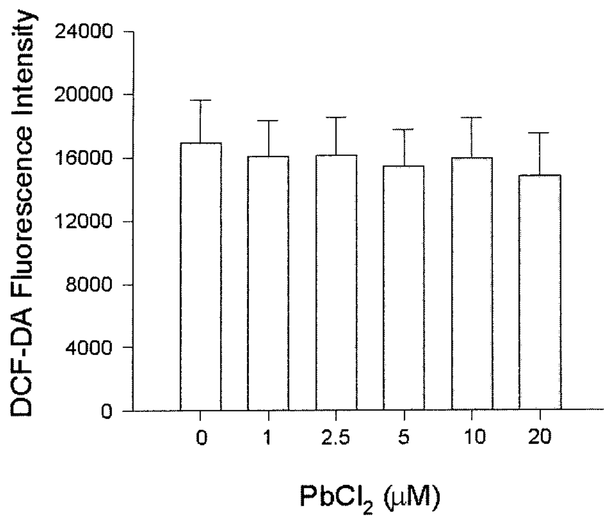


Fig. 2. Pre-treatment with $PbCl_2$ does not change ROS produc-tion in primary rat cortical astrocytes. Cells were stimulated for 48 h with various concentrations of $PbCl_2$. After thorough wash, the cells were stimulated with LPS (1 μg/ml) plus IFNγ (100 U/ml) for additional 48 h. The production of ROS in astrocytes was monitored by using H_2DCF-DA, as described in the Materials and Methods. All Data are expressed as a net increase of DCF fluorescence intensity and are plotted as the mean± S.E.M. (n=4). *$p<0.05$, significantly different from $PbCl_2$-untreated control.

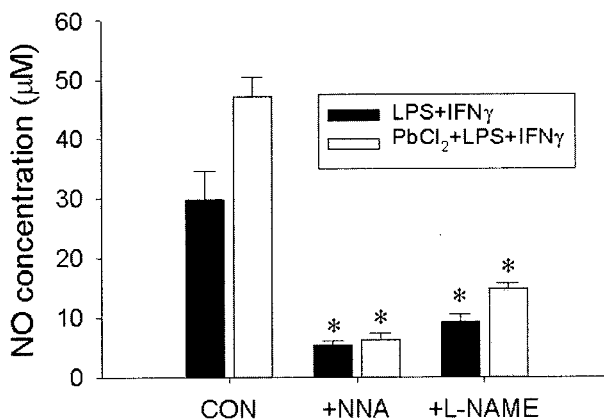
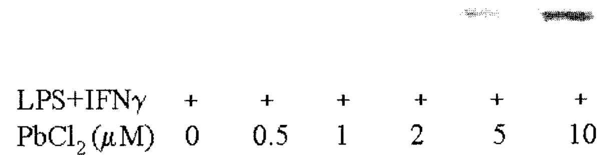


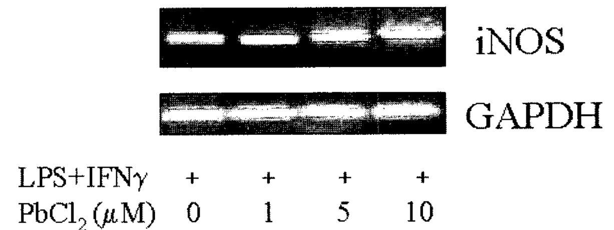
Fig. 3. Augmented NO production in immunostimulated astrocytes by Pb^{2+} was blocked by NOS inhibitors. $PbCl_2$ (5 μM)-pretreated (gray bar, 48 h) or -untreated (black bar) astrocytes were stimulated with LPS (1 μg/ml) plus IFNγ (100 U/ml) for 48 h in the presence or absence of NNA (800 μM) or L-NAME (1 mM). All Data are expressed as a net increase of NO production and are plotted as the mean±S.E.M. (n=4). *$p< 0.05$, significantly different from appropriate untreated control.

A



LPS+IFNγ + + + + + +
$PbCl_2$ (μM) 0 0.5 1 2 5 10

B



LPS+IFNγ + + + +
$PbCl_2$ (μM) 0 1 5 10

Fig. 4. Pb^{2+} potentiates the increased iNOS expression in LPS/IFNγ-treated astrocytes. Cells were pretreated for 48 h with $PbCl_2$ and then stimulated with LPS (1 μg/ml) plus IFNγ (100 U/ml) for additional 48 h(A) and 6 h(B), respectively. The expression levels of iNOS protein and mRNA were determined by Western blot (A) and RT-PCR (B). Data are representative of 5 independent experiments.

iNOS expression by Pb^{2+} in immunostimulated glial cells should be given special attention in several respects. First, glial cells act as a Pb^{2+} depot in the brain, sequestrating Pb^{2+} and preventing its contact with neurons, which are more sensitive than astrocytes to Pb^{2+}-induced toxicity. In cultures, astrocytes have been reported to accumulate Pb^{2+} up to four orders of magnitude higher than extracellular levels (Tiffany-Castiglioni, 1993). Second, glial cells play an essential role in the homeostatic regulation of the extracellular environment within the brain. Therefore, dysfunction of glial cells by Pb^{2+} may indirectly contribute to neurotoxicity by impairing proper function of glial cells.

Previously, Ding and his coworkers reported that lead promotes the generation of oxygen radicals such as superoxide anion and hydroxyl radical, and lipid peroxidation in cultured aortic endothelial cells (Ding *et al.*, 2000). In PC 12 cells, submicromolar lead increased ROS production within 24 h (Jadhav *et al.*, 2000). In the present study, however, Pb^{2+} did not change the ROS production in control as well as immunostimulated glial cells. No significant change of ROS production by Pb^{2+} could be attributed to the high antioxidant capacity of astrocytes (Raps *et al.*, 1989; Makar *et al.*, 1994; Dringen *et al.*, 1999; Ju *et al.*, 2000).

In the present study, other divalent cations such as Cu^{2+} or Zn^{2+} did not alter the immunostimulation-induced NO produc-

tion in rat primary glial cells. In contrast, Colasanti and his coworkers (Colasanti, 2000) previously reported that Cu^{2+} significantly increased LPS/IFNγ-induced NO production in C6 glioma cells. This difference could be caused by different experimental conditions including different cell types and different concentrations of LPS and IFNγ employed.

In contrast to our results, Garber and his coworkers (Garber and Heiman, 2002) reported that lead acetate attenuated NO production, but the experiments were performed in C6 glioma cells and the cells were pre-treated with Pb acetate for 3 h prior to cytokine stimulation for 24 h in the presence of Pb acetate.

Activation of glial cells by cytokines is commonly found in various neurological diseases such as ischemia, stroke, trauma, Alzheimers disease, multiple sclerosis and AIDS dementia (Shrikant *et al.*, 1996). Taken together, our present findings may indicate that lead intoxication would increase NO production in immunostimulated glial cells often observed in various neurodegenerative diseases, and thus directly or indirectly affect neuronal cell function and consequent brain function.

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