INTRODUCTION

Lead has been widely used in many products; for example, leaded gasoline, lead-based paint, and cans containing foods or alcoholic beverages. Exposure to high levels of environmental lead causes various public health problems, particularly among young children, because of its effects on the blood and brain, including disruption of nervous system communication (Gracia and Snodgrass, 2007). Recently, regulation of industrial and environmental levels of lead has been strengthened in many countries, but soil and water contamination is a persistent source of lead exposure in industrialized societies. Toxicity typically results from ingestion of food or water contaminated with lead, but may also occur after accidental ingestion of contaminated dust, soil, or lead-containing paints (Gracia and Snodgrass, 2007). Over 90% of lead absorbed after inhalation or oral ingestion is retained in the body and distributed to the bones (Links et al., 2001), where the half-life of lead is decades long. It was reported that individuals with baseline blood lead levels of 10 to 19 μg/dl suffer increased mortality from various causes: for example, mortality due to circulatory disease was increased by 10% and mortality due to cancer was increased by 46% relative to individuals with blood lead levels of less than 10 μg/dl (Lustberg and Silbergeld, 2002). Thus, blood lead level is positively associated with mortality due to circulatory disorders and cancers.

Lead is known to induce neurotoxicity, leading to lowered intelligence test scores, behavioral problems and decreased cognitive ability (Canfield et al., 2004; Laidlaw et al., 2005). Lead-related intellectual deficits are seen in children with blood lead levels of at least 10 μg/dl, though no evidence of a threshold was found (Lanphear et al., 2005). Schoolchildren with elevated blood lead levels due to both pre- and postnatal lead exposure are more likely to exhibit disruptive behavior in class (Leviton et al., 1993; Bellinger et al., 1994). Moreover, childhood exposure to lead is a risk factor for attention-deficit/hyperactivity disorder (ADHD) (Froehlich et al.,...
Glutamate is an essential amino acid in the central nervous system. Glutamate receptors affect the survival and maturation of cortical, mesencephalic, and cerebellar granule neurons (Blandini et al., 1996; Monti et al., 2002; Hirase I et al., 2003), and play a central role in learning and memory. Ca\(^{2+}\) influx through glutamate receptors due to excitotoxic and ischemic damage can trigger multiple intracellular cascades and cause damage to neuronal cells in the brain (Choi, 1988; Tymianski, 1996; Ying et al., 1997). Ionotropic glutamate receptors are mainly divided into two types, N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. NMDA receptors are composed of an obligatory NR1 subunit and accessory subunits from the NR2 or NR3 family and the latter subunits are expressed differentially during development. Each subunit plays a specific role, contributing to the subcellular localization and channel properties of NMDA receptors (Luo et al., 2011). Thus, changes of NMDA receptor subunit composition influence neuronal activity and survival. On the other hand, AMPA receptors are heteromeric complexes composed of four subunits (GluR1 to GluR4). Among the AMPA receptor subunits, GluR2 subunit shows high Ca\(^{2+}\) permeability to excitotoxicity (Liu and Zukin, 2007).

Lead is a potent, non-competitive and voltage-independent antagonist of NMDA receptor (Alkondon et al., 1989). It is reported that lead binding at the Zn\(^{2+}\)-binding site of NMDA receptor is dependent on the receptor composition, i.e., lead showed competitive inhibition at the Zn\(^{2+}\) binding site of NR2A, but not at the Zn\(^{2+}\) binding site of NR2B (Gavazzo et al., 2008). Moreover, lead alters NMDA receptor subunit composition. Expression of NR2A and NR1 is decreased (Nihei et al., 2000) and the expression of NR1 splice variant mRNA is altered (Guilarte et al., 2000) in rat hippocampus following exposure to lead. Further, lead exposure during synaptogenesis changes NMDA receptor expression at developing synapses (Neal et al., 2011). Thus, lead-induced changes of NMDA receptor subunit composition may result in disruption of downstream signaling. However, the effects of lead on AMPA receptors have not been investigated. Therefore, in the present work, we investigated the effect of lead on the viability and GluR2 expression of primary-cultured rat cortical neurons to test our hypothesis that decreased GluR2 expression is involved in lead-induced neuronal cell death.

**MATERIALS AND METHODS**

**Materials**

Eagle’s minimal essential salt medium (Eagle’s MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Nichirei Biosciences Inc. (Tokyo, Japan). Horse serum (HS) was purchased from Gibco (Life Technologies, Carlsbad, CA, USA). Trypan blue, D-(+)-glucose, NaHCO\(_3\), sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), glyceraldehyde, and paraformaldehyde were purchased from Wako (Tokyo, Japan). Lead acetate was purchased from EBISU (Osaka, Japan). HEPES was purchased from DOJINDO (Kumamoto, Japan). L-Glutamine, arabino-cyletosine, formaldehyde and anti-β-actin antibody (AC-15) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pentobarbital was purchased from Kyoritsu (Tokyo, Japan). Bromophenol blue was purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). Tris-HCl, nonidet P-40, EDTA, mercaptoethanol and Protease Inhibitor Cocktail was purchased from Nacalai Tesque (Kyoto, Japan). Anti-GluR2 antibody (MAB397) was purchased from Millipore (Billerica, MA, USA). Anti-N-cadherin antibody (sc-7939) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

**Cell culture**

The following procedures were performed under sterile conditions. The present study was approved by the university’s animal ethics committee of Hiroshima University. Primary cultures were obtained from cerebral cortex of fetal rats at 18 days of gestation. Fetuses were taken from pregnant Slc:Wistar/ST rats under pentobarbital anesthesia. The prefrontal part of the cerebral cortex was dissected with a razor blade, and cells were dissociated by gentle pipetting. Dissociated cells were plated on culture plates (4 × 10\(^5\) cells/cm\(^2\)). Cultures were incubated in Eagle’s MEM supplemented with 10% heat-inactivated FCS, L-glutamine (2 mM), D-(+)-glucose (11 mM), NaHCO\(_3\) (24 mM), and HEPES (10 mM). Cultures were maintained at 37°C in an atmosphere of humidified 5% CO\(_2\) in air. The cultures were incubated in MEM containing 10% FCS (days in vitro (DIV) 1-7) or 10% HS (DIV 8-11). The medium was exchanged every 2 days. Arabinocyletosine (10 μM) was added to inhibit the proliferation of non-neuronal cells after DIV 6. Cultures were
used for experiments at DIV 11. This protocol has been confirmed to produce cultures containing about 90% neurons by immunostaining for a neuron marker MAP2.

**Treatment of cultures**

Medium containing lead was changed at DIV 2, 4, 6, 8, and 10 and the neurons were exposed until DIV 11 for 9 days. In BDNF experiment, BDNF was added to the culture medium at DIV 2 and further added every day until DIV 10. Thus, the neurons were exposed also with BDNF for 9 days.

**Trypan blue assay**

After exposure to lead acetate, cell cultures were stained with 1.5% trypan blue for 10 min, then fixed with 10% formalin for 2 min, and rinsed with physiological saline. Unstained cells were regarded as viable and stained cells were regarded as dead. The viability of the cultures was calculated as the percentage ratio of the number of unstained cells to the total cells counted. Over 200 cells per cover slip were randomly counted.

**LDH assay**

LDH release was measured using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega®) according to the manufacturer’s protocol. After exposure to lead acetate, culture medium (50 μl) was transferred to a 96-well plate. Substrate mixture (50 μl) was added to each well and allowed to react for 30 min in the dark at room temperature. Stop solution (50 μl) was then added to each well, and the absorbance was read at 490 nm. The absorbance was normalized based on the absorbance of negative controls, which consisted of cells not exposed to lead.

**Western blotting**

After lead acetate treatment, cells were washed with PBS buffer and lysed in TNE buffer containing 50 mM Tris-HCl, 1% nonidet P-40, 20 mM EDTA, Protease Inhibitor Cocktail (1:200), 1 mM sodium orthovanadate, and 1 mM PMSF. The mixture was rotated at 4°C and centrifuged at 15,000 rpm, after which the supernatant was transferred to a microtube. The supernatant was added to sample buffer containing 100 mM Tris-HCl, 4% SDS, 20% glycerol, 0.004% bromophenol blue, and 5% mercaptoethanol, and then denatured at 95°C for 3 min. Protein was separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with blocking buffer containing 5% skim milk for 1 hr, and then incubated with anti-GluR2 (1:2,000) and anti-β-actin (1:4,000) overnight, and with secondary antibody for 1 hr. Other details were performed by the methods described previously (Hashida et al., 2011). The protein was detected with an enhanced chemiluminescence detection system (Chemi-Lumi One L, Nacalai Tesque (Kyoto, Japan)). Quantitative analysis was performed with digital imaging software (Image J, NIH (Bethesda, MD, USA)), and GluR2 protein levels were corrected on the basis of β-actin protein levels.

**Immunocytochemistry**

Cells were seeded in poly-D-lysine-coated 8-well chamber slides (BD BioCoat™) and incubated overnight. After treatment with 5 and 20 μM lead for 9 days, cells were washed with PBS(-) and fixed with 4% paraformaldehyde in PBS(-) for 15 min at room temperature. The slides were washed with PBS(-), blocked with 4 drops of Image-iT™ FX Signal Enhancer (Molecular Probes®) for 1 hr, and incubated with mouse anti-GluR2 (MAB397), which recognizes the N-terminal extracellular domain of GluR2 (1:250), and rabbit anti-N-cadherin (1:250) diluted in PBS(-) overnight at 4°C. Then, the slides were washed three times with PBS(-), and incubated with Alexa Fluor® 488-conjugated goat anti-mouse IgG (1:800, Molecular Probes®) and Alexa Fluor® 555-conjugated goat anti-rabbit IgG (1:800, Molecular Probes®) for 1 hr at room temperature in the dark. The slides were further washed three times with PBS(-), incubated with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:2,000, Molecular Probes®) and observed under a confocal laser scanning microscope (Olympus, FV-1000-D).

**Statistics**

All the experiments were performed at least three times and representative data are shown. Data are expressed as mean ± S.E.M. Statistical evaluation of the data was performed with ANOVA followed by Tukey’s test. A value of P < 0.05 was considered to be indicative of significance.

**RESULTS**

**Lead-induced cell death of cortical neurons**

First, we investigated neuronal cell death induced by long-term exposure to lead. Rat cortical neurons were exposed to 5 and 20 μM lead for 1, 3, 5, 7, and 9 days, and then the cell viability was examined by means of trypan blue assay (Fig. 1A) and LDH assay (Fig. 1B). Exposure of the cells to 5 μM lead for 9 days resulted in a decrease of the cell viability to 29% of the control, while...
exposure to 20 μM lead for 7 and 9 days decreased the cell viability to 67% and 19% of the control, respectively (Fig. 1A). Moreover, LDH release from cortical neurons was increased to 147% of the control after exposure to 20 μM lead for 9 days, although exposure to 5 μM lead had no effect (Fig. 1B).

**Effect of long-term exposure of lead on GluR2 expression**

We hypothesized that long-term exposure to lead would also decrease GluR2 expression, and would result in neuronal cell death. To examine this hypothesis, cortical neurons were exposed to 0.1-100 μM lead for 9 days and GluR2 protein expression was measured. As shown in Fig. 2A, a concentration-dependent decrease of GluR2 expression was seen upon exposure of the neurons to lead at concentrations above 1 μM and the decrease reached statistical significance at 5 μM. In 100 μM lead exposure, β-actin expression was also decreased, maybe due to drastic cell death (Fig. 2A). Moreover, cortical neurons were exposed to 5 and 20 μM lead for 1-9 days. A time-dependent decrease of GluR2 expression was observed in the neurons exposed to 5 and 20 μM lead. Exposure to 5 μM lead for 7 and 9 days significantly decreased the expression of GluR2 to 50% and 29% of the control (Fig. 2B), while exposure to 20 μM lead for 7 and 9 days significantly decreased the expression of GluR2 to 35% and 30% of the control (Fig. 2C). GluR2 subunits are largely expressed in cytoplasm, but some of them are expressed in plasma membrane and act as components of AMPA receptors. Next, GluR2 expression at the plasma membrane was examined by immunocytochemistry (Fig. 3). Membrane expression of GluR2, which was confirmed by co-localization with N-cadherin, a membrane protein marker, was observed in the control cells. Exposure to 5 and 20 μM lead for 9 days markedly decreased GluR2 expression, in accordance with the result of western blotting (Fig. 2). Moreover, co-localization of GluR2 and N-cadherin was also considerably reduced, while intracellular distribution of GluR2 was not altered in these cells. These results suggest that exposure to lead decreases GluR2 expression, leading to a decrease in plasma membrane GluR2.

**Amelioration of lead-induced GluR2 reduction and neuronal cell death by BDNF**

It has been reported that BDNF potently induces GluR2 promoter activity in SH-SY5Y cells, resulting in increased expression of GluR2 protein (Brené et al., 2000). Thus, we tested whether BDNF also increased GluR2 protein expression in cortical neurons and ameliorated lead-induced neuronal cell death. Although the GluR2 protein level was not increased, the decrease of GluR2 expression induced by exposure to 5 and 20 μM lead was partly reversed by 50 ng/ml BDNF (Fig. 4A). Concomitantly, the decrease of cell viability caused by exposure to 5 and 20 μM lead was also significantly ameliorated by 50 ng/ml BDNF (Fig. 4B).
DISCUSSION

In this study, cultured rat cortical neurons were exposed to lead to test our hypothesis that lead induces a decrease of GluR2 expression that in turn promotes neuronal cell death. We found that exposure to lead at 5-20 μM for 9 days decreased cell viability (Fig. 1A) and LDH release was increased after exposure to 20 μM lead for 9 days (Fig. 1B). Trypan blue-stained cells are regarded as dead, whereas LDH assay measures LDH release from membrane-disrupted cells. Thus, the differences in evaluation of neuronal cell death between trypan blue assay and LDH assay is considered to be due to the different endpoints used to determine cell death in the two assays.

The expression of GluR2, an AMPA-type glutamate receptor subunit, was significantly decreased by exposure to 5-20 μM lead for 7 days (Fig. 2). It was reported that exposure to 0.1-10 μM lead for 48 hr decreased cell proliferation and increased caspase-3 activity in human SH-SY5Y neuroblastoma cells (Chetty et al., 2005). Shinkai et al. (2010) reported that 5 μM lead induces endoplasmic
reticulum chaperones GRP78 and GRP94 via JNK-AP-1 pathway in vascular endothelial cells. Thus, the lead concentrations used in this study are similar to those used in other studies on the in vitro toxicities of lead. Incidentally, lead concentrations of up to 5 μM in blood have been reported in workers exposed to lead (Tomokuni et al., 1993). It is well known that lead induces hematotoxicity by inhibiting the activity of enzymes involved in heme biosynthesis such as δ-aminolevulinic acid dehydratase. It is believed to be the most sensitive to lead (Bottomley and Muller-Eberhard, 1988). However, δ-aminolevulinic acid dehydratase is inhibited only in the presence of comparatively high lead concentrations of 0.1-1 mM in human erythroblastic cultures (Rio et al., 2001). Thus, the concentrations used in this study are lower than those used in the above studies.

We have reported that long-term exposure of rat cortical neurons to tributyltin (TBT) decreases GluR2 expression, which results in increased Ca$$^{2+}$$ permeability of AMPA receptors, because the Ca$$^{2+}$$ permeability of AMPA receptors depends on whether or not they contain the GluR2 subunit (Nakatsu et al., 2009). It was reported that GluR2 knockdown rats showed neuronal cell death in hippocampal CA1 and CA3, and the neuronal cell death was reduced by injection of Nasp (an open channel blocker selective for Ca$$^{2+}$$-permeable, GluR2-lacking AMPA receptors) and CNQX (a competitive blocker of AMPA receptors) (Oguro et al., 1999), which suggests that GluR2 knockdown-induced neuronal cell death was mediated by Ca$$^{2+}$$-permeable, GluR2-lacking AMPA receptors. It has also been reported that knockdown of GluR2 exacerbates kainate-induced neuronal death (Friedman and Velísková, 1998; Friedman et al., 2003). Iihara et al. (2001) suggested that kainate-induced neuronal cell death in GluR2 knockdown animals involves altered Na$$^{+}$$ permeability as well as altered Ca$$^{2+}$$ permeability. It

![Fig. 3. Change of GluR2 protein expression on the plasma membrane induced by Pb$$^{2+}$$. Cortical neurons were exposed to 5 and 20 μM Pb$$^{2+}$$ for 9 days, and immunocytochemical staining was performed using a mouse anti-GluR2 antibody that recognizes the N-terminal extracellular domain of GluR2 (green) and a rabbit anti-N-cadherin antibody (red). Nuclear staining was performed using DAPI (blue). Yellow indicates colocalization between GluR2 and N-cadherin.](image-url)
is known that GluR2 knockdown mice also show behavioral changes, such as impaired novelty-induced exploratory activities, disrupted motor coordination, and reduced self-directed behaviors, compared with control mice (Jia et al., 1996). These findings are consistent with the idea that lead-induced GluR2 decrease can induce neuronal death via the increase of Ca$^{2+}$ permeability of AMPA receptors. However, further studies are needed to measure lead-induced Ca$^{2+}$ entry into neurons.

Expression of GluR2 can be induced by BDNF (Brené et al., 2000), and one of the neuroprotective effects of BDNF is supposed to be mediated by recovery of GluR2. We also investigated whether increasing GluR2 expression by exposure to BDNF results in amelioration of lead-induced neuronal cell death. We found that the lead-induced GluR2 decrease was partly reversed and neuronal cell viability was increased by exposure to 50 ng/ml BDNF (Fig. 4). Because GluR2 levels are thought to reach plateau in primary cortical neurons, BDNF may not increase basal GluR2 expression. These results support the hypothesis that lead causes neuronal cell death through decreasing GluR2 protein expression, though BDNF might rescue lead-induced neuronal death by other mechanism such as an activation of PI3-kinase-Akt pathway (Hetman et al., 1999). It should be confirmed that GluR2 overexpression recovers cell viability decreased by lead.

In conclusion, we investigated the influence of long-term lead exposure on cultured cortical neurons. Lead induced a decrease of GluR2 protein and caused neuronal cell death. The decrease of GluR2 expression was considered to cause the lead-induced neuronal cell death, because cell viability was restored by BDNF treatment, which elicits a recovery of GluR2. A decrease in the population of GluR2-containing AMPA receptors is associated with increased Ca$^{2+}$ influx. Our findings raise the possibility that GluR2 decrease in the brain is involved in lead-induced in vivo neurotoxicity, disorder of behavior, and impairment of cognitive function.

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REFERENCES


Blandini, F., Porter, R.H. and Greenamyre, J.T. (1996): Glutamate...
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and blood lead in workers exposed to lead. Ind. Health, 31, 51-57.