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Involvement of cyclin D1/CDK4 and pRb mediated by PI3K/AKT pathway activation in Pb^{2+}-induced neuronal death in cultured hippocampal neurons

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Abstract

Lead (Pb) is widely recognized as a neurotoxicant. One of the suggested mechanisms of lead neurotoxicity is apoptotic cell death. And the mechanism by which Pb^{2+} causes neuronal death is not well understood. The present study sought to examine the obligate nature of cyclin D1/cyclin-dependent kinase 4 (CDK4), phosphorylation of its substrate retinoblastoma protein (pRb) and its select upstream signal phosphoinositide 3-kinase (PI3K)/AKT pathway in the death of primary cultured rat hippocampal neurons evoked by Pb^{2+}. Our data showed that lead treatment of primary hippocampal cultures results in dose-dependent cell death. Inhibition of CDK4 prevented Pb^{2+}-induced neuronal death significantly but was incomplete. In addition, we demonstrated that the levels of cyclin D1 and pRb/p107 were increased during Pb^{2+} treatment. These elevated expression persisted up to 48 h, returning to control levels after 72 h. We also presented pharmacological and morphological evidences that cyclin D1/CDK4 and pRb/p107 were required for such kind of neuronal death. Addition of the PI3K inhibitor LY294002 (30 μM) or wortmannin (100 nM) significantly rescued the cultured hippocampal neurons from death caused by Pb^{2+}. And that Pb^{2+}-elicited phospho-AKT (Ser473) participated in the induction of cyclin D1 and partial pRb/p107 expression. These results provide evidences that cell cycle elements play a required role in the death of neurons evoked by Pb^{2+} and suggest that certain signaling elements upstream of cyclin D1/CDK4 are modified and/or required for this form of neuronal death.

Keywords: Cyclin-dependent kinase 4; Cyclin D1; Neuronal cell death; Pb^{2+}; Retinoblastoma protein (pRb); Phosphoinositide 3-kinase (PI3K)/AKT

Introduction

Lead (Pb) was one of the first metals used by humans, is highly persistent, is not involved in normal metabolism and is very toxic (Guity et al., 2002; Saleh et al., 2003). Chronic lead exposure has a variety of adverse effects on the health of developing humans and other animals (Regan, 1989; Silbergeld, 1992; Bellinger and Dietrich, 1994). The developing central nervous system is especially sensitive and vulnerable to Pb^{2+} toxicity (Reddy and Zawia, 2000; Chetty et al., 2001; Basha et al., 2003) so that, lead exposure produces neurotoxicity including behavioral, morphological and electrophysiological effects (Garza et al., 2006). Pb^{2+} can cause increased production of reactive oxygen species (ROS), oxidative stress, excitotoxicity (Guilarte, 1997; Savolainen et al., 1998b), DNA damage (Sieg and Billings, 1997; Bolin et al., 2006), and at still higher levels, even cause neuronal death (Oberto et al., 1996; Guilarte, 1997; Saito et al., 1998; Savolainen et al., 1998a; Yang et al., 1999; Loikkanen et al., 2003b). Several lines of evidences support the notion that such agent may cause two types of neuronal death, apoptosis (Franklin and Johnson, 1992) and (or) necrosis (Choi, 1988). This form of neural apoptosis is dependent upon selected death-signaling elements such as caspases, and Bax/Bcl-2 but is p53-independent (Sharifi et al., 2002; Loikkanen et al., 2003a). There are only a few studies, which have investigated and indicated an association between Pb^{2+} exposure and neural apoptosis (Oberto et al., 1996; Fox et al., 1997, 1998; He et al., 2000,

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2003; Sharifi et al., 2002), and the mechanism by which Pb^{2+} causes neuronal death is not well understood.

Several factors are involved in the induction of neuronal cell death (apoptosis) including activator protein-1 (AP-1) transcription factors (c-Fos and c-Jun), c-Jun-N-terminal kinase-3, caspases, the Bcl-2 family, and phosphoinositide 3-kinase (PI3K)/AKT pathway (Pettmann and Henderson, 1998). In addition, previous evidence suggested a relationship between the cell cycle and apoptosis both in non-neuronal and neuronal cells. In the nervous system, although most of the cells escape from the cell cycle, up-regulation of cyclin D1 has been reported in dying neurons (Freeman et al., 1994; Kranenburg et al., 1996; Timsit et al., 1999), and its overexpression also induces neuronal cell death (Kranenburg et al., 1996). Cyclin D1, a member of the G1 cyclins, plays an important role in the G1 phase progression of the cell cycle in proliferating cells via activation of cyclin-dependent kinase 2 (CDK2), CDK4, or CDK6. The cyclinD/CDK4/6 complexes induce the phosphorylation of retinoblastoma (pRb) protein and the release of E2F, which trigger G1 cell cycle progression. In addition, numerous reports indicated that chemical inhibitors of CDKs and expression of dominant negative forms of CDK4 and CDK6 suppressed the death of cultured postmitotic neurons exposed to select death insults including excitotoxins (Chopp et al., 1992; Crumrine et al., 1994), hypoxia and ischemia (Tomasevic et al., 1998; van Lookeren Campagne and Gill, 1998; Timsit et al., 1999; Rashidian et al., 2005), loss of trophic support (Park et al., 1997b, 1998b; Migheli et al., 1999), or β-amyloid peptide (Copani et al., 1999; Giovanni et al., 1999). In this study, we identify that cyclin D1/CDK4, and its down-stream signal pRb are required for Pb^{2+} induced neuronal death, and suggest that Pb^{2+} maybe activate the cyclin D1/CDK4/pRb signaling through the phosphoinositide 3-kinase (PI3K)/AKT pathway.

Experimental procedures

Cell culture. Rat hippocampal neurons were cultured from postnatal day 0 rats as described previously (Park et al., 1997a). The neurons were suspended in DMEM (containing 10% heat-inactivated bovine serum) and plated into 24-well dishes (approximately 200,000 cells/well) before being maintained in neurobasal medium (Invitrogen, Carlsbad, CA) with B27 serum-free supplements, L-glutamine (0.5 mM), and penicillin–streptomycin for 16–18 h. After 5–6 days in culture, cells were used for metabolic studies.

Cell treatment and viability assay. Lead acetate (Sigma) was dissolved in MilliQ-purified water. Serum-starved cells were then exposed to lead acetate, and lead acetate plus CDK4 inhibitor, LY294002 and wortmannin (Sigma) respectively for 6–72 h in serum-free media. The inhibitors were replenished every 6 h at appropriate times of culture under the conditions described above. Cells were lysed by a detergent-containing lysing solution that dissolves cell membranes and cytoplasm and that provided a uniform suspension of single, intact nuclei. The latter were quantified by counting in a hemacytometer. Broken or damaged nuclei were not included in the counts. All experimental points are expressed as a percentage of cells plated on day 0 and are reported as the means ± S.E. ($n=8$).

Western blot analysis. Hippocampal neurons were dissociated and cultured as described above. In experiments to determine the effects of protein kinase inhibitors, serum-deprived cells were pretreated with 30 μM LY294002 and 100 nM wortmannin respectively, for 30 min before the addition of Pb^{2+}. LY294002 or wortmannin were replenished every 6 h. At appropriate times during lead acetate treatment, the neurons were harvested in sample buffer, and 50 μg of protein were loaded onto SDS-polyacrylamide gels and transferred onto nitrocellulose membrane as described previously. Blots were probed with anti-phospho-Rb antibody (Santa Cruz, 1:1000 dilution), anti-cyclin D1 (Sigma, 1:300 dilution), anti-CDK4 (Santa Cruz, 1:300), anti-phospho-AKT (Ser473) (Santa Cruz, 1:1000) and anti-AKT (Santa Cruz, 1:1000).

Terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL). TUNEL staining was performed with the In Situ Cell Death Detection kit (Promega). Endogenous peroxidase activity was blocked by incubating with 0.3% H_2O_2 in methanol for 30 min. Sections were incubated in 0.1% Triton X-100 in 0.1% sodium citrate for 10 min at 4 °C. After blocking with 5% serum, 50 μl of TUNEL solution containing terminal deoxynucleotidyltransferase and biotin–dUTP mixture was applied to sections for 1 h at 37 °C. Sections were then washed three times in PBS and visualized with the Cy-3-conjugated streptavidin (Promega). Positive control sections were immersed in DNase I solution for 10 min at room-temperature before equilibration in TdT buffer. The sections were observed under a fluorescent microscope with appropriate excitation/emission filter pairs.

Immunohistochemical staining. Paraformaldehyde-fixed sections performed similar to those described previously for TUNEL staining. The sections were then

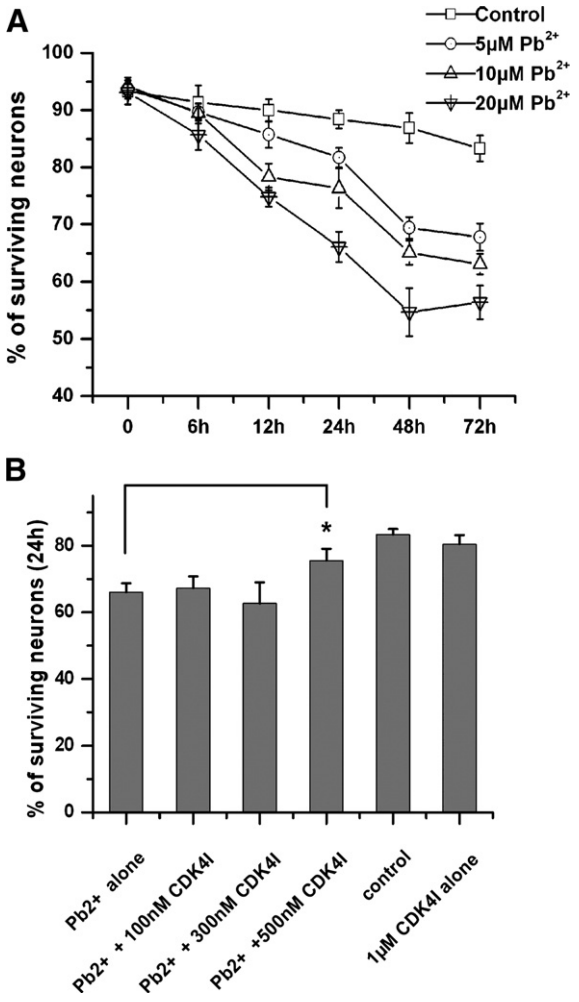

Fig. 1. The CDK4 inhibitor protects hippocampal neurons from death evoked by Pb^{2+}. A: After the indicated times cell viability was analyzed as described in Experimental procedures. B: Effects of various dose of CDK4 inhibitor on the survival of Pb^{2+}-treated (20 μM) hippocampal neurons. Each data point is the mean ± S.E. ($n=8$) and is expressed relative to the number of neurons present in each culture at the time of drug treatment. The * (asterisk) indicates significance relative to Pb^{2+} treatment alone (Student's t test).