Persistent activation of ERK1/2 by lead acetate increases nucleotide excision repair synthesis and confers anti-cytotoxicity and anti-mutagenicity

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Lead, a possible human carcinogen, affects signal transduction pathways in many aspects, yet exhibits low mutagenicity in human cells. In this study, we explore whether signaling pathways including the four MAPKs and AKT affect DNA repair and mutagenicity in the exposure of mammalian cells to lead acetate [Pb(II)]. Pb(II) increased the phosphorylated ERK1/2 and phosphorylated AKT but not the phosphorylated ERK5, phosphorylated p38 and JNK activity in human non-small cell lung adenocarcinoma CL3 cells. The duration of ERK1/2 activation was much longer than AKT activation and these two signals were independently activated by Pb(II) in CL3 cells. Intriguingly, a MKK1/2 inhibitor PD98059 (25–50 µM) markedly suppressed ERK1/2 activation and greatly promoted the hprt mutation frequency and cytotoxicity in Pb(II)-treated CL3 cells. Conversely, inhibition of the AKT signal by wortmannin did not exhibit such effects. Inhibition of the persistently activated ERK1/2 in Pb(II)-treated diploid human fibroblasts by PD98059 also markedly increased the mutagenicity and cytotoxicity. The Pb(II)-induced mutagenicity and cytotoxicity were significantly higher in nucleotide excision repair (NER)-deficient UVL-10 rodent cells than their counterpart AT3-2 cells; also, ERK1/2 activation by Pb(II) was observed in AT3-2 but not UVL-10 cells. Furthermore, cellular NER synthesis was enhanced by Pb(II) exposure, which was markedly suppressed by PD98059. Activation of ERK1/2 by expressing a constitutively active form of MKK1 in CL3 cells also elevated cellular NER synthesis. Together, these results indicate that persistent activation of ERK1/2 signaling by Pb(II) enhances cellular NER synthesis, thereby conferring anti-cytotoxicity and anti-mutagenicity.

Introduction

Lead is an ubiquitous toxic contaminant of our environment, which was evaluated as a possible human carcinogen (group 2B) in 1987 by the International Agency for Research on Cancer (1) (based on sufficient animal data and insufficient human data). More recently, epidemiologic studies of lead smelter or battery workers provided some evidence of an increased risk of lung, stomach and bladder cancers; however, the results may be biased by confounders such as tobacco smoking and arsenic (2–5). Lead acetate [Pb(II)] causes kidney, brain and lung cancers in experimental rodents and acts synergistically with other carcinogens (1,6,7). Pb(II) increases gene mutations in cultured rodent cells (8–12), yet, the induction levels are much weaker than typical carcinogens. Pb(II) does not enhance gene mutations in diploid human fibroblasts, although it induces anchorage-independent phenotypes in the same cells (13). Pb(II) does not cause DNA strand breaks in HeLa cells (14), however, in vitro studies have demonstrated that Pb(II) is able to interact with the phosphate backbone of nucleic acids (15) and induce DNA strand breaks and 8-hydroxydeoxyguanosine adducts (16). Due to its weak genotoxicity and co-genotoxicity with UV and alkylating carcinogens, the Pb(II) genotoxicity has been attributed to indirect mechanisms such as interference with DNA repair processes (10,14).

The four subfamilies of mammalian mitogen-activation protein kinase (MAPKs), i.e. the extracellular signal-regulated kinases (ERK1/2), the c-JUN N-terminal kinases (JNKs), the p38 kinases and ERK5 (also termed big MAP kinase 1), as well as AKT (also termed as protein kinase B) are vital signaling transducers differentially activated in response to a wide diversity of extracellular stimuli including growth factors, cytokines and environmental stresses (17–26). Activation of MAPKs is regulated through a three-kinase module composed of a MAPK, a MAPK kinase (MKK) and a MKK kinase (MKKK) (17–23). These MAPK modules are connected to the cell surface receptor and activated through interaction with a family of small GTPases and MKKK kinases. Activation of MAPKs requires a dual-phosphorylation of the Thr and Tyr residues within the motif Thr–Glu–Tyr (ERK1/2 and ERK5), Thr–Pro–Tyr (JNK) and Thr–Gly–Tyr (p38) in the subdomain VIII of the catalytic domain. In general, the activated ERKs control cell proliferation and differentiation (17–19), whereas, the stimulated JNK and p38 pathways regulate growth arrest, apoptosis, cell survival, transformation, proliferation and invasion (17,20–23). The particular function regulated by MAPKs is likely to depend on the cell type, the stimulus and the duration and strength of kinase activities. On the other hand, AKT is recruited to the plasma membrane by phosphatidylinositol 3-kinase (PI3K)-dependent phospholipid binding and full activation of AKT required phosphorylation at Thr308 and Ser473 residues (24–26). Activation of AKT results in increasing cellular proliferation and protection from apoptosis through phosphorylation and inactivation of several effectors including Bad, caspase-9, the forkhead family of transcription factors, GSK-3, p27 and p21 (24–26).

Pb(II) has been recently reported to activate ERK1/2 and JNK in a rat pheochromocytoma cell line PC-12 (27), and ERK1/2 but not AKT signaling in a human astrocytoma cell line 1232N1 (28). Conversely, Pb(II) does not activate ERK1/2 and JNK, while it stimulates p38 phosphorylation.
and subsequently Hsp27 phosphorylation in bovine adrenal chromaffin cells (29). Whether Pb(II) can activate these signals in cell types other than neural-derived cells and what are the physiological roles of their activation remains unknown. Here we show that Pb(II) activates ERK1/2 persistently and AKT transiently, but does not stimulate ERK5, JNK and p38 in a human non-small cell lung adenocarcinoma cell line, CL3. We further demonstrate that inhibition of the activated-ERK1/2 but not AKT signaling greatly increases Pb(II) cytotoxicity and mutagenicity. Moreover, we provide evidence to reveal that the activated-ERK1/2 signal is essential for the enhanced cellular nucleotide excision repair (NER) synthesis in Pb(II)-treated cells, which may account for the low mutagenicity of this metal in mammalian cells.

Materials and methods

Cell culture

The CL3 cell line established from a non-small-cell lung carcinoma tumor was provided by Dr P.-C. Yang at the Department of Internal Medicine and Clinical Pathology, National Taiwan University Hospital, Taipei. The human diploid fibroblast line HF2 was given by Dr W.-N.Wen at the Institute of Biochemistry, National Taiwan University, Taipei. The AT2-3 (NER proficiency) and UVL–10 (ERCC1 deficiency) lines derived from Chinese hamster ovary (CHO) cells were provided by Dr G.M.Adamar at MD Anderson Cancer Center, Smithville, Texas (30–32). CL3, HF2 and CHO cells were cultured in RPMI-1640, DMEM and McCoy’s 5A media (Gibco, Life Technologies, Grand Island, NY), respectively, supplemented with sodium bicarbonate (2.2%, w/v), l-glutamine (0.25%, w/v), penicillin (100 units/ml), streptomycin (100 µg/ml) and fetal calf serum (10%). CL3 and CHO cells were maintained at 37 °C in a humidified incubator containing 5% CO2 in air, while HFW cells were cultured in a 10% CO2 incubator.

Treatment

Cells in exponential growth were plated before serum starvation for 16–18 h. Lead acetate (Merk, Darmstadt, Germany) was dissolved in Milli-Q-purified water (Millipore, Bedford, MA). Serum-starved cells were then exposed to lead acetate for 15 min–24 h in serum-free media. In experiments to determine the effects of protein kinase inhibitors, serum-deprived cells were pre-treated with PD98059 (Calbiochem, San Diego, CA), a MKK1/2 inhibitor, for 1 h or for 24 h in serum-free media. Immediately after treatment, cells were washed with phosphate-buffered saline (PBS) and trypsinized for the determination of cell numbers using a hemocytometer. The cell number was measured with an UVX radiometer (UVP Inc., CA).

Cytoxicity assay

Immediately after treatment, cells were washed with phosphate-buffered saline (PBS) and trypsinized for the determination of cell numbers using a hemocytometer. The cells were plated at a density of 100–200 cells per 60 mm Petri dish in triplicate for each treatment. The cells were then cultured for 7–14 days and cell colonies were stained with 1% crystal violet solution (in 30% ethanol). Cytoxicity was determined to be the number of colonies in the treated cells divided by the number of colonies in the untreated control (12).

Mutagenicity assay

The Pb(II)-treated or untreated cells were maintained in exponential growth for 7 days to allow for the expression of resistance to 6-thioguanine (6-TG). One million cells from each treatment were plated onto 100 mm Petri dishes in a selective medium containing 40 µM (CL3 and HF2) or 66 µM (AT3-2 and UVL–10) of 6-TG, followed by incubation for 7–14 days. Plating efficiency of cells at the time of selection was also assayed in a non-selective medium to correct the observed hprt mutant frequency. The hprt mutant frequency was calculated to be the total number of 6-TG resistant colonies divided by the total number of clonable cells at selection time (12).

Preparation of whole cell extract (WCE)

The Pb(II)-treated or untreated cells were rinsed twice with cold PBS and lysed in the WCE buffer containing 20 mM HEPES, pH 7.6, 75 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na3VO4, 50 mM NaF, 0.5 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin and 100 µg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride. The cell lysate was rotated at 4°C for 30 min, centrifuged at 10 000 r.p.m. for 15 min and the precipitates were discarded. The BCA protein assay kit (Pierce, Rockford, IL) was adopted to determine protein concentrations using bovine serum albumin as a standard.

Western blot analysis

Equal amounts of proteins in WCE from each set of experiments were subjected to western blot analyses as described previously (33). The polyclonal antibodies specific against phospho-ERK1/2 (Thr202/Tyr204) (#9101), phospho-p38 (Thr180/Tyr182) (#9211) and phospho-AKT (Ser473) (#9271) were purchased from Cell Signaling (Beverly, MA). The polyclonal antibody against phospho-ERK5 (Thr218/Tyr220) (#44–612) was purchased from BIOSOURCE International (Camarillo, CA). The polyclonal antibody against ERK2 (#sc-154), ERK5 (#sc-5626), p38 MAP kinase (#sc-535), JNK1 (#sc-571), AKT (#sc-8312), c-FOS (#sc-52) and α-tubulin (#sc-8035) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody reaction was detected using the enhanced chemiluminescence detection procedure according to the manufacturer’s recommendations (NEN, Boston, MA). To re-probe the membrane with another primary antibody, antibodies in the blot were stripped from membranes by a solution containing 2% SDS, 62.5 mM Tris–HCl, pH 6.8, and 0.7% (w/v) β-mercaptoethanol at 50°C for 15 min. The relative protein intensities on blots were quantitated using a computing densitometer equipped with the ImageQuant analysis program (Molecular Dynamics, Sunnyvale, CA).

JNK kinase assay

JNK in WCE (50 µg proteins) was reacted with GST-cJUN(1–79) (5 µg) and glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech, Arlington Heights, IL) and the kinase activity was performed by transferring [γ32P]ATP (6 000 Ci/mmol) to the substrate GST-cJUN(1–79) as described previously (33).

Transfection

Cells (4×105) were plated in a 60 mm dish one-day before transfection. Plasmid (5–15 µg) containing a constitutively active form of MKK1 (AN3/S218E/ S222D; MKK1-CA) (34), kindly provided by Dr N.G.Ahn at the Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado, was transfected into CL3 cells by calcium phosphate co-precipitation. After incubation for 6 h, the cells were washed with PBS, kept cultured in complete media for 2 days and then subjected to the preparation of WCE and repair synthesis assay.

NER synthesis

The efficiency of cellular NER synthesis was measured according to the procedure of Dr R.D.Wood (35,36) with modifications. Briefly, the pUC19 plasmid substrates were prepared by alkaline lysis method and stored at −20°C. The purified plasmid substrates (250 ng/ml in deH2O) were irradiated with UV (25 nm, 400 J/m2) at a radiation intensity of 1–1.5 J/m2/s. NER synthesis reaction mixtures (50 µl) contained 60 µg of proteins derived from human WCE, 250 ng of UV-irradiated or un-irradiated plasmid substrates, 20 µM each of dGTP, dCTP and dTTP, 8 µM of [γ32P]dATP (3000 Ci/mmol), 2 mM ATP, 45 mM HEPES-KOH, pH 7.5, 60 mM KCl, 7.5 mM MgCl2, 0.9 mM dithiothreitol, 0.4 mM EDTA, 3.4% glycerol and 18 µg bovine serum albumin. Reactions were performed at 30°C for 1 h and terminated by adding EDTA to a final concentration of 20 mM. The samples were then treated with 80 µg/ml RNaseA for 10 min and 190 µg/ml proteinase K and 0.5% SDS for 30 min at 37°C. The plasmid DNA in the reaction mixtures was purified by phenol/chloroform extraction and ethanol precipitation, linearized with BamHI and subjected to agarose gel (0.8%) electrophoresis. The plasmid DNA in gel was stained with ethidium bromide (0.5%) and visualized under near-UV transillumination. The gel was then dried and subjected to autoradiography. The band intensities were measured with a computing densitometer equipped with the ImageQuant analysis program.

Determination of intracellular lead level

Cells were exposed to various Pb(II) concentrations in serum-free medium for 24 h. Following treatment, the cells were washed three times with PBS and the numbers of cells were determined. One million cells were centrifuged and the cell pellet was sonicated in Milli-Q-purified water. Total cellular Pb level was analyzed by an inductively coupled plasma-mass spectrometer (ICP-MS; SCIEX ELAN 5000, Perkin Elmer, Norwalk, CT). The ICP-MS conditions were as follows: power of 1000 W, plasma flow rate of 15 l/min, auxiliary flow rate of 0.8 l/min, carrier gas flow rate of 0.8 l/min and sample flow rate of 1 ml/min.

Results

Pb(II) elicits ERK1/2 and AKT phosphorylation in CL3 cells

The ability of Pb(II) to activate the four MAPKs was investigated by exposing CL3 cells to various concentrations of lead acetate in serum free medium for 15 min–24 h. The activation

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of ERK1/2, ERK5 and p38 kinase was determined by western blots using antibodies specific to recognize phospho-ERK1/2, phospho-ERK5 and phospho-p38, respectively. The JNK kinase activity was performed by in vitro kinase assay using GST-cJUN(1–79) as a substrate. As shown in Figure 1, exposure of cells to Pb(II) for 24 h increased ERK1/2 phosphorylation in a dose-dependent manner, e.g. 10–100 μM Pb(II) enhanced the phosphorylated ERK1/2 levels 3.1–4.5 fold of the untreated control. Pb(II) also induced the phosphorylated ERK1/2 levels in a time-dependent manner (15 min–24 h; data not shown). In contrast, exposure of CL3 cells to Pb(II) (10–500 μM) for 15 min, 1 or 24 h did not activate ERK5, p38 and JNK, although these MAPKs could be activated in the same cell line by positive control stimuli (Figure 1 and data not shown). Additionally, the endogenous protein levels of the four MAPKs were unaltered by Pb(II) (Figure 1).

We also investigated the ability of Pb(II) to activate AKT, a potent survival signal, by western blots using antibody specific to recognize phospho-AKT(Ser473). Figure 2A shows that Pb(II) could activate the phospho-AKT in CL3 cells, however, the activated levels were variable as the exposure time increased. To determine the duration of ERK and AKT signals activated by Pb(II), after a 24-h exposure to 30 μM Pb(II), CL3 cells were washed with PBS and allowed recovery in serum-free media for 0–8 h before WCE isolation. Figure 2B shows that the phospho-ERK1/2 activated by Pb(II) remained at high levels during the recovery times. Conversely, the Pb(II)-activated AKT diminished rapidly after removing the metal from media (Figure 2B). Pb(II) did not significantly influence the endogenous ERK2 and AKT protein levels during the time-course and recovery experiments (Figure 2). The above results imply that Pb(II) activates ERK1/2 persistently and AKT transiently in CL3 cells.

Pb(II) independently elicited ERK1/2 and AKT phosphorylation
It has been reported that AKT can phosphorylate and inactivate RAF, which is an upstream ERK1/2 activator (37,38). To inspect the relationship between the ERK1/2 and AKT signaling pathways elicited by Pb(II), CL3 cells were exposed to Pb(II) in the presence of PD98059, an inhibitor of the ERK1/2 upstream kinases MKK1/2, or wortmannin, an inhibitor of the AKT upstream activator PI3K, and then subjected to examination of the phospho-ERK1/2 and phospho-AKT levels. PD98059 (50 μM) and wortmannin (100 nM) completely blocked phospho-ERK1/2 and phospho-AKT in Pb(II)-treated cells, respectively (Figure 3). Wortmannin did not influence the ERK1/2 activation and PD98059 did not affect the AKT activation elicited by Pb(II) in CL3 cells (Figure 3). The results suggest that Pb(II)-elicited ERK1/2 and AKT signals do not communicate with each other.

Inhibition of ERK suppresses the c-fos levels induced by Pb(II)
In response to growth factors and mitogens, the ERK1/2 activity is required for the expression of the immediate
wortmannin for 30 min before co-exposure to Pb(II) for 24 h or pretreated with 50 µM PD98059. The levels of ERK1/2 and AKT activation were determined from three independent experiments as described in Figure 2.

As shown in Figure 4A, Pb(II) increased c-FOS protein levels through phosphorylation and activation of early gene c-fos through phosphorylation and activation of transcriptional activators TCF and SRF (39,40). We therefore examined the ability of Pb(II) to induce c-FOS and the involvement of ERK1/2 signal. CL3 cells were exposed to 30 µM Pb(II) for various times and the protein levels of c-fos were determined by western blot analysis. As shown in Figure 4A, Pb(II) increased c-FOS protein levels. In contrast, wortmannin did not alter the low mutagenicity of Pb(II) in CL3 cells, while wortmannin does not. Cells were pretreated with PD98059 (30 µM) for 1 h or wortmannin (100 nM) for 30 min before exposure to various concentrations of Pb(II) for 24 h. Alternatively, cells were pretreated with various concentrations of PD98059 for 1 h before exposure to 300 µM Pb(II) for 24 h. The cytotoxicity and mutagenicity were performed by colony-forming ability (A) and 6-TG resistant (B and C) assays, respectively. Results were obtained from four to fourteen experiments and bars represent SEM. Total numbers of viable cells examined were 1.01–4.95×10⁶ in each treatment for 6-TG assay. **P < 0.01 using Student’s t-test for the comparison between cells exposed to Pb(II) and Pb(II) plus PD98059. The ERK1/2 activation (D) was examined as described in Figure 1, and the western blot shown is one representative of four independent experiments.

In serum-free medium and then subjected to the colony-forming ability and 6-TG assays. Approximately 40% of the cells survived when they were exposed to 500 µM Pb(II), which was reduced to 20% by co-exposure to PD98059 (Figure 5A, P = 0.01). In contrast, co-treatment with wortmannin did not affect the Pb(II)-induced cytotoxicity (Figure 5A). Neither PD98059 nor wortmannin alone exhibited a cytotoxic effect in these experiments (Figure 5A). After treatment with Pb(II) and/or inhibitors, CL3 cells were cultured for 7 days at exponential growth before selection for the hprt mutagenesis. As shown in Figure 5B, 300–500 µM Pb(II) did not induce the hprt mutation frequency in CL3 cells. Intriguingly, 50 µM PD98059 co-treatment dramatically increased the hprt mutation frequency in CL3 cells exposed to Pb(II) (Figure 5B, P = 0.01). Conversely, wortmannin did not alter the low mutagenicity of Pb(II) in CL3 cells (Figure 5B). To further illustrate the correlation between ERK1/2 inactivation and Pb(II) mutagenesis, CL3 cells were exposed to various concentrations of PD98059 for 1 h before treatment of 300 µM Pb(II) for 24 h. As shown in Figure 5C and 5D, PD98059 at 25–50 µM markedly enhanced the hprt mutation frequency in Pb(II)-treated cells, in which the ERK1/2 activity was completely suppressed. Conversely, wortmannin did not completely suppress the ERK1/2 activity showed no effect on the low mutagenicity in Pb(II)-treated cells.

The phenomenon that persistent activation of ERK1/2 participates in anti-cytotoxicity and anti-mutagenicity in Pb(II)-treated cells was further studied in a diploid human fibroblast line HFW. Western blot analysis showed that Pb(II) (10–500 µM) could dose-dependently activate ERK1/2 in HFW cells (Figure 6A). Again, PD98059 (50 µM) significantly
elevated the cytotoxicity and mutagenicity in Pb(II)-treated HFW cells (Figure 6B and 6C). Recently, PD98059 has been demonstrated to inhibit the activation of MKK5→ERK5 as well as MKK1/2→ERK1/2 pathways (42, 43). However, Pb(II) (10–500 µM) did not activate ERK5 in HFW cells (data not shown). The above results indicate that activation of the ERK1/2 signaling pathway protects human cells from cytotoxicity and mutagenicity upon Pb(II) exposure.

**NER participates in preventing Pb(II)-induced cytotoxicity and mutagenicity**

NER is a major error-free DNA repair process to remove a broad variety of DNA lesions caused by environmental carcinogens (44–46). The genotoxicity of Pb(II) has been attributed to indirect mechanisms such as interference with NER because it enhances the genotoxicity of strong mutagens such as UV (10, 14). However, evidence that Pb(II) alone affects the NER pathway has never been demonstrated. We therefore examined the cytotoxicity and the hprt mutation frequency in Pb(II)-exposed cells using a rodent UVL-10 cell line that is defective in NER and its counterpart AT3-2 cells (30, 31) to reveal the involvement of NER in preventing Pb(II) genotoxicity. UVL-10 cells are unable to produce ERCC1 protein due to a point mutation located at exon 5 of its encoded gene (32), thereby failing in incision 5’ to the site of base damage (44). As shown in Figure 7, UVL-10 cells were significantly more sensitive than AT3-2 cells to Pb(II)-induced cytotoxicity and mutagenicity. The results suggest that Pb(II) may induce DNA lesions that can be repaired through the NER pathway. Intriguingly, ERK1/2 activation by Pb(II) was observed in AT3-2 but not UVL-10 cells (Figure 7C), despite the endogenous ERK1/2 activity being 2.4-fold higher in the latter. The results suggest that ERK1/2 activation may be correlated to NER capability in cells exposed to Pb(II).

**Persistent activation of ERK1/2 enhances NER synthesis**

We next adopted a NER synthesis assay (35, 36) to explore whether Pb(II)-activated ERK1/2 signal is involved in regulating DNA repair processes. CL3 cells were left untreated or treated with 10–100 µM Pb(II) for 24 h in the presence or absence of PD98059 and then allowed recovery in serum-free media for 8 h before extraction of the proteins. The proteins in the WCE were incubated with UV-irradiated plasmid DNA, 4 dNTP, and α-32P[dCTP] to examine the efficiency of NER synthesis. As shown in Figure 8, the capability of WCE derived from the Pb(II)-treated cells to incorporate nucleotides into UV-damaged DNA was higher than that derived from the untreated control cells. Furthermore, PD98059 markedly decreased the Pb(II)-stimulated NER synthesis (Figure 8). Quantitative analysis showed that cells treated with 30 µM Pb(II) increased the NER synthesis by 166% of the untreated cells; in the presence of PD98059 this level was 22% lower than that derived from the untreated control cells. The results indicate that Pb(II) exposure elevates cellular NER synthesis through the ERK1/2 pathway.

To further examine the role of the ERK1/2 signal in NER synthesis, we manipulated the ERK1/2 activity by transfection of MKK1-CA, a constitutive active mutant of MKK1 vector, into CL3 cells and allowed expression for 2 days before preparation of WCE for the NER synthesis assay. As shown in Figure 9, the WCE derived from cells transfected with 5–15 µg of MKK1-CA had elevated phospho-ERK1/2 and exhibited markedly higher NER synthesis than that derived from cells expressing a control vector. Taken together, the
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Fig. 8. Pb(II) increases the efficiency of NER synthesis in CL3 cells in an ERK1/2-dependent manner. Cells were left untreated or pretreated with PD98059 (50 µM) for 1 h before exposure to 10–100 µM of Pb(II) for 24 h in serum-free medium. The cells were then washed twice with PBS and kept cultured in serum-free medium for 8 h before WCE preparation. The NER synthesis efficiency of equal amounts of proteins (60 µg) in WCE was determined by reaction with pUC19 plasmid (250 ng) that had been irradiated with UV (400 J/m²) as described in Materials and methods. Untreated pUC19 plasmid (-UV) was incubated with WCE derived from untreated cells to serve as a negative control. In (A), upper panel: autoradiograph of gels, showing the incorporation of α-[32P]dCTP. Lower panel: photograph of the same gels stained with ethidium bromide showing equal amounts of DNA used in each reaction. In (B), the relative activities were determined by densitometric analysis, which were calculated from the average of four to eight independent experiments and were normalized by arbitrarily setting the densitometry of control cells to 100%.

Fig. 9. Expression of MKK1-CA, a constitutively active form of MKK1, enhances the efficiency of NER synthesis in CL3 cells. Cells (4 x 10⁵) were plated in a 60 mm dish 1 day before transfection. Vectors pcDNA3 or MKK1-CA (5–15 µg) were transfected into CL3 cells and allowed expression for 2 days. The WCE was harvested to determine the efficiency of NER synthesis as described in Figure 8. An example of gel pattern is shown in (A) and the quantitative results averaged from four independent experiments are shown below the autoradiograph. The levels of phospho-ERK1/2 and ERK2 in equal amounts of proteins derived from each WCE are also shown in (B).

above results indicate that activation of ERK1/2 enhances cellular NER synthesis and that the low mutagenicity of Pb(II) in NER-proficient mammalian cells may be due mainly to the sustained-ERK1/2 signal to enhance the error-free NER synthesis.

PD98059 does not affect the intracellular Pb levels

The levels of Pb accumulated in cells may influence toxicity. We therefore measured the amounts of Pb accumulation in the presence or absence of PD98059 using ICP-MS. CL3 cells were exposed to 300–500 µM of Pb(II) for 24 h, washed three times with PBS and subjected to the analysis of the intracellular Pb amounts. As shown in Figure 10, similar levels of Pb were accumulated in the presence or absence of PD98059. This result indicates that the ability of PD98059 to enhance the cytotoxicity and mutagenicity and decrease NER synthesis in Pb(II)-treated cells is not due to differential intercellular Pb levels.

Discussion

Previous reports have indicated that Pb(II) exhibits weak genotoxicity in cultured rodent cells and does not cause mutations in human cells (8–14,47,48). However, in vitro evidence has demonstrated that this non-essential toxic metal can destabilize DNA helical structure and induce DNA strand breaks and oxidative DNA adducts (15,16). These contradictory results suggest that the etiology of Pb(II) genotoxicity is rather complex. Because Pb(II) enhances the genotoxicity of strong
mutagens such as UV and alkylating carcinogens, it has been proposed that Pb(II) impedes NER machinery (10,14). Nonetheless, evidence of cellular NER affected by Pb(II) alone has never been demonstrated. Here we show for the first time that NER synthesis is indeed elevated in Pb(II)-exposed human cells and that Pb(II) induces significantly higher cytotoxicity and mutagenicity in the NER-deficient than in the NER-proficient rodent cells. These results clearly suggest that exposure of mammalian cells to Pb(II) stimulates NER and confers anti-mutagenicity. In the presence of H$_2$O$_2$, Pb(II) dose-dependently induces 8-hydroxydeoxyguanosine adducts in calf thymus DNA, which can be prevented by singlet oxygen scavengers (16). Cellular NER machinery removes many kinds of base damage including oxidative DNA adducts (44–46). Whether Pb(II) can induce oxidative DNA adducts in NER-deficient cells deserved further investigation.

Recent studies on neural-derived cells have shown that Pb(II) affects signal transduction pathways including ERK1/2 (27,28), however, the physiological role remains largely unknown. In this study, we found that Pb(II) could activate ERK1/2 among the four MAPKs and AKT survival signals in human CL3 cells. We further demonstrated that inhibition of the persistent activated-ERK1/2 by PD98059 markedly potentiated the cytotoxicity and mutagenicity of Pb(II) in human CL3 and HFW cells. Conversely, blockade of the transient activated-AKT in CL3 cells did not affect the low mutagenicity of Pb(II). Intriguingly, Pb(II) could stimulate ERK1/2 in the NER-proficient but not the NER-deficient rodent cells. Moreover, the Pb(II)-induced NER synthesis was diminished by inhibiting the persistent activated-ERK1/2 in CL3 cells; also, constitutive expression of MKK1 → ERK1/2 signal could significantly increase NER synthesis. Taken together, these observations indicate that Pb(II) persistently activates ERK1/2 which triggers NER, thereby conferring anti-cytotoxicity and anti-mutagenicity in mammalian cells.

ERK1/2 exemplify one class of MAPKs that undergoes activation by a range of stimuli including growth factors, cytokines, cell adhesion, tumor-promoting phorbol esters and oncogenes (19). It is well known that ERK1/2 activation is necessary for cell growth because it phosphorylates and activates numerous substrates involved in nucleotide synthesis, gene transcription, protein synthesis and cell cycle progression (19). Recently, three global NER enzymes involved in the recognition of and binding to damaged DNA, i.e. hHR23A, hHR23B and replication protein A2, have been identified as novel targets which are posttranslationally modified by the ERK1/2 pathway using functional proteomics and mass spectrometry techniques (49). The hHR23B that tightly complexes with XPC to stimulate the initiation step of NER (44–46) is rapidly proteolytically processed and phosphorylated in response to ERK1/2 signaling induced by UV (49). Accordingly, ERK1/2 may regulate the repair efficiency by posttranslational modification of enzymes involved in NER. Alternatively, ERK1/2 may phosphorylate transcription factors to upregulate the expression of genes involved in NER and thereby enhance the repair efficiency. In human cells, NER is composed of at least 30 proteins involved in DNA damage recognition, dual incision of the DNA strand containing a lesion, DNA synthesis and ligation to replace error-free an excised 25–30 oligonucleotide (44–46). The role of ERK1/2 signaling in regulating the expression of NER genes and posttranslational control of NER proteins warrants further investigation.

Both the ERK1/2 and p38 pathways contribute to TCF activation and c-fos transcription in response to UV (50). Here, we also observed that c-FOS induced by Pb(II) required ERK1/2 and was possibly independent of p38. This is consistent with the finding that sustained phospho-ERK docking to the DEF domain of c-FOS protein resulted in phosphorylation and prolongation of its biological effect (41). Our present finding that ERK1/2 could be involved in preventing genotoxicity is also consistent with previous reports showing that c-FOS play a role in cellular defense systems, in which mouse fibroblasts lacking c-fos are hypersensitive to a wide variety of genotoxic agents in the induction of cytotoxicity, apoptosis and chromosomal breakages (51,52). Recently, ERK1/2 signaling has been implicated in protecting apoptosis and micronuclear formation induced by Cd (33,53), enhancing cellular viability and recovery from the G2/M cell cycle checkpoint arrest upon ionizing radiation (54) and reducing DNA strand breakage and apoptosis induced by hyperoxia (55). Moreover, a recent report has shown that ERK1/2 activated upon ionizing radiation is associated with increased expression of ERCC1 and XRC1, repairing of apurinic sites and decreased micronucleus formation (56). All the above data suggest that ERK1/2 signal activated by certain DNA damage agents can function in providing protection from genomic instability. On the other hand, activation of the ERK1/2 through constitutive expression of oncoproteins such as Mos and Ras greatly enhances chromosome instability (57–59). The finding that ERK1/2 participated in chromosome instability caused by Mos has been associated with the loss of p53 function to induce cell cycle arrest and apoptosis in mouse embryo fibroblasts (57). However, ERK1/2-mediated chromosome instability induced by Ras is also observed in thyroid PCCCL3 cells containing wild-type p53 (59), yet these cells are resistant to apoptosis upon ionizing radiation (60). The contradictory role of ERK1/2 signaling induced by DNA damage agents and oncoproteins in maintaining genome integrity is obviously an interesting issue for further exploration.

Previous reports have indicated that Pb(II) can substitute for Ca(II) and bind with Ca(II) binding proteins (61,62). In CL3 cells, Pb(II) could increase intracellular Ca(II) levels, however, PD98059 did not alter the Ca(II) levels induced by Pb(II) (data not shown). Moreover, pretreatment CL3 cells with a membrane-permeable form of Ca(II) chelator, (acetoxyethyl)-1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (20 µM) did not affect Pb(II)-induced ERK1/2 activation (data not shown). ERK1/2 activation by Pb(II) in CL3 cells may thus be independent of intracellular Ca(II) levels. Pb(II) can activate protein kinase C (PKC) in many neural-derived cells (63,64). The PKC activity is implicated in the induction of AP-1 DNA binding activity and c-fos expression by Pb(II) in PC-12 cells (65,66). More recently, PKCa$_\alpha$ is identified as an upstream signal for the activation of ERK1/2 in human astrocytoma cells exposed to Pb(II) for 15–30 min; however, longer exposure (24 h) results in down-regulation of both PKCa and ERK1/2 (28). In contrast to the finding in human astrocytoma cells (28), we observed that longterm Pb(II) exposure persistently activated ERK1/2 in human CL3 cells. Whether PKC plays a role in ERK1/2 activation in our system is currently under investigation.

Noticeably, contradictory to the finding of WCE derived from Pb(II)-exposed cells, our unpublished data showed that the efficiency of NER synthesis was inhibited by adding Pb(II) in vitro, which is consistent with a previous observation (67).
This phenomenon together with the finding that inhibition of ERK1/2 could not affect Pb levels in CL3 cells (Figure 10) suggest that once entering the cells the metal itself may not directly interfere with NER machinery, while in a cell-free system Pb(II) may interact with NER proteins and decrease their enzymatic activities. However, an in vitro study has shown that Pb(II) does not affect the DNA binding activity of mouse XPA protein (68), a NER metalloprotein that preferentially binds to damaged DNA (44–46). Pb(II) may block other NER proteins in a cell-free system, nevertheless, this does not reflect the physiological role of Pb(II).

Exposure to Pb(II) could induce cellular NER synthesis, however, our unpublished data showed that co-exposure of CL3 cells to benzo[a]pyrene diol epoxide and Pb(II) synergistically enhanced mutagenicity and cytotoxicity in CL3 cells. Intriguingly, no activation of the ERK1/2 signal was observed in this co-treatment (Lin et al., manuscript in preparation). These results suggest that the co-genotoxicity of Pb(II) is due partly to down-regulation of the ERK1/2 activity. It is therefore important to notice that although Pb(II) itself can activate ERK1/2 to prevent genotoxicity, complex signaling transduction pathways would be generated when Pb(II) was combined with other environmental carcinogens that may result in a different cell fate.

In conclusion, we have demonstrated for the first time that persistent activation of ERK1/2 by Pb(II) is essential for the stimulation of NER synthesis conferring anti-cytotoxicity and anti-mutagenicity in mammalian cells. Conversely, the AKT signal transiently induced by Pb(II) does not influence the genotoxicity of Pb(II). Although Pb(II) itself is capable of inducing an error-free repair mechanism, multifaceted DNA damage checkpoint signals can be stimulated when there is a combination of exposures to other environmental mutagens, which may decrease the protecting effect generated by Pb(II). Moreover, we identify a novel role of ERK1/2 in regulating DNA repair machinery and guarding genome integrity.

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