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Cadmium activates extracellular signal-regulated kinase 5 in HK-2 human renal proximal tubular cells

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ABSTRACT

We examined the effects of cadmium chloride ($CdCl_2$) exposure on the phosphorylation and functionality of extracellular signal-regulated kinase 5 (ERK5), a recently identified member of the mitogen-activated protein kinase (MAPK) family, in HK-2 human renal proximal tubular cells. Following exposure to $CdCl_2$, ERK5 phosphorylation increased markedly, but the level of total ERK5 was unchanged. ERK5 phosphorylation following $CdCl_2$ exposure was rapid and transient, similar to the time course of ERK1/2 phosphorylation. Treatment of HK-2 cells with the MAPK/ERK kinase 5 inhibitor, BIX02189, suppressed $CdCl_2$-induced ERK5 but not ERK1/2 phosphorylation. The $CdCl_2$-induced increase of phosphorylated cAMP response element-binding protein (CREB) and activating transcription factor-1 (ATF-1), as well as the accumulation of mobility-shifted c-Fos protein, were suppressed by BIX02189 treatment. Furthermore, BIX02189 treatment enhanced cleavage of poly(ADP-ribose) polymerase and increased the level of cytoplasmic nucleosomes in HK-2 cells exposed to $CdCl_2$. These findings suggest that ERK5 pathway activation by $CdCl_2$ exposure might induce the phosphorylation of cell survival-transcription factors, such as CREB, ATF-1, and c-Fos, and may exert a partial anti-apoptotic role in HK-2 cells.

1. Introduction

Cadmium is an occupational and environmental pollutant that damages various organs, especially renal proximal tubular cells [1]. It has also been reported to induce apoptosis in the proximal tubules of experimental animals [2], as well as LLC-PK_1 porcine and HK-2 human proximal tubule epithelial cell lines [3,4]. However, the signaling pathways responsible for cadmium-induced damage and anti-apoptosis have not been fully elucidated.

Mitogen-activated protein kinases (MAPKs) are evolutionarily conserved enzymes that transmit extracellular signals to critical intracellular regulatory targets [5,6]. Extracellular signal-regulated kinase 5 (ERK5), also known as big MAPK 1 (BMK1), is a recently identified member of the mammalian MAPK family [7]. Like other MAPKs (e.g., ERK1/2, c-Jun NH_2-terminal kinase [JNK], and p38), ERK5 activation requires dual phosphorylation of threonine and tyrosine residues in the kinase domain by specific MAPK kinase (MAPKK). ERK5 is twice the size of other MAPKs (~100 kDa) and contains an N-terminal kinase domain that is 51% homologous with ERK2, as well as a unique C-terminal extension that contains a transactivation domain, a nuclear localization sequence, a nuclear export sequence, and two proline-rich regions [8,9]. The ERK5 pathway has been implicated in cell survival, anti-apoptotic signaling, angiogenesis, cell motility, differentiation, and cell proliferation [10]. Although ERK5 is reportedly activated by MAPK/ERK kinase 5 (MEK5), a member of MAPKK family, in response to growth factors, serum, oxidative stress, and hyperosmolarity [8,10], little attention has been paid to the role of the ERK5 pathway in cellular damage induced by environmental stresses. To our knowledge, the effects of cadmium exposure on ERK5 phosphorylation and its pathological significance in proximal tubule epithelial cells have not yet been examined.

In the present study, we assessed phosphorylated ERK5 and ERK1/2 in HK-2 cells exposed to cadmium chloride ($CdCl_2$). ERK5 pathway activation induces the phosphorylation of many transcription factors, including cAMP response element-binding protein (CREB) and c-Fos [10,11]. We used BIX02189, a pharmacological inhibitor of MEK5 [12], to determine the contribution of ERK5 activation to CREB and c-Fos phosphorylation in $CdCl_2$-treated HK-2 cells. In addition, the protective role of the ERK5 pathway in $CdCl_2$-induced apoptosis was examined in HK-2 cells pretreated with BIX02189.

Abbreviations: ATF-1, activating transcription factor-1; $CdCl_2$, cadmium chloride; CREB, cAMP response element-binding protein; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEK, MAPK/ERK kinase; PARP, poly(ADP-ribose) polymerase.

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2. Materials and methods

2.1. Chemicals

$CdCl_2$ was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BIX02189 was purchased from Selleck Chemicals (Houston, TX). Antibodies raised against phospho-ERK5 (Thr218/Tyr220), ERK5 (D23E9), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), ERK1/2, phospho-CREB (Ser133) (87G3), CREB (48H2), and poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against c-Fos (4) and actin (I-19) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Cell Count Reagent SF was from Nacalai Tesque (Kyoto, Japan).

2.2. Cell culture and treatments

HK-2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in D-MEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Invitrogen Corp., Carlsbad, CA) in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. For each experiment, exponentially growing HK-2 cells were seeded 4×10^5 cells/well in 6-well culture plates or 1×10^4 cells/well in 96-well culture plates, cultured for 1 day, and deprived of serum for 24 h before the experiments. $CdCl_2$ was dissolved in water and sterilized by filtration. Cells were incubated with serum-free medium containing an appropriate concentration of $CdCl_2$ for 1 to 16 h at 37 °C. BIX02189 was dissolved in dimethyl sulfoxide (DMSO). After preincubation in serum-free medium containing DMSO (0.1%) or BIX02189 (5, 10, 20, or 50 μM) for 1 h, HK-2 cells were treated with 50 μM $CdCl_2$ for 2 or 4 h, or with 20 or 50 μM $CdCl_2$ for 16 h.

2.3. Western immunoblotting

Cells were washed with phosphate-buffered saline and lysed with sodium dodecyl sulfate (SDS)-polyacrylamide gel Laemmli sample buffer. The cell lysates were sonicated and then boiled for 5 min. Protein concentrations were determined using the RC DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of protein (5 or 10 μg) were subjected to SDS-10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Buckinghamshire, England). The membrane was blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. The membrane was then incubated overnight at 4 °C with the primary antibody and the protein was detected with a Phototope-HRP Western blot detection kit (Cell Signaling Technology).

2.4. Nucleosome assay

After preparing the cytoplasmic fraction, histone-associated DNA fragments (mono- and oligonucleosomes) were assayed with a Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions.

2.5. WST-8 assay

Cell viability was determined using a WST-8 cytotoxicity assay. This assay is based on the conversion of tetrazolium salt, WST-8, to the highly water soluble formazan by viable cells. We added 10 μl Cell Count Reagent SF containing 5 mM WST-8 to each well of 96-well culture plates. After incubation for 2 h at 37 °C, the absorbance of each well was measured at 450 nm with a reference wavelength at 655 nm.

2.6. Statistics

The results are expressed as the mean ± standard error of mean (S.E.M.). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons tests. $P < 0.05$ was considered statistically significant.

3. Results

Western immunoblotting for phospho-ERK5 (Thr218/Tyr220) confirmed that phosphorylated ERK5 was detected at the same molecular weight (115-kDa) in HK-2 cells treated with epidermal growth factor and those with $CdCl_2$ (data not shown). Following exposure to 50 μM $CdCl_2$, the level of phosphorylated ERK5 increased after 1 h, peaked at 2 h, and then gradually declined (Fig. 1). However, the level of total ERK5 did not change during the 5-h incubation period. Similarly, the levels of phosphorylated ERK1/2 (ERK2/p42 and ERK1/p44) increased after 1 h and peaked at 2 h, whereas the level of total ERK1/2 was not changed by $CdCl_2$ exposure. The phospho-CREB antibody used in the present study also detected the phosphorylated form of the CREB-related protein activating transcription factor-1 (ATF-1). In contrast to ERK5 and ERK1/2, the levels of the phosphorylated forms of CREB and ATF-1 increased after 1 h and continued to increase over the entire incubation period. After 3 or 4 h incubations, we observed greater levels of c-Fos protein with lower electrophoretic mobility, which was suggestive of increased c-Fos phosphorylation [13]. We did not observe any significant changes in total CREB or actin levels in $CdCl_2$-treated cells.

When HK-2 cells were incubated with 1–50 μM $CdCl_2$ for 2 h, phosphorylated ERK5 and ERK1/2 increased in cells treated with concentrations higher than 10 μM (Fig. 2). After a 4-h incubation,

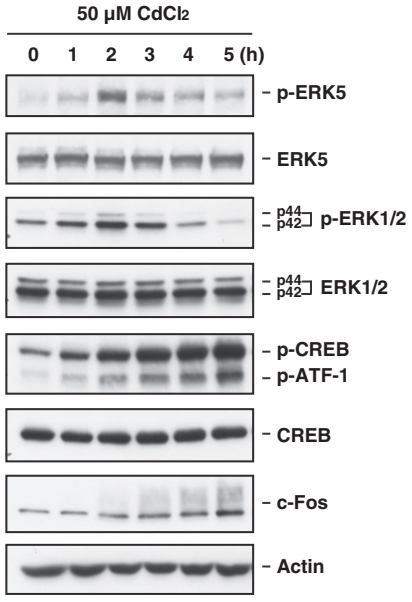

Fig. 1. Time-course of cadmium-induced accumulation of phosphorylated ERK5, phosphorylated ERK1/2, phosphorylated CREB, and c-Fos proteins in HK-2 cells incubated with 50 μM $CdCl_2$ for 1–5 h. Cell lysates were subjected to Western immunoblotting using antibodies against phospho-ERK5, ERK5, phospho-ERK1/2, ERK1/2, phospho-CREB, CREB, c-Fos, and actin. Results are representative of at least four experiments.

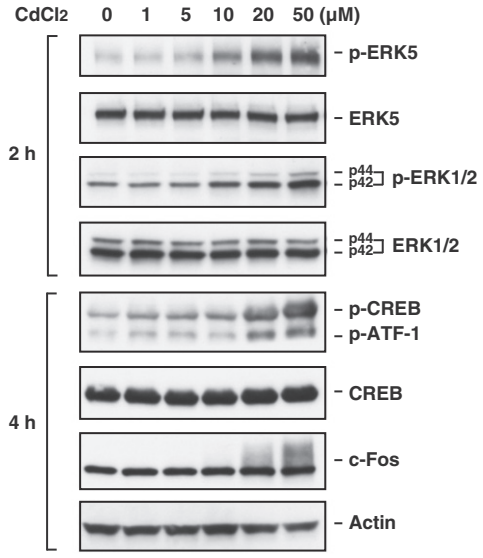

Fig. 2. Dose-dependent effect of cadmium-induced accumulation of phosphorylated ERK5, phosphorylated ERK1/2, phosphorylated CREB, and c-Fos proteins in HK-2 cells incubated with 0, 1, 5, 10, 20, or 50 μM $CdCl_2$ for 2 (top four panels) or 4 h (bottom four panels). Cell lysates were subjected to Western immunoblotting using antibodies against phospho-ERK5, ERK5, phospho-ERK1/2, ERK1/2, phospho-CREB, CREB, c-Fos, and actin. Results are representative of at least four experiments.

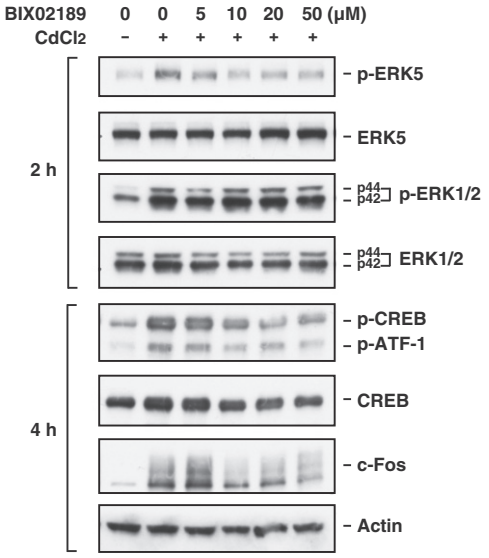

Fig. 3. Effects of BIX02189 on cadmium-induced accumulation of phosphorylated ERK5, phosphorylated ERK1/2, phosphorylated CREB, and c-Fos proteins in HK-2 cells. Cells were preincubated with 0.1% DMSO or 5, 10, 20, or 50 μM BIX02189 for 1 h, then incubated with or without 50 μM $CdCl_2$ for 2 (top four panels) or 4 h (bottom four panels). Cell lysates were subjected to Western immunoblotting using antibodies against phospho-ERK5, ERK5, phospho-ERK1/2, ERK1/2, phospho-CREB, CREB, c-Fos, and actin. Results are representative of at least three experiments.

the phosphorylated forms of CREB, ATF-1, and c-Fos increased in cells treated with 20 μM $CdCl_2$ and higher. The total levels of ERK5, ERK1/2, CREB, and actin were not changed at any concentration after 2 or 4 h incubations. In HK-2 cells exposed to 1, 5, 10, 20, or 50 μM $CdCl_2$ for 4 h, cell viabilities determined by WST-8 assay were 107.6 ± 4.6%, 113.2 ± 4.0%, 112.2 ± 1.0%, 108.5 ± 4.6%, and 98.9 ± 6.1%, respectively (percentage of the value of control cells without $CdCl_2$ exposure, mean ± S.E.M. of three experiments). Thereafter, cells were incubated with 20 or 50 μM $CdCl_2$.

At 2 h, treatment of HK-2 cells with 5–50 μM BIX02189 suppressed $CdCl_2$ (50 μM)-induced ERK5 phosphorylation in a dose-dependent manner, whereas the level of total ERK5 was unchanged in this range (Fig. 3). On the other hand, $CdCl_2$-induced phosphorylation of ERK1/2 was not affected by BIX02189. At 4 h, $CdCl_2$ (50 μM)-induced accumulation of phosphorylated CREB and ATF-1, and accumulation of mobility-shifted c-Fos protein were suppressed by treatment with BIX02189 at 10 or 20 μM. BIX02189 treatment did not affect the level of total ERK1/2, total CREB, or actin.

To examine the possible role of ERK5 activation in cellular damage, HK-2 cells were exposed to 20 or 50 μM $CdCl_2$ for 16 h. Cell viabilities were 104.7 ± 1.3% for 20 μM and 49.2 ± 4.0% for 50 μM $CdCl_2$ exposure (percentage of the value of control cells without $CdCl_2$ exposure, mean ± S.E.M. of three experiments). Following exposure to 20 or 50 μM $CdCl_2$ for 16 h, the caspase-3 substrate PARP was cleaved into 89-kDa fragments, indicating HK-2 apoptosis (Fig. 4A, lanes 2 and 3). PARP cleavage was further enhanced by treatment with BIX02189 (20 μM) in cells exposed to 20 μM but not 50 μM $CdCl_2$ (Fig. 4A, lanes 5 and 6). Consistent with these findings, BIX02189 (20 μM) increased the level of cytoplasmic nucleosomes in HK-2 cells treated with 20 μM $CdCl_2$ for 16 h (Fig. 4B).

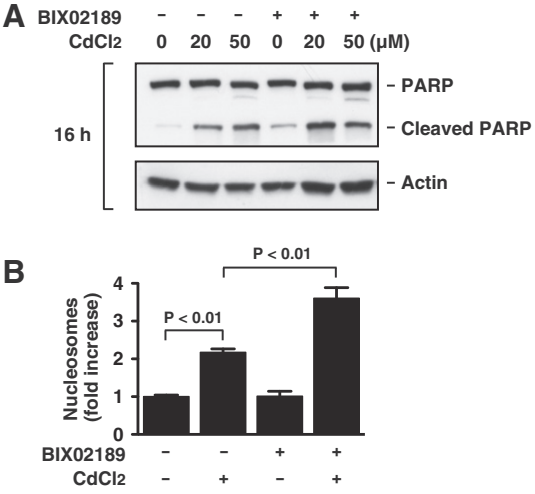

Fig. 4. Effects of BIX02189 on cadmium-induced apoptosis in HK-2 cells. (A) PARP cleavage. Cells were preincubated with 0.1% DMSO or 20 μM BIX02189 for 1 h, then incubated with 0, 20, or 50 μM $CdCl_2$ for 16 h. Cell lysates were subjected to Western immunoblotting using antibodies against PARP and actin. Full-length and cleaved forms of PARP were detected at 116- and 89-kDa, respectively. Results are representative of at least four experiments. (B) Cytoplasmic nucleosomes. Cells were preincubated with 0.1% DMSO or 20 μM BIX02189 for 1 h, then incubated with or without 20 μM $CdCl_2$ for 16 h. The cytoplasmic fraction was used for a nucleosomes ELISA. Each value (mean ± S.E.M., n = 5–7) represents the fold increase with respect to untreated control (without BIX02189 or $CdCl_2$). Results are representative of four experiments.

4. Discussion

Cadmium exposure markedly augmented the phosphorylated form of ERK5 in HK-2 cells, but the level of total ERK5 did not change. ERK5 phosphorylation peaked at 2 h and then reduced following a time course similar to that of ERK1/2 phosphorylation. As has been reported previously [13], levels of phosphorylated forms of MAPK family members JNK and p38 began to increase after 2 h, and continued to rise as incubation time increased (data not shown). On the other hand, no significant reduction of cell viability was found when HK-2 cells were exposed to 1–50 μM $CdCl_2$ for 4 h. Similar to ERK5, ERK1/2 was originally shown to be important for cell survival, whereas JNK and p38 were implicated in apoptotic cell death [14]. The collective data suggest that

cellular stress initiated by cadmium exposure might transmit two functionally distinct signals in proximal tubule epithelial cells, i.e., rapid but transient ERK1/2 and ERK5 phosphorylation and delayed but prolonged JNK and p38 phosphorylation. The upstream activator of ERK5 is MEK5, which is phosphorylated by MAPK kinase kinases (MAPKKK or MEKK) 2 and 3, whereas ERK1/2 is phosphorylated by MEK1 and 2, which themselves are phosphorylated by Raf and Mos [15]. Therefore, how the renal epithelial cell line differentially senses and activates ERK1/2 and ERK5 signaling pathways in response to cadmium exposure remain to be determined.

Recently, BIX02188 and BIX02189 were identified as novel MEK5 inhibitors. Both compounds suppress MEK5 catalytic activity and are selective against several kinases, including the closely related kinases, MEK1, MEK2, ERK2, and JNK2, but BIX02189 is more potent [10,12]. In the present study, BIX02189 inhibited ERK5 phosphorylation in a dose-dependent manner in $CdCl_2$-exposed HK-2 cells without affecting ERK1/2 phosphorylation. It has also been reported that BIX02189 ($\sim$30 μM) selectively suppresses ERK5 phosphorylation in sorbitol-treated HeLa cells [12], rat PC12 pheochromocytoma cells treated with nerve growth factor [16], neonatal rat cardiomyocytes treated with isoproterenol [17], and rat C6 glioma cells treated with basic fibroblast growth factor [18]. However, $CdCl_2$-induced phosphorylation of p38 but not JNK was suppressed in HK-2 cells treated with BIX02189 (data not shown). Although the possibility of nonselective inhibitory effects on other kinases cannot be completely excluded, BIX02189 appears to preferentially inhibit the ERK5 pathway relative to ERK1/2 in cells treated with various stimuli, including cadmium.

Treatment of HK-2 cells with BIX02189 suppressed $CdCl_2$-induced accumulation of phosphorylated forms of CREB and ATF-1 and mobility-shifted c-Fos protein, suggesting that ERK5 pathway activation induces the phosphorylation of these transcription factors involved in cell proliferation and survival [19,20]. Consistent with our findings, ERK5 is reported to be the predominant mediator of CREB phosphorylation in rat dorsal root ganglion neurons [21] and A549 human pulmonary adenocarcinoma cells [22], while ERK1/2 induces CREB phosphorylation by activating a ribosomal protein S6 kinase (Rsk) family member in COS cells [23]. Furthermore, activation of the MEK5/ERK5 pathway causes the phosphorylation and stabilization of c-Fos in COS cells [24], and an in vitro kinase assay showed that ERK5 phosphorylates c-Fos at Ser32 and Thr232 [25]. In contrast, another study demonstrated that activation of ERK1/2, but not ERK5, is sufficient for c-Fos phosphorylation in human embryonic kidney 293 (HEK293) cells [26]. We also found that treatment with the MEK1/2 inhibitor U0126 and the p38 inhibitor SB203580 suppressed $CdCl_2$-induced phosphorylation of c-Fos at Ser362 and Ser374 in HK-2 cells [13]. Collectively, in addition to the p38 pathway [27], the data suggest that the ERK1/2 and ERK5 pathways might individually or cooperatively regulate the phosphorylation of transcription factors, such as CREB, ATF-1, and c-Fos, depending on cell and stimuli type.

BIX02189 treatment also enhanced PARP cleavage and increased the level of cytoplasmic nucleosomes in HK-2 cells exposed to 20 μM but not 50 μM $CdCl_2$. These findings suggest that $CdCl_2$-induced activation of the ERK5 pathway might play a partial anti-apoptotic role in HK-2 cells. However, the anti-apoptotic function of ERK5 might not be sufficient to reduce severe renal proximal tubular cell damage caused by a relatively high dose of $CdCl_2$ (50 μM). The ERK5 pathway is reportedly involved in protecting against a variety of stimuli that induce apoptotic cell death in a number of different cell types [28–31]. Additional experiments are required to identify the target molecules responsible for cell survival through ERK5 activation and its downstream transcription factors in renal proximal tubular cells following exposure to cadmium and other nephrotoxic compounds.

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