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Connexin 43 mediates changes in protein phosphorylation in HK-2 cells during chronic cadmium exposure

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ABSTRACT

Connexin 43 (Cx43) is believed to play a role in the mechanisms of toxicity of many chemical species, include cadmium (Cd). In this study, human renal proximal tubule (HK-2) cells were exposed to Cd (1 μM, 10 days). Of the 584 protein residues detected using a Phospho Explorer antibody microarray (PEX100), more than half changed their levels of phosphorylation after chronic Cd exposure. Cx43 siRNA attenuated Cd-induced apoptosis and inhibited proliferation, while also attenuating changes in the levels of phosphorylation of many protein residues. According to DAVID Bioinformatics Resources analysis and KEGG PATHWAY database, AKT signal pathway may be the important one. Focusing on the AKT pathway confirmed that Cx43 mediated increased levels of p-PTEN$^{Ser380/Ser382/Thr383}$ and decreased levels of p-AKT^{Thr308}, p-AKT^{Tyr326}, p-ASK1^{Ser83}, and p-p27^{Thr187}, thereby possibly contributing to the Cd-induced apoptosis and inhibited proliferation. These results suggested that AKT pathway was the dominant pathway involved in Cx43-mediated chronic Cd toxicity.

1. Introduction

Changes in protein phosphorylation can directly affect biological activity, alter protein–protein interactions, and impact the formation of molecular complexes, and intra- and extracellular signaling pathways (Pawson et al., 2002). When encountering an environmental stimulant, all living cells will perceive and interact with their environmental signals to regulate cell growth, differentiation, metabolism, and apoptosis by altering their levels of protein phosphorylation, as well as further changes in signal transduction. Therefore, recognizing systematic changes in the levels of phosphorylated residues may offer an opportunity to view a wide range of variations in signal pathways for environmental stimulation and, thereby, better identify biomarkers and therapeutic targets.

Cadmium (Cd) is an environmental contaminant that is involved in the onset of many diseases. The kidneys are major target organs for chronic Cd exposure, with epidemiological studies, animal experiments, and *in vitro* tests suggesting that renal proximal tubules are among the main target sites (Gennari et al., 2003; Liang et al., 2012; Zhang et al., 2013). Although toxic effects of Cd on renal proximal tubules involve alteration of cellular signaling cascades, no systematic examinations have appeared of the changes in the phosphorylation status of residues along the signal pathway during long-term, low-dose, chronic exposure of human renal proximal tubule cells to Cd.

Connexins (Cxs) and Cx-formed hemichannels and gap junction channels have also been implicated in many diseases (Kumar and Gilula, 1996; Contreras et al., 2004). Cx-formed gap junction channels and hemichannels permit the direct transfer of small molecules between adjoining cells or between intra- and extracellular environments (Kumar and Gilula, 1996; Sala et al., 2016). Thus, Cxs are believed to play a crucial role in the regulation of cell signal pathways and in maintaining cell homeostasis by keeping cell signals at equilibrium among channel-connected cells. In addition, Cxs can interact with structural proteins and cell signaling molecules to influence cell growth, death, and function in a channel-independent manner (Kalra et al., 2006; Abed et al., 2015). In the kidneys, various subtypes of Cxs are expressed along the nephron (Abed et al., 2015). Human proximal tubules express several Cx isoforms, including Cx43, the levels of which might be associated with chronic kidney disease in human samples (Abed et al., 2014). Nevertheless, whether Cx43 mediates Cd-induced changes in protein phosphorylation has not been established with certainty.

In this study, we used the Phospho Explorer antibody microarray (PEX100) and Cx43 small interfering RNA (siRNA) to investigate the

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Cx43-mediated changes in protein phosphorylation along cell signal pathways during chronic Cd exposure.

2. Materials and methods

2.1. Materials and reagents

Cadmium chloride ($CdCl_2$) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY, USA). RPMI-1640 medium and PBS were purchased from Corning (Manassas, VA, USA). An EdU cell proliferation kit was obtained from RiboBio (Guangzhou, China). An annexin V-FITC apoptosis detection kit and PI/RNase Staining Buffer were purchased from BD Biosciences (San Diego, CA, USA). The Phospho Explorer Antibody Array (PEX100) was obtained from Full Moon BioSystems (Sunnyvale, CA, USA). Rabbit monoclonal antibodies against Bax, Bcl-2, $p27^{Kip1}$ and p38, $p\text{-}AKT^{Ser437}$, $p\text{-}PTEN^{Ser380/Ser382/Thr383}$, PTEN, and β-tubulin and rabbit polyclonal antibodies against Cx43, p53, cyclinD1, $p\text{-}AKT^{Thr308}$, and $p\text{-}ASK1^{Ser83}$ were purchased from Cell Signaling Technology (Cambridge, MA, USA). Rabbit polyclonal antibodies against $p\text{-}AKT^{Tyr326}$ and $p\text{-}p27^{Thr187}$ were purchased from Abcam (Cambridge, MA, USA). The kits and chemicals for western blotting were obtained from Beyotime Company of Biotechnology (Shanghai, China).

2.2. Cell culture and Cd treatment

Human renal proximal tubular epithelial (HK-2) cells, obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), were used in this experiment. The HK-2 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (Beyotime, Shanghai, China) in a humidified incubator with 5% CO_2 at 37 °C. $CdCl_2$ was dissolved in aseptic distilled water to give 200 mM stock solutions. In a preliminary experiment, HK-2 cells were exposed to low-dose Cd (1 μM) (Olszowski et al., 2015) for 2 days or 6 days; no differences in cell apoptosis or proliferation rates appeared in the control and Cd-exposed groups (data not shown). The HK-2 cells were then exposed to Cd (1 μM) for 10 days.

2.3. Determination of gap junction intercellular communication (GJIC)

GJIC was evaluated using a dye transfer assay, as described previously (Gielen et al., 2013). In brief, Calcein AM and DiI dye (Dojindo Laboratories, Kumamoto, Japan) were dissolved in DMSO to form stock solutions, which were diluted in PBS to acquire the treating concentrations. Donor cells that had not received any treatment were stained with 5 μM Calcein AM and 10 μM DiI, then gently trypsinized and resuspended in complete medium. The donor cells were then loaded at a 1:100 ratio onto a confluent plate of unlabeled recipient cells, which has been treated according to the experimental design. The plates were rinsed with RPMI-1640 medium to remove any unattached donor cells. The cells were visualized under an epifluorescence microscope. GJIC levels were determined by quantifying donor (DiI dye–stained) neighboring cells that had displayed evidence for the transfer of Calcein AM.

2.4. Transfection of small interfering RNA (siRNA)

Human Cx43 siRNA was obtained from RiboBio (Guangzhou, China). The primers of this Cx43 siRNA were (forward) 5′-GGCTAA TTACAGTGCAGAA-3′ and (reverse) 3′-dTdT CCGAUUAAU GUCACGUCUU-5′. Non-silencing control siRNA (NC siRNA) was provided by RiboBio (Guangzhou, China). siRNA transfection was performed according to the instructions of the manufacturer of Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA). After 9-day $CdCl_2$ exposure, the cells with or without Cd exposure were transfected with NC siRNA (1 μM or 0 μM) or Cx43 siRNA (S1 or S0) for 12 h, cultured in medium containing $CdCl_2$, washed with PBS, and then exposed again to $CdCl_2$ for 4 h to complete the 10-day period of exposure.

2.5. EdU proliferation assay

An EdU Cell Proliferation Assay Kit was used to detect cell proliferation, according to the manufacturer's protocol. Briefly, EdU was added 2 h prior to cell fixation, permeabilization, and staining. Hoechst 33342 (1×) was used to stain the cell nuclei. Cell proliferation rates were quantified by counting the number of EdU-positive (green) cells relative to the total number of cells (blue nuclei).

2.6. Cell cycle assay

The distribution of the cell cycle was analyzed by using flow cytometry. After the cells were harvested and fixed, the fixed cells were stained with PI/RNase Staining Buffer. The cell populations in the G0/G1, S, and G2/M phases among 10,000 cells were measured using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). The data were analyzed using CellQuest software (BD Bioscience).

2.7. Annexin V-FITC/PI apoptosis assay

An annexin V-FITC apoptosis detection kit was used to measure cell apoptosis, according to the manufacturer's protocol. The fluorescence intensity of the cells (10,000 cells/sample) was measured using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). FACS data were analyzed using FlowJo software (v. 7.6.3, 2011, BD Biosciences).

2.8. Phospho explorer antibody microarray analysis of phosphoproteins

A Phospho Explorer antibody microarray (PEX100) was used in this study. Experiments were performed according to an established protocol; detection of the phosphorylation ratio was performed by Wayen Biotechnologies. The PEX100 chip contains 1318 antibodies, covering 584 phosphorylation sites of 452 key proteins involved in many signaling pathways. Each of the antibodies has two replicates to calculate the mean, standard deviation (SD) and coefficient of variation (CV). In the present data, the CV of approximately 80% of the antibodies was less than 20%. Each phosphorylation residue has paired antibodies: one to identify the phosphorylation state and the other to identify the non-phosphorylated state. The extent of protein phosphorylation was measured as a ratio of the "phospho" and "unphospho" values.

2.9. Determination of protein level by western blot

The equivalent proteins were separated on 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline Tween 20 and probed with various primary and secondary antibodies. After incubation, the membranes were visualized using an ECL detection kit.

2.10. Statistical analyses

All data are presented as means ± SD. Statistical analysis was performed using the statistical package SPSS19.0 for Windows. Two-tailed Student's *t*-tests were used; values of P of less than 0.05 were considered statistically significant.

3. Results

3.1. Cx43 siRNA attenuated Cd-induced apoptosis and inhibited proliferation

The HK-2 cells were exposed to the 1 μM $CdCl_2$ for 10 days. The Cd

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