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RESEARCH ARTICLE

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Time-dependent response of A549 cells upon exposure to cadmium

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Abstract

Cadmium is considered one of the most harmful carcinogenic heavy metals in the human body. Although many scientists have performed research on cadmium toxicity mechanism, the toxicokinetic process of cadmium toxicity remains unclear. In the present study, the kinetic response of proteome in/and A549 cells to exposure of exogenous cadmium was profiled. A549 cells were treated with cadmium sulfate ($CdSO_4$) for different periods and expressions of proteins in cells were detected by two-dimensional gel electrophoresis. The kinetic expressions of proteins related to cadmium toxicity were further investigated by reverse transcription-polymerase chain reaction and western blotting. Intracellular cadmium accumulation and content fluctuation of several essential metals were observed after 0–24 hours of exposure by inductively coupled plasma mass spectrometry. Fifty-four protein spots showed significantly differential responses to $CdSO_4$ exposure at both 4.5 and 24 hours. From these proteins, four expression patterns were concluded. Their expressions always exhibited a maximum abundance ratio after $CdSO_4$ exposure for 24 hours. The expression of metallothionein-1 and ZIP-8, concentration of total protein, and contents of cadmium, zinc, copper, cobalt and manganese in cells also showed regular change. In synthesis, the replacement of the essential metals, the inhibition of the expression of metal storing protein and the activation of metal efflux system are involved in cadmium toxicity.

KEYWORDS

cadmium, kinetic processes, metal homeostasis, protein expression, time-dependent change

1 | INTRODUCTION

Cadmium (Cd) is a transition metal and is non-essential to the human body. Cd and Cd compounds are widely distributed in the living environment and are dangerous environmental toxicants (Satarug, Garrett, Sens, & Sens, 2010). A trace amount of Cd is found naturally in the earth, but it may accumulate in specific environments because of industrial practices (Ammendola, Cerasi, & Battistoni, 2014). Chronic exposure to Cd can cause damage to the lung, kidney, bone, liver, immune system and reproductive organs and then lead to different types of cancer (Adams, Passarelli, & Newcomb, 2012; Hartwig, 2013). At present, Cd compounds have been classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC) (IARC, 1993).

The toxicity of Cd originates mainly from its strong binding affinity to metal-sensitive groups, such as thiol or histidyl moieties in cells (Bae & Chen, 2004; Hall, 2002). It can replace these essential metal

ions such as, zinc (Zn), copper (Cu), manganese (Mn) and iron bound to the enzymes and other biomolecules, interfere with the homeostasis of essential metals, trigger reactive oxygen species formation and disrupt the cellular function of biologically important molecules (Adiele, Stevens, & Kamunde, 2012; Kitamura & Hiramatsu, 2010; Zhang et al., 2018). Cells respond to Cd exposure with multi-strategies. Cysteine-rich proteins such as metallothionein (MT) in cells exposed to Cd can initiate thiol-mediated defense mechanisms to chelate competitively the Cd, activate metal efflux systems and buffer reactive oxygen species (Ammendola et al., 2014; Kim, Kim, & Seo, 2015; Schwager, Lumjiaktase, Stockli, Weisskopf, & Eberl, 2012). Moreover, cells can also export or compartmentalize Cd into specific organelles, such as the vacuole (Tamás, Labarre, Toledano, & Wysocki, 2005). In addition, the expression and modification of proteins, transcription of different genes and metabolism of the organism also play a number of important roles in the response to Cd (Bae & Chen, 2004).

MT is an intracellular cysteine-rich protein with a low molecular weight that has a selective capacity to bind heavy metal ions, such as Zn, Cd, Cu and Mn. It has also been known to regulate Zn and Cu homeostasis (Karin, 1985; Miles et al., 2000). MT-1 is associated with the detoxification mechanism of Cd (Asselman et al., 2012). After Cd exposure, organisms are induced to express high levels of MT, which can then bind with biologically toxic Cd through sulfhydryl of cysteine (Vallee, 1995). This is one of the most important defense mechanisms against Cd toxicity (Lee et al., 2010).

However, MT-null and wild-type mice show similar Cd absorption, implicating that other forms of Cd transport may be more important than the MT-complexed form (Liu, Liu, & Klaassen, 2001). The possible role of Ca^{2+} channels in cellular uptake of Cd has also been demonstrated (Choong, Liu, & Templeton, 2014; Thévenod, 2010). SLC11A2 (DMT1), a proton-coupled divalent metal transporter with a preference for iron, has been implicated in Cd uptake and toxicity in mammals (Bressler et al., 2007). SLC39A8 (ZIP8), a Zn transporter, has also been found to be a major portal for Cd uptake into cells (Napolitano et al., 2012). ZIP8 expression is the highest in alveolar cells. Hence, cigarette smoke or other Cd contaminants are transported into alveolar cells by ZIP8, which most likely plays a pivotal role in Cd-induced human cancer (He, Wang, Hay, & Nebert, 2009). The relatively direct evidence for the molecular mechanism of this in intact animals has been reported (Dalton et al., 2005).

Although the toxicity of Cd has been extensively studied (Guo et al., 2017; Xu et al., 2012), the mechanisms by which mammalian cells protect themselves against this toxic metal ion are very complex and not well understood. Proteomics, or the systematic analysis of the proteins expressed by a genome, is a powerful tool for not only describing the complete proteome of an organelle, cell, organ or tissue levels, but also comparing proteomes affected by different physiological conditions (Luque-Garcia, Cabezas-Sanchez, & Camara, 2011). The identification of changes in individual proteins or a group of proteins associated with heavy metal exposure could provide insight into the biomolecular mechanisms of metal toxicity and identify potential candidate metal-specific protein markers of exposure and response. The environmental proteomic analyses of the effects of Cd have been performed (Luque-Garcia et al., 2011). For example, differentially expressed proteins resolved by two-dimensional gel electrophoresis (2DE) showed that the thioredoxin (TRX) system is essential for Cd tolerance. TRX stimulates cysteine (Cys) and glutathione biosynthesis and promotes oxidative stress (Vido et al., 2001). Study of the cellular responses of *Schizosaccharomyces pombe* to cadmium sulfate ($CdSO_4$) using amino acid-coded mass tagging and liquid chromatography tandem mass spectrometry (MS/MS) suggested that, *S. pombe* produces a significantly higher level of inorganic sulfide to immobilize cellular Cd as a form of cadmium sulfide (CdS) nanocrystallites capped with glutathione and/or phytochelatins as an alternative mechanism for the detoxification of Cd (Bae & Chen, 2004). In previous studies, we have examined the time-dependent changes in the expression of proteins and the proteome involved in Zn homeostasis, and we elucidated the mechanism of the process. Our results showed that A549 cells present a kinetic response to exogenous zinc sulfate ($ZnSO_4$) stress in four conservatively time-dependent manners and that exogenous $ZnSO_4$ more predominantly reduced the expression of proteins in cells after 24 hours than 9 hours (Zhao et al., 2014; Zhao et al., 2015). In the present work, to unclose further the difference of response mechanisms of different cells to various metals ions, we profiled the toxicokinetic response of the proteome in A549 cells exposed to extracellular $CdSO_4$ at different time points using 2DE coupled with silver staining. We also investigated the time-dependent expression patterns of differentially expressed proteins in A549 cells after exposure to $CdSO_4$. Moreover, we studied the toxicokinetic changes in the concentrations of proteins, Cd content and the expression of key proteins related to Cd toxicity in cells using the Bradford essay, inductively coupled plasma MS (ICP-MS), western blot or reverse transcription-polymerase chain reaction (RT-PCR) to elucidate further the mechanisms involved in Cd toxicity.

2 | MATERIALS AND METHODS

2.1 | Chemicals and materials

Fetal bovine serum (KGY009) and incomplete culture medium supplemented with L-glutamine (KGM1640SF) were supplied by KeyGen Biotech (Nanjing, China). The Bradford protein assay kit, Cell Counting Kit-8, western blocking buffer, primary antibody dilution buffer and secondary antibody dilution buffer were from Beyotime Institute of Biotechnology (Haimen, China). Trypsin was from Promega (Madison, WI, USA). Non-linear immobilized pH gradient (IPG) strips were purchased from GE (Piscataway, NJ, USA). The chemicals used for 2DE were purchased from Amresco (Solon, OH, USA). $CdSO_4 \cdot 8/3H_2O$ was purchased from Aladdin (Shanghai, China). All water used in experiments was Millipore Milli-Q filtered at a resistivity ≥18.25 MΩ/cm. The culture dishes and polyvinylidene difluoride membranes used were from Millipore (Bedford, MA, USA). The primary antibodies used were anti-actin antibody (AA128; Beyotime), anti-MT antibody [UC1MT] (ab12228; Abcam, Cambridge, MA, USA), heat shock protein 90 alpha (Hsp90α) (D1A7) rabbit monoclonal antibody (CST no. 8165) and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (D21H11) rabbit monoclonal antibody (CST no. 8443) (both Cell Signaling Technology, Danvers, MA, USA). The secondary antibodies, including goat antirabbit IgG-horseradish peroxidase

(sc-2004) and goat antimouse IgG-horseradish peroxidase (sc-2005), were also purchased from Santa Cruz Biotechnology (Dallas, TX, USA). TRIzol was purchased from Invitrogen (Carlsbad, CA, USA). The First-Strand complementary DNA (cDNA) Synthesis Kit and Taq DNA Polymerase were purchased from Thermo Fisher (Waltham, MA, USA).

2.2 | Cell culture and exposure

A549 cells (human lung adenocarcinoma cell line) were purchased from KeyGen Biotech and were maintained in incomplete culture medium supplemented with 10% fetal bovine serum, 80 units/mL penicillin and 0.08 mg/mL streptomycin at 37°C in a 5% humidified carbon dioxide (CO_2)-enriched atmosphere. All cell samples were prepared using at least three replicates for an experiment. A stock solution of $CdSO_4$ with 8/3-hydrate ($CdSO_4{\cdot}8/3H_2O$) was prepared. A549 cells were exposed to $CdSO_4$ dissolved in culture medium for different periods or at different concentrations. Detailed descriptions of some of the methods used in this text are found in Supporting Information, Text S1.

2.3 | Cell viability assays

Cells were exposed to various concentrations of $CdSO_4$ and incubated in culture medium for 4.5 or 24 hours. The Cell Counting Kit-8 was used to test the viability of A549 cells after different time-courses of exposure or after exposure to various doses of $CdSO_4$ in accordance with the previous description of the experiments performed for Zn (Zhao et al., 2014).

2.4 | Preparation of protein samples

Whole proteins in cells were prepared by resuspending the cell pellets in 200 μL lysis buffer and then vortexing the solution vigorously for 3 min at 4°C. The lysates were sonicated for 1 minute. The supernatants were clarified and recovered after centrifugation at 15 000 *g* for 30 minutes at 4°C. The concentrations of the protein extracts were determined using the Bradford method.

2.5 | Two-dimensional gel electrophoresis and image analyses

Protein separation was carried out using a GE Healthcare (Pittsburgh, PA, USA) IPGphor isoelectric focusing (IEF) and an Ettan Dalt six electrophoresis system. IEF was performed using 24 cm precast non-linear IPG strips (pH 3–10). Then, 200 μg whole cell proteins prepared using the above method was mixed with 450 μL rehydration buffer and loaded on to IPG strips by in-gel rehydration at room temperature overnight. IEF was performed using a step-wise voltage increase procedure at 20°C. After IEF, the IPG strips were subjected to a two-step equilibration. Separation in the second dimension was performed using 1 mm thick 12% polyacrylamide gels in Tris-glycine buffer.

All samples including controls were analyzed in triplicate, and nine gel pieces were visualized by silver nitrate staining. Spot detection and quantification were carried out using PDQuest 8.0 analysis software (Bio-Rad, Hercules, CA, USA). The ratios of protein abundance were obtained by comparing the mean abundance from triplicate gels of the corresponding differentially expressed proteins after exposure for 4.5 or 24 hours to Cd with their controls using gel analysis software. Spots with at least twofold differential expression (ratio values were higher than 2 and lower than 0.5 for up- and downregulated proteins, respectively) between the $CdSO_4$ treated and control groups, and $P < 0.05$ resulting from ANOVA were considered significant. These spots were subsequently subjected to differential expression analysis and protein identification.

2.6 | Protein identification

Protein spots of interest were manually excised from gels. The excised gel pieces were washed, destained, shrunk and digested in-gel according to previous methods (Zhao et al., 2015). The supernatants of the trypsin-digested mixtures were collected. All supernatants derived from the peptide extracts were mixed and then were completely dried. The extracted peptide samples were analyzed on a 5800 Plus matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometer Analyzer (Applied Biosystems, Foster City, CA, USA).

Proteins were successfully identified based on a 95% or greater confidence interval of their scores using the MASCOT V2.3 search engine (Matrix Science Ltd., London, UK) to query the human protein National Center of Biotechnology Information database.

2.7 | Determination of cadmium and other metal contents

The cells were digested for 7 hours using nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) (2:5, *v*/v). Each sample to be detected was prepared with 2 mL 2% HNO_3. The whole Cd, Zn, Cu, cobalt and Mn contents were determined using a Perkin-Elmer SCIEX Elan 9000 ICP-MS (Überlingen, Germany).

2.8 | Western blot analysis

Thirty micrograms of proteins from the whole cell extracts was fractionated on 12% acrylamide gels using sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli's method, and the proteins were electrotransferred on to polyvinylidene difluoride membranes using a Mini P-4 electrotransfer apparatus (Cavoy, Beijing, China). The membranes were washed with phosphate-buffered saline containing 0.1% (*v*/v) Tween-20 and incubated with the respective primary antibody overnight at 4°C. Then, the membranes were incubated with the appropriate secondary antibody for 1 hour at room temperature. After several washes, the membrane was incubated with Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA), and the immune complexes were detected using the enhanced chemiluminescence assay (CLINX, Shanghai, China). Scanning densitometry and the quantitative analysis of immunoblot data were performed using dedicated Gel Image Analysis software (CLINX). β-actin was used as an internal control.

2.9 | Reverse transcription-polymerase chain reaction

Total RNA was prepared from A549 cells using TRIzol reagent according to the manufacturer's instructions. RNA samples (2 μg each) were reverse-transcribed as described in the instructions of the First-Strand cDNA Synthesis Kit. Next, 1 μL of the resulting cDNA solution was used for PCR. The genes were amplified in a 20 μL reaction solution using TC9600-G (Labnet, Edison, NJ, USA). After the reaction, the reverse transcriptase enzyme was inactivated by heating at 95°C for 5 minutes, and then the reaction went through 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds, with a final extension step of 72°C for 10 minutes. A reduced glyceraldehyde phosphate dehydrogenase primer was used as an internal control, and the amplifications were quantified in triplicate. The sequences of the PCR primers used for analysis of the genes of interest are summarized in Supporting information, Table S1. An aliquot (10 μL) of each reaction was analyzed by agarose gel electrophoresis and ethidium bromide staining.

2.10 | Statistical analyses

All measurements were repeated at least three times, and the data are expressed as the means ± SD. Statistical significance for the comparison of two groups was assessed, unless otherwise specified, using one-way ANOVA with the Turkey-Kramer multiple comparison post-hoc test. Differences that were considered statistically significant are indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ vs. unexposed controls.

3 | RESULTS

3.1 | Cell viability

The viability of A549 cells after exposure to various concentrations of $CdSO_4$ for 4.5 or 24 hours was assayed (Figure 1). Our statistical analyses indicated that the viability of cells significantly declined after 24 hours of exposure to 75 μM $CdSO_4$, whereas a significant difference in cell viability was observed at an elevated concentration of $CdSO_4$ (i.e., 150 μM) after exposure for 4.5 hours. Elevated $CdSO_4$ concentrations resulted in greater cell death. Moreover, the viability of A549 cells was lower after exposure to $CdSO_4$ for 24 hours than after 4.5 hours at the same concentration. It was found that more cells died after exposure to 75 μM $CdSO_4$ for 24 hours than 4.5 hours by two-way ANOVA. The viability of A549 cells declined by less than 5%, and was not significantly different between cells exposed to 25 μM $CdSO_4$ for 4.5 or 24 hours, indicating that 25 μM $CdSO_4$ represents a subcytotoxic metal concentration. Subtoxic $CdSO_4$ could trigger regulatory mechanisms of defense against Cd stress. Therefore, 25 μM $CdSO_4$ administered for appropriate periods of time was used for further analysis.

3.2 | Proteome expression patterns

To investigate the kinetic response of the proteome of A549 cells exposed to $CdSO_4$ stress, cells were exposed to 25 μM $CdSO_4$ for 4.5 or 24 hours, and the whole cell proteins were subjected to highly sensitive silver nitrate staining in 2DE gels in triplicate. A representative 2DE gel after $CdSO_4$ exposure is shown in Figure 2. Comparisons of the three groups of gels (controls and $CdSO_4$ exposure for 4.5 or 24 hours) were performed. A total of 18 034 spots were detected on these gels, and 2685 matches were mapped to a reference gel. Compared to the controls, 58 and 64 protein spots (a total of 122 protein spots) showed significantly differential expression ($P < 0.05$, fold change >2) after exposure to 25 μM $CdSO_4$ for 4.5 or 24 hours, respectively. All of the 122 differentially expressed protein spots were divided into two groups, as summarized in Supporting information, Figure S1 and Table S2. Fifty-four protein spots showed significantly different responses to $CdSO_4$ exposure at both 4.5 and 24 hours (Supporting information, Figure S1). Therefore, these proteins were used as our main study criteria. In addition, 14 other protein spots (Supporting information, Table S2) showed significantly different responses to $CdSO_4$ exposure at both 4.5 and 24 hours. There was a total of 68 unique differentially expressed proteins spots in the two groups of samples (4.5 or 24 hours vs. controls), which are indicated in Figure 2 (including the match numbers).

The mean abundance of triplicate samples of each spot of the 54 protein spots after exposure to $CdSO_4$ for 4.5 or 24 hours is presented in Figure 3. The differential expression patterns of the 54 protein spots were classified into four models accordingly (patterns 1, 2, 3 and 4). The meanings of these four patterns are illustrated by the corresponding histograms presented in Figure 4. The height of each column in histograms indicates the mean abundance of all differentially expressed protein spots in the corresponding groups. Notably, patterns 2 and 3 were the most prevalent for the 54 proteins (68.5%), whereas patterns 1 and 4 account for a small proportion of proteins (31.5%).

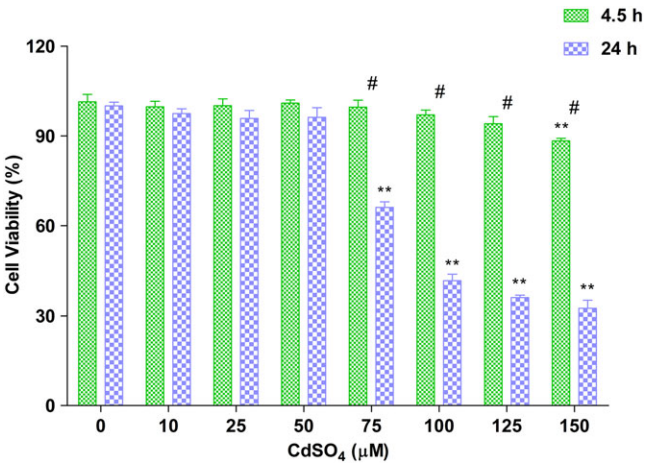

FIGURE 1 Viability of A549 cells after exposure to $CdSO_4$ for 4.5 or 24 h. Viability of A549 cells was assessed using Cell Counting Kit-8 after exposure with various doses of $CdSO_4$ for 4.5 or 24 h. Cell viability showed kinetic changes and was significantly decreased after exposure to 150 μM $CdSO_4$ for 4.5 h and to 75 μM $CdSO_4$ for 24 h. More cells died after exposure to 75 μM $CdSO_4$ for 24 h than 4.5 h by two-way ANOVA analysis. #$P < 0.001$, 4.5 h vs. 24 h by two-way ANOVA analysis [Colour figure can be viewed at wileyonlinelibrary.com]

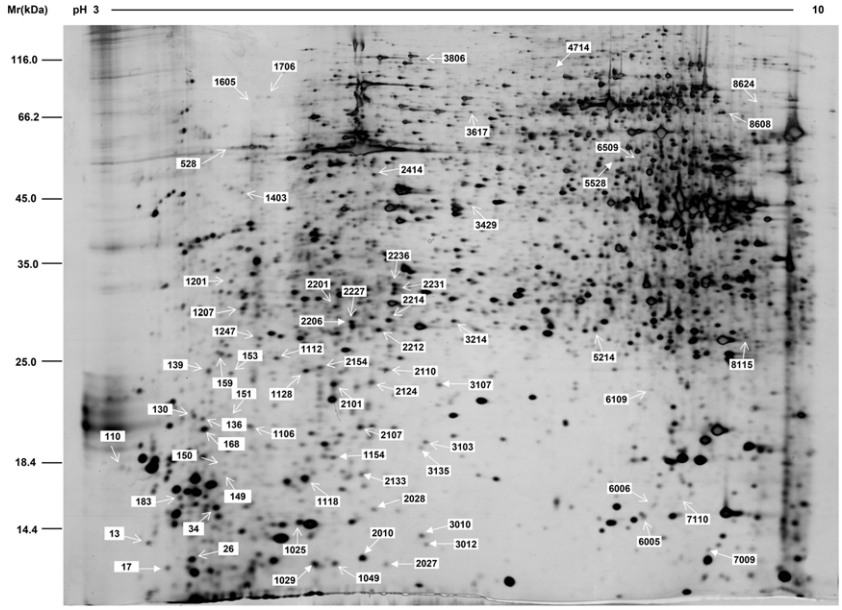

FIGURE 2 Representative two-dimensional gel electrophoresis gels of soluble proteins in A549 cells stained with $AgNO_3$. A549 cells were incubated with 25 μM $CdSO_4$ for 4.5 or 24 h. Controls were not exposed to $CdSO_4$. Whole cell proteins were extracted, and 200 μg proteins were separated by isoelectric focusing in a 24 cm immobilized pH gradient gel strip containing a broad non-linear pH gradient of 3–10, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a vertical 12% gel. Differentially expressed proteins that changed in response to 25 μM $CdSO_4$ for 4.5 or 24 h are illustrated with different colors. These proteins in A549 cells showed a twofold or greater change in abundance versus controls ($P < 0.05$)

3.3 | Functional classification of differentially expressed proteins

A matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometer was used to identify Cd-responsive proteins that exhibit significantly differential expression patterns compared with controls in response to Cd exposure. Among 122 differentially expressed protein spots, 53 were successfully identified by MS/MS (Supporting information, File S1). Most of these proteins have been previously implicated in various intracellular physiological activities (Kälin et al., 2011; Liu et al., 2012; Trepel, Mollapour, Giaccone, & Neckers, 2010). The differentially expressed proteins that we identified were categorized according to the PANTHER Classification System (http://pantherdb.org/). Among these proteins, 38 had a reliable "hit" within the system. These proteins were classified according to their biological processes (Supporting information, Figure S2) and were predominantly involved in the categories of metabolic processes, cellular processes, developmental processes and cellular component organization or biogenesis. Other biological processes accounted for a small percentage of the proteins identified, which included the categories of localization, response to stimulus, biological regulation, multicellular organismal process, immune system process, apoptotic process and reproduction. Moreover, the protein hits were classified according to their molecular function (Supporting information, Figure S2). Catalytic activity, binding and structural molecule activity were

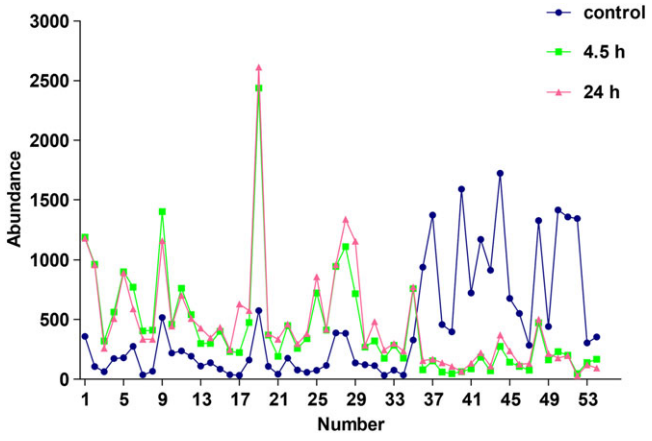

FIGURE 3 Abundances of differentially expressed proteins that repeatedly exhibited after $CdSO_4$ exposure for different periods. These proteins showed significantly differential expression compared with controls after exposure to 25 μM $CdSO_4$ for both 4.5 and 24 h. Numbers 1–12 indicate proteins that were upregulated after $CdSO_4$ exposure and for which the abundance ratios compared with controls at 4.5 h were higher than that at 24 h (Ratio4.5 h > Ratio24h). Numbers 13–35 indicate proteins that were upregulated after $CdSO_4$ exposure and for which the abundance ratios compared with controls at 4.5 h were lower than that at 24 h (Ratio4.5 h < Ratio24h). Numbers 36–49 indicate proteins that were downregulated after $CdSO_4$ exposure and for which the abundance ratios compared with controls at 4.5 h were larger than that at 24 h (Ratio4.5 h < Ratio24h). Numbers 50–54 indicate proteins that were upregulated after $CdSO_4$ exposure and for which the abundance ratios compared with controls at 4.5 h were higher than that at 24 h (Ratio4.5 h > Ratio24h) [Colour figure can be viewed at wileyonlinelibrary.com]

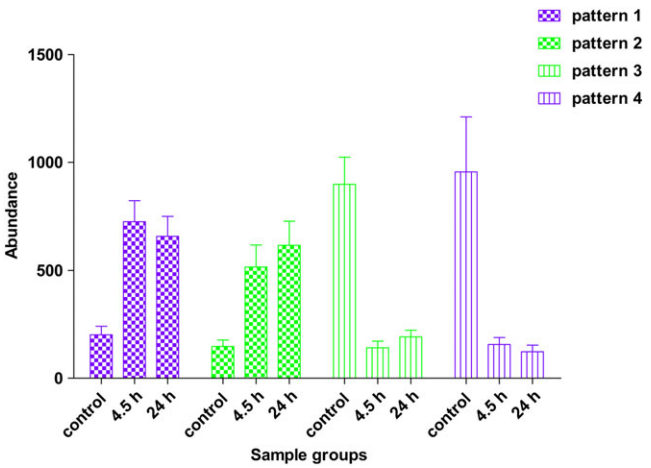

FIGURE 4 Expression patterns of differentially expressed proteins that repeatedly exhibited after $CdSO_4$ exposure for different periods. These proteins showed significantly differential expression compared with controls after exposure to 25 μM $CdSO_4$ for both 4.5 and 24 h [Colour figure can be viewed at wileyonlinelibrary.com]

the most common molecular functions of the differentially expressed proteins. The categories of enzymatic regulatory activity, receptor activity and transporter activity accounted for a smaller number of the molecular functions of these proteins.

3.4 | Concentrations of differential protein

The concentrations of proteins in whole cells exposed to $CdSO_4$ for 1, 2, 3, 6, 9, 12 or 24 hours were determined by the Bradford method (Figure 5). The protein concentrations changed in a time-dependent manner. The total expression of proteins in whole cells increased gradually after $CdSO_4$ exposure. Statistical analysis revealed a significant difference in expression of whole cell proteins after exposure to $CdSO_4$ for 3 hours.

3.5 | Heat shock protein 90α and hnRNPA1 expression patterns

The proteins Hsp90α and hnRNPA1 were found to respond to metal exposure (Barque, Abahamid, Chacun, & Bonaly, 1996; Padmini & Rani, 2011; Zhao et al., 2014; Zhao et al., 2015). The kinetic responses and differential expression patterns of Hsp90α and hnRNPA1 were observed when A549 cells were incubated with $CdSO_4$ for 1, 3, 6, 9, 12 or 24 hours. The relative abundances of proteins in cells exposed to $CdSO_4$ compared to an internal reference protein were analyzed and normalized. These differential expression patterns are shown in Figure 6. Exposure to $CdSO_4$ continually induced the expression of Hsp90α. A different time course of hnRNPA1 protein expression after $CdSO_4$ exposure was observed. The expression of hnRNPA1 showed a peak valley after 3 hours. Statistical analysis revealed a significant increase in the abundance of Hsp90α after exposure to $CdSO_4$ for 4.5 hours and a significant decrease in the abundance of hnRNPA1 after exposure for 3 hours.

3.6 | Intracellular cadmium accumulation

To investigate further the cross-talk between toxic and essential metals, the accumulation of intracellular Cd and the change in the contents of several essential heavy metals, including Zn, Cu, Co and Mn, in A549 cells at the same time were analyzed by ICP-MS after exposure to $CdSO_4$ for 3, 4.5, 6, 9, 12 or 24 hours. The data are shown in Supporting information, Figure S3. The metal concentrations in cells after exogenous Cd exposure for different periods compared with controls were analyzed. Cd concentration in cells increased continually with long periods of exposure. Significant differences in the Cd concentration of whole cells after 3 h of exposure were detected. The concentration of Zn reached a minimum at 4.5 hours and then increased. Subsequently, the Zn concentration began to decrease at 4.5 hours again. After 24 hours, the Zn concentration increased again. The Zn concentrations in cells after exposure to Cd were always smaller than that of the controls. The Cu concentration in cells dramatically peaked at 3 hours after Cd exposure. Then Cu concentration decreased gradually and minimized at 12 hours. After that, the Cu concentration increased again, although it was still lower than that of the controls. The changes in cobalt concentrations were found to have a trend similar to Cu, but the cobalt concentrations were higher than the controls before 12 hours. Additionally, the concentrations at 24 hours were not significantly different from the controls. After exogenous Cd exposure, the Mn concentrations gradually declined and the lowest point came at 12 hours. Then, Mn concentrations increased again.

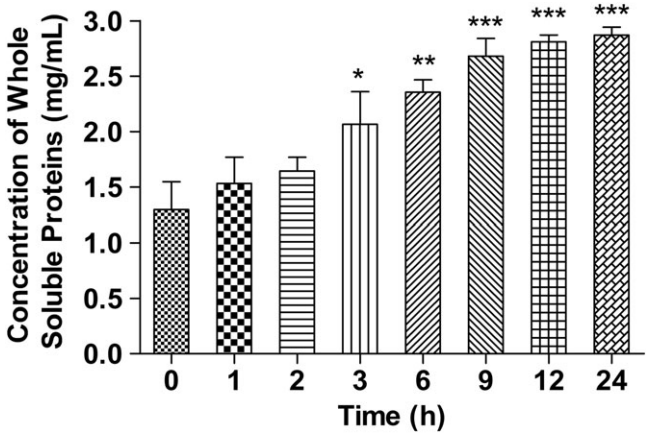

FIGURE 5 Differential concentrations of proteins in A549 cells. Concentrations of proteins in cell exposed to 25 μM $CdSO_4$ for different periods were determined, and kinetic changes were observed

3.7 | Kinetic expression of MT-1 and ZIP-8

The change in expression of the metal binding protein MT-1 at the gene and protein level in cells after exposure to $CdSO_4$ for 1, 3, (4.5 for gene level), 6, 9, 12 or 24 hours was investigated by western blotting and RT-PCR, as shown in Figure 7A. The expression of MT-1 in gene level sharply decreased to a minimum after exposure to exogenous Cd for 4.5 hours. Then it increased gradually, but the expression of MT-1 was always lower than that of the controls before exposure at 24 hours. At 24 hours, the expression of MT-1 at the gene level dramatically increased and was higher than that of the controls. Compared to the change in gene expression, the change in the level of protein expression of MT-1 was small, and the expression of MT-1 at the protein level was always lower than that of the controls, even at 24 hours. In addition, the change in expression of the metal transporter protein ZIP-8 gene in cells after exposure to $CdSO_4$ for 1, 3, 4.5, 6, 9, 12 or 24 hours was also analyzed by RT-PCR, as shown in Figure 7B. The expression of ZIP-8 at the gene level decreased to a minimum after exposure to exogenous Cd for 3 hours and then, it increased gradually and was always higher than that of the controls.

4 | DISCUSSION

Environmental proteomics has been a powerful tool for the assessment of toxicity and risk of environmental pollutants. This promising proteomic technology is also very helpful to explore the underlying molecular mechanism of Cd toxicity in the present study.

Cell viability changed in a time-dependent manner after exposure to $CdSO_4$, and A549 cells kinetically responded to exogenous Cd exposure. A longer exposure time resulted in greater cell death. Additionally, our investigations indicated that the expression of Cd-

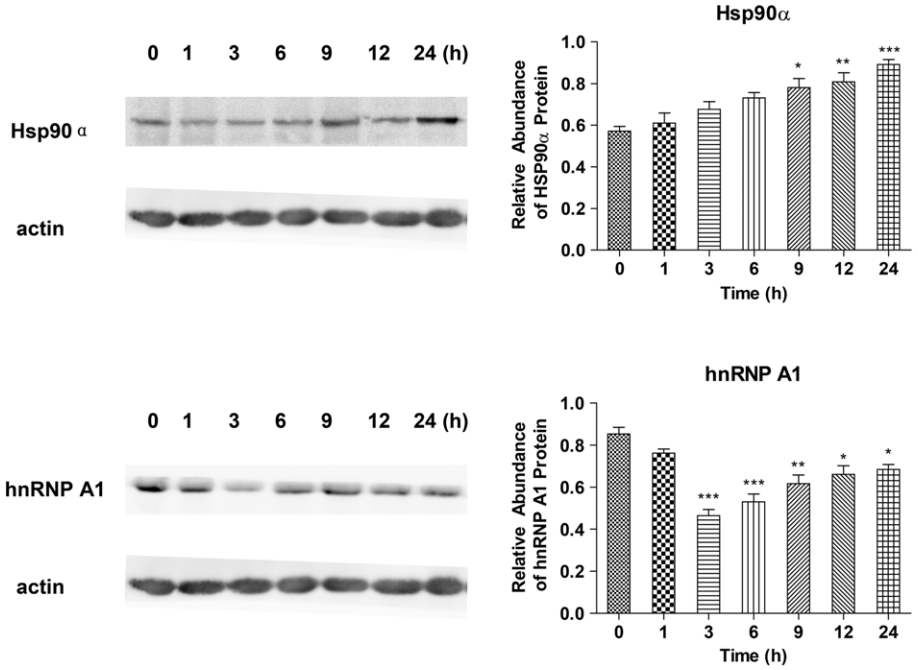

FIGURE 6 Expression patterns of Hsp90α and hnRNPA1 at the protein level. Abundances of proteins changed in time-dependent manner. Western blot (upper) and protein band density (lower) analyses of Hsp90α and hnRNPA1 protein levels in A549 cells after different periods of exposure to 25 μM $CdSO_4$ were analyzed. Expression patterns of Hsp90α and hnRNPA1 at the protein level corresponded to prevailing patterns 2 and 3, respectively

responsive proteins exhibits apparent variation in protein and messenger RNA (mRNA) levels at 4.5 hours during exposure for 24 hours to $CdSO_4$. Therefore, the 4.5 and 24 hour time-points of $CdSO_4$ exposure were selected to compare the differential expression of the Cd-responsive proteome of A549 cells and to elucidate the Cd-responsive kinetic process and signaling pathways in A549 cells.

The differential expression of Cd-responsive proteins was robustly reproducible after exposure for different lengths of time and varied according to one of four kinetic expression patterns. The classification of the proteins that repeatedly differentially expressed after $CdSO_4$ exposure for different periods indicated that a longer period of exposure mostly further increased the expression of the up- and downregulated proteins. These similar phenomena were also found among the other 14 significantly differentially expressed proteins after either 4.5 or 24 hours of $CdSO_4$ exposure. Pattern 2 (upregulated with steady induction) and pattern 3 (downregulated with rapid initial repression and a subsequent slight rise) were found to be the most prevalent expression patterns. The abundance of the upregulated proteins changing in pattern 2 exhibited maximum values at 24 hours, while the abundance of the downregulated proteins changing in pattern 3 exhibited minimum values at 4.5 hours. Therefore, larger abundance ratios or higher expression of proteins was almost always obtained after 24 hours of $CdSO_4$ exposure. These findings indicated that exposure time longer than 4.5 hours increased the abundance of most differentially expressed proteins, irrespective of the up- or downregulation after $CdSO_4$ exposure. Moreover, more protein spots displayed differential expression after 24 hours (64 spots) of $CdSO_4$ exposure than after 4.5 hours (58 spots), which also suggested that this duration of $CdSO_4$ exposure is more advantageous for the observation and analysis of the differentially expressed proteome. Although determining the functional significance of these proteins will require further investigation, these proteins undoubtedly play specific roles in Cd homeostasis. These results meant that a longer exposure time is more helpful in discovering the more important differentially expressed proteins and elucidating the molecular mechanism of Cd toxicity.

Among the differentially expressed proteins, the population of upregulated proteins accounted for a large proportion of the 68 proteins after $CdSO_4$ exposure and is slightly more than two-fold of the population of downregulated proteins. Additionally, the expressions of 11 protein spots among the other 14 proteins (Supporting information, Table S2) were also induced after $CdSO_4$ exposure. These data suggest that most of the proteins in A549 cells were induced by Cd exposure. Moreover, the concentrations of proteins after $CdSO_4$ exposure for different lengths of time were always higher than that of the controls and gradually increased to a maximum value at 24 hours. Furthermore, a larger decline in the abundance of downregulated proteins than increase in the abundance of upregulated proteins is shown in Figure 4 after 4.5 and 24 hours of $CdSO_4$ exposure. These findings further confirmed our conclusion.

The change in the abundance of a differentially expressed protein always had the same direction of either up- or downregulation after $CdSO_4$ exposure for 4.5 hours and 24 hours. The conservative changes just with different ratios coupled with the four expression pattern could facilitate the prediction and rationalization of the time-dependent differential expression of uncharacterized proteins that respond to $CdSO_4$ exposure. For example, Hsp90α and hnRNPA1 were found to be significantly up- and downregulated, respectively, after $CdSO_4$ exposure for 24 hours. The present study showed that

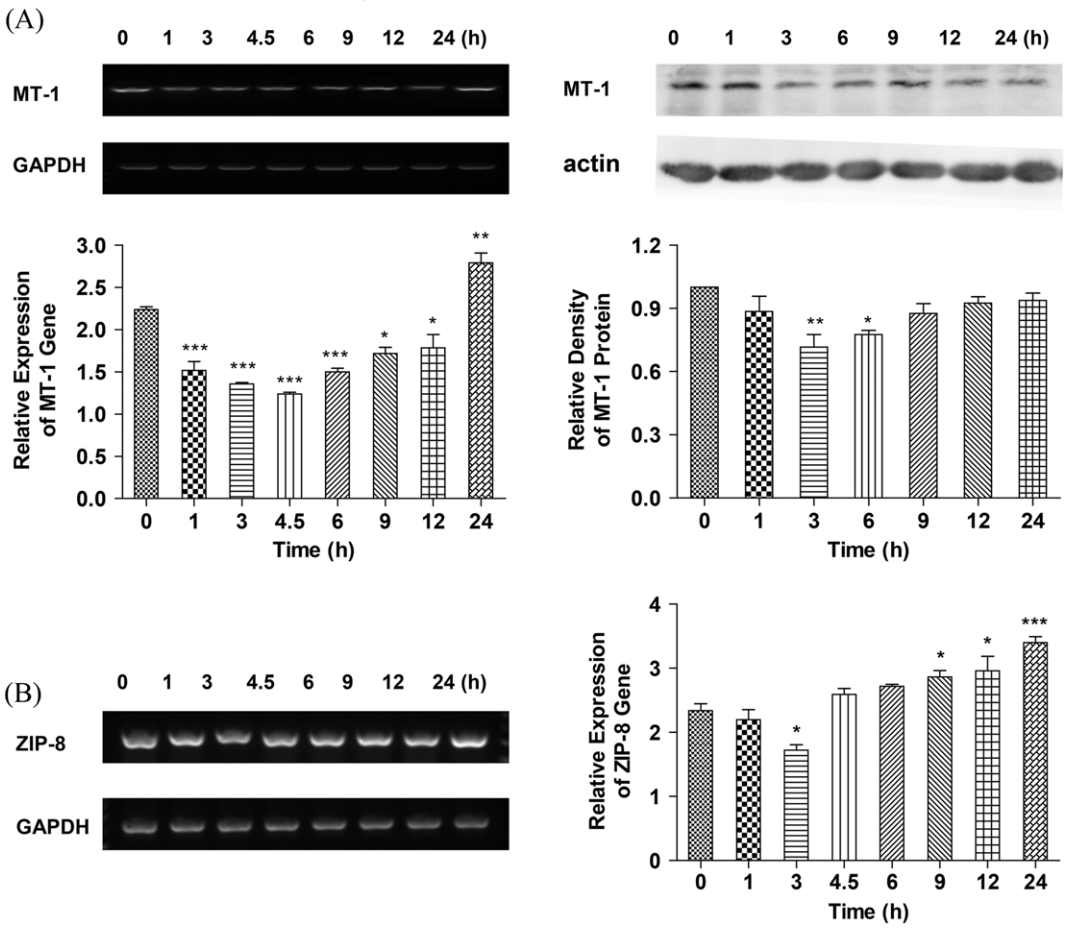

FIGURE 7 Kinetic changes in MT-1 and ZIP-8 in cells exposed to $CdSO_4$. A549 cells were exposed to 25 μM $CdSO_4$ for different stimulation periods. Both, A, reverse transcription-polymerase chain reaction and western blot analyses of MT-1, and B, reverse transcription-polymerase chain reaction analysis of ZIP-8 were performed. Band density was analyzed with Gel Image Analysis software. GAPDH and actin were used as the internal control in gene and protein levels, respectively. Means ± SD were calculated from at least three independent samples. Data were normalized. GAPDH, glyceraldehyde phosphate dehydrogenase; MT-1, metallothionein-1

changes in the expression of the two proteins after $CdSO_4$ exposure over time conformed to patterns 2 and 3, respectively.

With the increase in Cd content, the concentrations of essential metals in the cells fluctuated in different ways and showed some inflection points after exogenous Cd exposure. Cellular damage by Cd appeared to be tightly related to its ability to interfere with the homeostasis of essential metals, including Zn, Cu, Co and Mn. After Cd exposure, the decrease in the concentration of the essential metals and the increase in the concentration of Cd proved that the replacement of essential metal ions such as Zn, Cu and Mn resulted in Cd toxicity. Interestingly, Cd exposure mainly increased the cobalt content in cells, which suggested that cobalt has a very different cross-talk with toxic Cd than the other essential metals. Perhaps A549 cells capture the exogenous cobalt in the cell culture medium after many steps. These complicated and delicate kinetic processes were closely associated with Cd toxicity, although the mechanism of this association still needs further exploration.

As a metal binding and storing protein, MT-1 plays a critical role in the mechanism of Cd toxicity (Costa, Chicano-Gálvez, López Barea, Delvalls, & Costa, 2010). These essential metals, such as Zn, Cu, cobalt and Mn, similar to Cd, bind to MT-1 with a relatively strong affinity (Liu et al., 2014). The analysis of the gene expression of MT-1 by RT-PCR showed that the expression of the MT-1 gene was induced by Cd exposure after 24 hours, which agrees with previous reports (Lee et al., 2010; Vallee, 1995). However, the lower expression of MT-1 before 24 hours of Cd exposure, as compared to the controls, and its minimum at 4.5 hours were observed. These findings indicated that Cd exposure was likely to induce initially the displacement of essential metals by Cd and inhibit the expression of MT-1, particularly before the critical 4.5 hour mark of exposure to Cd. MT-1 responded to Cd exposure in a more subtle way at the protein level than at the gene level, which indicated that the response of the MT-1 protein may be involved in more complicated signaling processes than the MT-1 gene. The kinetic response of ZIP-8 as a major portal for Cd uptake into cells to Cd exposure was investigated to describe further the process of Cd toxicity of A549 cells. When cells were exposed to Cd for a short period (less than 4.5 hours), the expression of ZIP-8 was suppressed. This probably resulted from the influx of exogenous Cd and the release of essential metals. In our previous study, the situation was totally different as most of the proteins showed a lower expression after 24 hours of $ZnSO_4$ treatment than 9 hours (Zhao et al., 2015). It is likely that cells present a distinct response to Cd ions from Zn ions. Importantly, we found that Cd replaced intracellular Zn, and the expression of

proteins abided by adverse patterns compared to overdosed Zn treatment. According to this, the efflux of Zn may cover a large proportion in the process of protein changes, and could be essential in the mechanism of Cd toxicity. Time of treatment should also be taken into consideration because the expression of proteins experienced a significant change between 4.5 and 9 hours. However, Bae and Chen observed a different result using $CdSO_4$ in the treatment of *Schizosaccharomyces pombe*, which is similar to our previous Zn experiment (Bae & Chen, 2004). On the other hand, they found that a large number of proteins involved in protein biosynthesis were upregulated, which were not observed in our experiment. Therefore, it is highly possible that human cells do not share the same detoxification mechanism with yeast. Zhang et al. found that the majority of differentially expressed proteins were downregulated by Cd (Zhang, Xu, Zou, & Pang, 2015). Although no statistical analysis of protein changes was made between 1 and 5 day results, the repression of protein expression could still be concluded. Considering brown algae were used in the experiment, it is rational to suggest that higher animals may have a more advanced detoxification strategy for Cd.

In conclusion, the influence of Cd on A549 cells is described as follows. After A549 cells were exposed to exogenous Cd, Cd entered cells, replaced the intra−/extracellular essential metals rapidly and inhibited the expression of the metal storing protein MT-1. As a defense against Cd exposure, the cell activated the metal efflux systems, which resulted in the decrease in the essential metal content of the cells. The expression of ZIP-8 was reduced at the same time to relieve Cd stress. After that, Cd damaged the normal physiological function of A549 cells and resulted in the massive influx of Cd and overexpression of MT-1 and ZIP-8. It was revealed by an environmental proteomics-based strategy in this work that A549 cells presented a different kinetic response to exogenous Cd exposure from Zn exposure in four similar time-dependent ways. The expression of most differentially expressed proteins showed an increase after a long period of $CdSO_4$ exposure. Furthermore, the replacement and efflux of essential metals were found to be important processes in Cd toxicity. These findings facilitate the discovery of differentially expressed proteins after exogenous Cd exposure and help the elucidation for the mechanism of Cd toxicity.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

HZL, LM and WJZ (Wei-juan Zheng) designed the research; WJZ (Wen-jie Zhao), ZJZ, ZYZ and QS conducted the research; XH offered experimental technical guidance; WJZ (Wen-jie Zhao) and ZJZ analyzed data; HZL, WJZ (Wen-jie Zhao) and ZJZ wrote this manuscript. All authors have read and approved the final version of the manuscript.

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SUPPORTING INFORMATION

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