Changes in DNA-binding activity of transcription factors in the kidney of mice exposed to cadmium

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ABSTRACT — Cadmium (Cd) is a toxic heavy metal, long-term exposure to which causes renal damage associated with disruption in gene expression. Transcription factors whose activities were altered in the kidneys of mice exposed to Cd for 3 months were assessed using protein/DNA-binding assays. Female C57BL/6J mice were exposed to 300 ppm Cd in the diet for 3 months. Nuclear extracts of kidney were used for protein/DNA-binding assays. The concentration of Cd was approximately 100 ppm in mouse kidney, a level that did not induce renal toxicity. Among the 345 transcription factors evaluated, five transcription factors showed over a two-fold increase in their activities and 14 transcription factors showed a half-fold change in their activities after Cd exposure. These findings may provide new information about the causative transcription factors associated with Cd renal toxicity.

Key words: Cadmium, Kidney, Transcription factors, DNA-binding activity

INTRODUCTION

Cadmium (Cd) is a toxic heavy metal that adversely affects various tissues (Fujisawa et al., 2012; Järup and Åkesson, 2009; Lee et al., 2019). The kidney is considered to be a primary target tissue as Cd gets accumulated with chronic exposure through dietary uptake (Fujisawa et al., 2012; Järup and Åkesson, 2009; Lee et al., 2019). Our previous studies demonstrated that apoptosis induced by disruption of gene expression is the main cause of Cd renal toxicity (Lee et al., 2016, 2017; Tokumoto et al., 2011). Gene expression is regulated by transcriptional activities organized by the assembly of transcription factor complexes in promoter regions (Hatzis and Talianidis, 2002; Lemon and Tjian, 2000). We have also identified various transcription factors that show altered expression after Cd exposure in mouse and human proximal tubular cells (Lee et al., 2017; Tokumoto et al., 2014). Furthermore, FOXF1, YY1, and ARNT are upstream transcription factors involved in Cd-induced apoptosis (Lee et al., 2016, 2017). Previous reports have established the transcription factors related to Cd toxicity using cultured cells. Cd exposure for 12 months results in the accumulation of the metal to as much as 200 ppm in the mouse kidney (Tokumoto et al., 2011). This Cd concentration causes renal toxicity in the mice. Thus, identification of the transcription factors associated with tissue injury before the appearance of renal failure is important. We elucidated the transcription factors related to Cd renal toxicity in mice exposed to dietary Cd for 3 months.

MATERIALS AND METHODS

Animals and Cd treatment

Four-week-old female C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and routinely bred in the vivarium of the laboratory animal facility of Aichi Gakuin University (Nagoya, Japan). All protocols were performed according to the regulations on animal experimentation at Aichi Gakuin University. The mice were housed in cages in a ventilated animal room at a controlled temperature of 23 ± 1°C, relative humidity of 45 ± 15%, and a 12-hr light/dark cycle. Five-week-old mice were randomly assigned to control and experimental groups, with 5-6 animals per group. Control and Cd-exposed groups were fed standard laboratory chow and chow containing 300 ppm Cd (Oriental-BioService, Kyoto, Japan), respectively, and allowed access to tap water ad libitum. After 3 months of Cd exposure, kidney, liver, serum, and urine samples were collected from each
mouse under ether anesthesia.

**Cd concentration measurement**

Kidney and liver were digested with nitric acid and hydrogen peroxide. Subsequently, inorganic residues were dissolved in ultrapure water, and Cd was analyzed using atomic absorption spectrophotometry (200 series AA; Agilent Technologies, Santa Clara, CA, USA).

**Renal toxicity evaluation**

The activity of N-acetyl-β-D-glucosaminidase (NAG) in the urine was examined using the NAG Test Shionogi Kit (Shionogi & Co. Ltd, Osaka, Japan). NAG activity was determined spectrophotometrically based on the level of products generated from the reaction of NAG and its substrate (575 nm). NAG activity was normalized with creatinine (Cre) level in the urine. Values of blood urea nitrogen (BUN) in the serum were examined. An automatic dry-chemistry analyzer system (Spotchem EZ SP-4430; Arkray, Kyoto, Japan) was used to determine BUN and Cre levels.

**Hepatic toxicity evaluation**

Activities of glutamic oxaloacetic transaminase (GOT) and glutamate pyruvate transaminase (GPT) in the serum were assessed to evaluate hepatic toxicity. An automatic dry-chemistry analyzer system (Spotchem EZ SP-4430; Arkray) was used to determine the activities.

**Nuclear extraction**

The nuclei were extracted with a Nuclear Extraction Kit (Panomics; Affymetrix, Santa Clara, CA, USA). After Cd exposure, the kidney was minced in ice-cold phosphate-buffered saline (Nissui, Tokyo, Japan), and lysed on ice for 10 min in extraction buffer A. The buffer included protease inhibitor, phosphatase inhibitor, and dithiothreitol (DTT). The nuclei were collected by centrifugation at 14,000 × g for 3 min at 4°C. The pellet was resuspended in extraction buffer B containing protease inhibitor, phosphatase inhibitor, and DTT and incubated at 4°C for 60 min. The mixture was then centrifuged at 14,000 × g for 5 min at 4°C. Supernatants were collected and total protein concentration was measured using a Quick Start Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

**Assay analysis**

Protein/DNA-binding assays made use of the TransSignal™ Combo Protein/DNA Array (Affymetrix). Twenty micrograms of nuclear extracts were mixed with biotin-labeled probe mix, and the mixture was incubated at 15°C for 30 min. Protein-bound probes were then isolated from the non-bound probed using Spin Columns. The protein-bound probes were eluted with column elution buffer and denatured at 95°C for 3 min. The eluted probes were then added to hybridization buffer and hybridized to array membranes spotted with consensus sequences complemented with probes at 42°C overnight. The membranes were washed twice in 2 × SSC (Saline sodium citrate)/0.5% SDS (Sodium dodecylsulfate) at 42°C for 20 min and then twice in 0.1 × SSC/0.5% SDS at 42°C for 20 min.

**Detection**

The membranes were blocked with 1× blocking buffer. Biotin-labeled probes were detected with streptavidin-horseradish peroxidase diluted 1:10,000. Images were acquired using the LAS-4000 device (GE Healthcare, Little Chalfont, UK). Spot density was evaluated using ImageQuantTL software (GE Healthcare).

**RESULTS AND DISCUSSION**

Body weights of the Cd-exposed mice were monitored during the exposure period. After 3 months of Cd exposure, the body weights of the control and Cd-exposed mice were 23.22 ± 1.26 g and 23.86 ± 1.80 g, respectively, without any considerable differences between the two groups (Fig. 1A). The Cd concentration in the kidney was measured using atomic absorption spectrophotometry. Only negligible amounts of Cd were detected in the kidney of the control mice, but the Cd concentration in the kidney of the exposed mice was 97.56 ± 9.48 µg/g (Fig. 1B). Cd concentration in the liver of the exposed mice was 63.41 ± 5.80 µg/g liver (Fig. 1C). NAG activities in the urine and BUN values in the serum were examined to assess renal toxicity. The BUN values in the serum and NAG activities in the urine did not differ between the control and Cd-exposed groups (Fig. 1D, E). Activities of GOT and GPT in the serum were examined to assess hepatic toxicity. GOT activity in the serum of mice was slightly increased after Cd exposure; however, GPT activities remained unchanged (Fig. 1F, G). The mice exposed for 3 months exhibited accumulation of Cd in the liver and kidney; nevertheless, only slight hepatic toxicity was observed. Our previous study demonstrated that Cd exposure for 12 months showed Cd concentration by 174.74 µg/g kidney; in addition, the Cd accumulation induced apoptosis in the mouse kidney (Tokumoto et al., 2011). Therefore, the transcript changes in the kidney of mice exposed to Cd for 3 months may be causative factors for Cd-induced renal dysfunction.

Subsequently, nuclear extract was prepared from
the kidney of Cd-treated mice. We incubated the nuclear extracts with a mix of 345 biotin-labeled DNA probes, which corresponded to transcription factor response elements (cis-elements). After the probes were allowed to bind specific transcription factors, we separated the protein/DNA complexes from the free probes using spin columns. Cis-elements bound to the transcription factors were eluted using elution buffer, and the number of cis-elements was determined using protein/DNA-binding assay membranes on which elements hybridizing with the cis-elements present were pre-spotted. Cd altered the binding of transcription factors to the cis-elements when compared with the control group (Fig. 2). The density of each spot means the DNA-binding activity of transcription factor. For example, the spot on first column and second row indicates the binding activity of AP-2 tran-

Transcription activity changes in the kidney of mice exposed to cadmium

Fig. 1. Toxicity evaluation of mice exposed to cadmium for 3 months. (A) Changes in the body weight of mice after chronic exposure to Cd. (B and C) Cd accumulation in the kidney (B) and liver (C) of mice exposed to the metal for 3 months. The tissues were digested with nitric acid and hydrogen peroxide, and the Cd content was measured by atomic absorption spectrophotometry. (D) BUN level in the serum. (E) NAG activity in the urine. NAG activity was normalized based on the creatinine (Cre) level in the urine. (F and G) Activities of GOT (F) and GTP (G) in the serum. Values are expressed as mean ± SD (n=4-5). * Significantly different from the control group, p < 0.05, Bonferroni’s multiple t-test after ANOVA.

Fig. 2. Protein/DNA binding assay with nuclear extracts from the kidney of mice exposed to Cd. DNA-binding activity of the transcription factors in the kidney of mice exposed to Cd using protein/DNA-binding assays. The nuclear extracts from the control and Cd-exposed groups (n = 4 - 5) were pooled and used for protein/DNA binding assays. Spots along the right side and bottom of the membranes were biotinylated for normalization.
The density of the spot for AP-2 in Cd-treated sample is decreased compared to that of control sample. Overall, Cd caused a more than 2-fold increase in the binding activity of five transcription factors (Table 1) and a less than 0.5-fold decrease in the binding activity of 14 transcription factors (Table 2). Our previous study suggested that a 3-hr Cd treatment suppresses the binding activity of HFH-8 (FOXF1) and HIF-1 in HK-2 human proximal tubular cells (Lee et al., 2017). However, a 3-month exposure elevated the activity of HFH-2 and HIF-1 in the mouse kidney. The activities of several transcription factors were dynamically altered. Further studies are essential to elucidate how the screened transcription factors are involved in Cd-induced renal toxicity.

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**Conflict of interest** The authors declare that there is no conflict of interest.

### Table 1. Transcription factors with increased binding activity in response to Cd exposure. Transcription factors showing ≥ 2-fold increase are listed.

| Name   | Description                  | Protein(s)          | Accession number                          | Ratio |
|--------|------------------------------|---------------------|-------------------------------------------|-------|
| Thy-1BP| Thy-1 binding protein        | Sp1; NF-YA; NF-YB   | NP_038700; NP_001104302; NP_035044         | 2.97  |
| GATA-1 | GATA binding protein-1       | GATA-1              | NP_032115                                 | 2.67  |
| HFH-2  | Forkhead box D3              | HFH-2               | NP_034555                                 | 2.64  |
| HIF-1  | Hypoxia-inducible factor 1   | HIF-1a              | NP_001300848                              | 2.49  |
| Surf-2 | Surf-1                       | SURF2               | NP_038706                                 | 2.35  |

### Table 2. Transcription factors with decreased binding activity in response to Cd exposure. Transcription factors showing ≤ 0.5-decrease are listed. N/A, not applicable for unidentified proteins.

| Name    | Description                                | Protein(s)          | Accession number                          | Ratio |
|---------|--------------------------------------------|---------------------|-------------------------------------------|-------|
| MEF1    | MDR1 promoter enhancing factor 1           | MEF1                | N/A                                       | 0.01  |
| RAR/DR-5| Retinoic acid receptor                     | RAR-alpha; RAR-beta; RAR-gamma | NP_001169999; NP_001276689; NP_001036192 | 0.01  |
| USF-1   | Upstream transcription factor              | USF1                | NP_001292605                              | 0.01  |
| AFP-1   | Alpha-fetoprotein                          | Alpha-1 fetoprotein | NP_034149                                 | 0.03  |
| AP-2    | Transcription factor 3                     | AP-2 alpha          | NP_00116420                               | 0.04  |
| E47     | Transcription factor 3                     | E2-alpha            | NP_001157619                              | 0.13  |
| HiNF    | Histone gene transcription factors         | HiNF-P              | NP_751894                                 | 0.21  |
| L-III BP| Pyruvate kinase L gene binding protein III | TEF-3               | NP_001074448                              | 0.21  |
| LF-A1   | Liver-specific TF                          | LF-A1               | N/A                                       | 0.23  |
| YB-1    | Glutathione S-transferase Yb subunit       | HNF-1               | NP_033353                                 | 0.23  |
| MSP-1   | Mutant SP-1                                | N/A                 | N/A                                       | 0.29  |
| MT-Box  | Tentative new binding domain, located in hTERT promoter | N/A | N/A | 0.31 |
| PAX-3   | Paired box protein-3                       | Pax-3               | NP_001152992                              | 0.45  |
| Freac-2 | Forkhead-related activator 2               | Foxf2               | NP_034355                                 | 0.49  |
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