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Letter

Cadmium-induced protein ubiquitination in *UBA80* knockdown HK-2 cells

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ABSTRACT — Cadmium (Cd) is a toxic heavy metal that is particularly damaging to proximal tubular cells of the kidney. Cd-induced renal toxicity is associated with perturbation of the ubiquitin proteasome system. Our previous study demonstrated that Cd increased gene expression of ubiquitin-coding genes, *UBB*, *UBC*, and *UBA80*. Notably, knockdown of the polyubiquitin gene *UBB* by siRNA transfection significantly decreased ubiquitinated protein levels that had been increased by Cd treatment. The present study showed that in contrast to *UBB*, knockdown of the monoubiquitin gene *UBA80* did not diminish the Cd-induced protein ubiquitination in HK-2 cells. Taken together, our results suggest that polyubiquitin rather than monoubiquitin is preferably engaged in Cd-induced accumulation of ubquitinated proteins in HK-2 cells.

Key words: Cadmium, HK-2 cells, Renal toxicity, Ubiquitinated protein, Ubiquitin-coding genes

INTRODUCTION

Cadmium (Cd) is a nephrotoxic environmental heavy metal (Järup *et al.*, 1998; Fujiwara *et al.*, 2012). The ubiquitin-proteasome system, a cellular protein degradation system, is involved in Cd-induced renal toxicity (Tokumoto *et al.*, 2011; Lee *et al.*, 2016). Our recent study demonstrated that Cd triggered renal toxicity through protein ubiquitination in human proximal tubular (HK-2) cells (Lee *et al*., 2015). In addition, it was found that Cd increased gene expression of the ubiquitin-coding genes *UBB*, *UBC*, and *UBA80* (also known as *RPS27A*). Among four ubiquitin-coding genes, there are two genes (*UBB* and *UBC*) encoding polyubiquitin and two genes (*UBA80* and *UBA52*) encoding monoubiquitin fused to ribosomal proteins (Lee *et al*., 2015). Remarkably, *UBB* was strongly associated with Cd-induced protein ubiquitination. However, the involvement of UBA80 in Cd-induced protein ubiquitination remains unclear. Therefore, we examined the ubiquitinated protein levels in *UBA80* knockdown HK-2 cells.

MATERIALS AND METHODS

Cell culture

HK-2 cells were purchased from ATCC (Manassas, MA, USA), and cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 25 U/mL penicillin (DS Pharm, Osaka, Japan), 25 μg/mL streptomycin (DS Pharm), 1% Insulin-Transferrin-Selenium-X (Gibco), 10 ng/mL EGF (epidermal growth factor; Sigma-Aldrich), and 5 ng/mL hydrocortisone (Sigma-Aldrich), at 37°C in a humidified incubator containing 5% CO_2.

Cell viability

HK-2 cells were seeded in 96-well plates at a density of 2.5×10^4 cells/cm^2 and cultured until confluent. The culture medium was discarded and cells were treated with Cd ($CdCl_2$) (Wako Pure Chemical Industries, Osaka, Japan) in serum-free culture medium for 6 or 24 hr. After treatment, culture medium was replaced with fresh 10% FBS-DMEM/F-12 containing MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide;

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DOJINDO Laboratories, Kumamoto, Japan] and incubated for another 4 hr at 37°C. After removing the medium, 100 µL dimethyl sulfoxide (Wako Pure Chemical Industries) was added to MTT formazan. Absorbance at 570 nm was measured by the DTX880 multimode detector (Beckman Coulter Inc., Brea, CA, USA).

siRNA transfection

Silencer® Select pre-designed siRNAs were purchased from Ambion (Grand Island, NY, USA). The siRNAs were as follows: s52245 and s12339 (for human *UBA80*). Control siRNA (Silencer® Select Negative Control #1 siRNA) was also purchased from Ambion. The transfection of siRNA was performed using Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY, USA). After the siRNA mixture was incubated for 15 min with Lipofectamine RNAiMAX and Opti-MEM® I Reduced Serum Medium (Opti-MEM; Gibco), HK-2 cells were transfected with the siRNA mixture (1 nM siRNA/sequence, 0.2% Lipofectamine RNAiMAX, 10% Opti-MEM) for 48 hr.

RNA extraction and real-time RT-PCR

HK-2 cells transfected with siRNA were washed twice with ice-cold phosphate-buffered saline (PBS) (Nissui, Tokyo, Japan), and total RNA extracted with the Quick Gene RNA cultured cell kit S (Fujifilm, Tokyo, Japan) according to the manufacturer's protocol. RNA quantitation and purity were measured using the BioSpec-nano (Shimadzu Biotech, Kyoto, Japan). cDNA was generated from total RNA using the PrimeScript reverse transcription (RT) Reagent Kit (Perfect Real Time) (Takara Bio, Shiga, Japan). Real-time PCR was performed with the SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio), and a Thermal Cycler Dice Real Time System (Takara Bio). PCR conditions were as follows: 10 sec hot start at 95°C followed by 40 cycles of 5 sec at 95°C and of 30 sec at 60°C. Gene expression was normalized to *GAPDH* mRNA levels. The oligonucleotide sequences of the primers were as follows: sense, 5′-GCACCGTCAAGGCTGAGAAC-3′, and antisense, 5′-TGGTGAAGACGCCAGTGGA-3′, for human *GAPDH*; sense, 5′-TCACTCTGGAGGTGGA-3′, and antisense, 5′-CCCTCAGGCGCAGGAC-3′, for human *UBB*; sense, 5′-AAAGAGTCCACCTTGCACCTG-3′, and antisense, 5′-ACCTCAAGGGTGATGGTCTTG-3′ for human *UBC*; sense, 5′-TCGTGGTGGTGCTAAGAAAA-3′, and antisense, 5′-TCTCGACGAAGGCGACTAAT-3′ for human *UBA80*; sense, 5′-AGGAGGGTATCCCACCTGAC-3′, and antisense, 5′-CAGGGTGGACTCTTTCTGGA-3′ for human *UBA52*.

Western blotting

After treatment, cells were washed twice with ice-cold PBS and harvested in RIPA buffer [25 mM Tris (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate 0.1% SDS; Thermo Fisher Scientific, Waltham, MA, USA]. Protein concentrations were measured using the BCA protein assay kit (Thermo Fisher Scientific). Proteins were electrophoresed on gradient (4%-15%) SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA), transferred to polyvinylidene fluoride membranes (ATTO, Tokyo, Japan), and probed with antibodies against ubiquitin (P4D1) (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA), or β-actin (1:1,000; American Research Products, Waltham, MA, USA). The membrane was subsequently incubated with horseradish peroxidase-conjugating (HRP) secondary antibodies (1:10,000; GE Healthcare, Tokyo, Japan), and proteins detected by enhanced chemiluminescence using ImmunoStar® Zeta (Wako Pure Chemical Industries). Chemiluminescent images were captured using the Image Quant LAS 500 (GE Healthcare) device.

Statistical analysis

Statistical analyses were performed using ANOVA and Student's *t*-test ($P < 0.05$).

RESULTS AND DISCUSSION

The cytotoxicity of Cd against HK-2 cells is shown

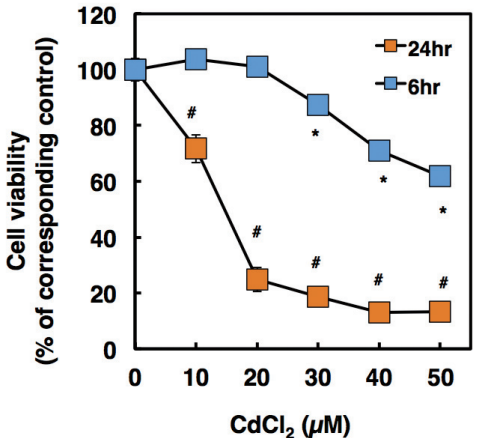

Fig. 1. The viability of HK-2 cells after Cd treatment. HK-2 cells were cultured for 48 hr and then treated with Cd in serum-free culture medium for 6 hr or 24 hr. Cell viability was measured by MTT assay. Values are means ± S.D. (n = 5). *Significantly different from the control group after 6 hr of treatment. #Significantly different from the control group after 24 hr of treatment, $P < 0.05$.

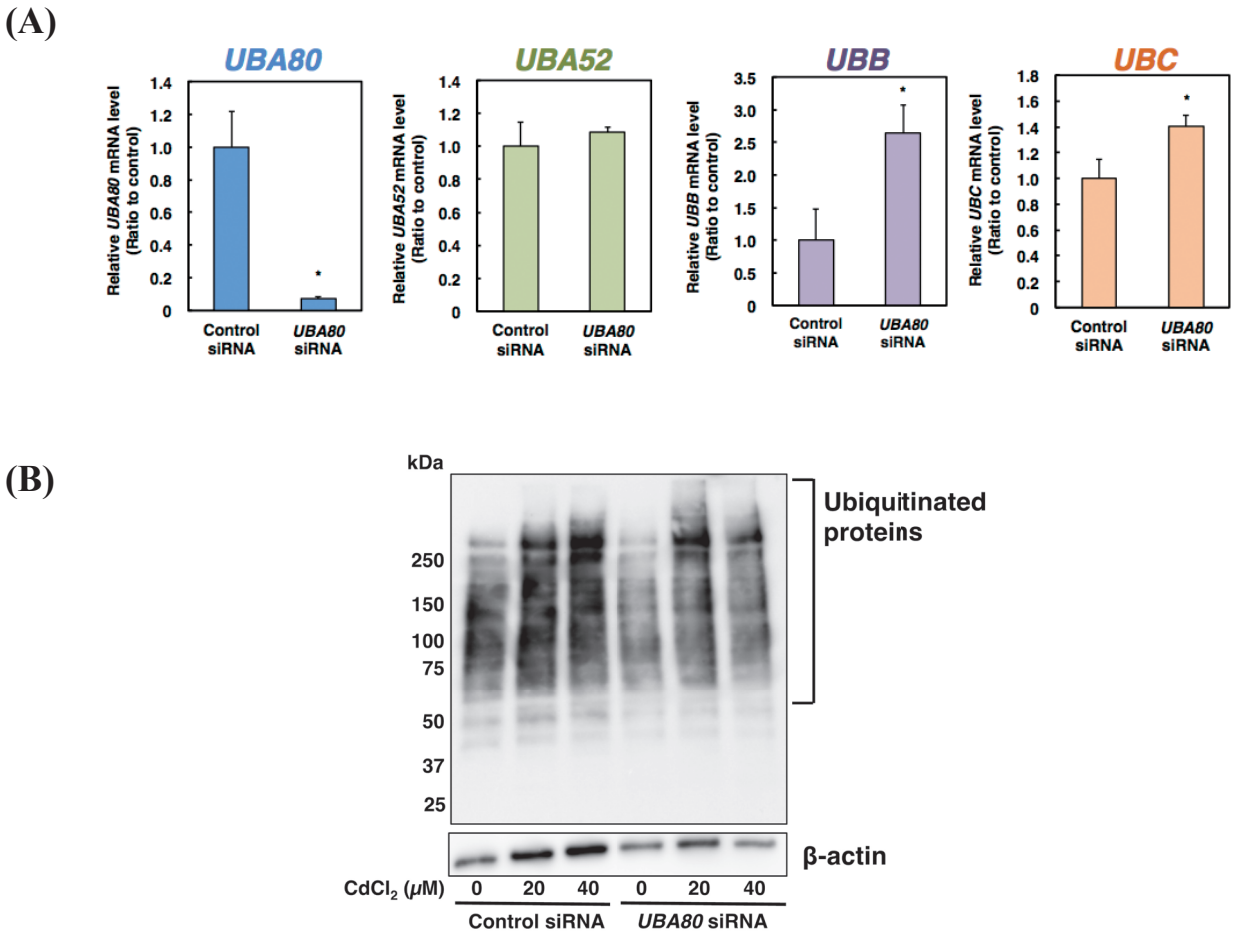

Fig. 2. Effect of *UBA80* siRNA on Cd-induced protein ubiquitination in HK-2 cells. (A) The knockdown efficiency of *UBA80* in HK-2 cells following *UBA80* siRNA treatment. *UBA80* siRNA was added to HK-2 cells and cells were incubated for 48 hr. The levels of *UBA80* and other ubiquitin-coding genes (*UBA52*, *UBB*, and *UBC*) were measured using real-time RT-PCR. The mRNA levels were normalized to *GAPDH* mRNA levels. Values are means ± S.D. (n = 3). *Significantly different from the control siRNA group, $P < 0.05$. (B) Effect of *UBA80* siRNA on Cd-induced protein ubiquitination in HK-2 cells. HK-2 cells were treated with control siRNA or *UBA80* siRNA for 48 hr. Cells were then treated with Cd in serum-free culture medium for 6 hr. Whole cell lysates were analysed by western blot for ubiquitinated proteins. β-actin was used as a loading control.

in Fig. 1. In this study, 30 μM Cd was the lowest dose that after 6 hr exhibited a significant toxic effect on HK-2 cells. After treatment for 24 hr, cytotoxicity was apparent from 10 μM Cd. Treatment with 40 μM Cd for 6 hr and 24 hr showed cytotoxicity complementary to our previous study (Lee *et al.*, 2015). We then investigated the effect of *UBA80* knockdown by siRNA transfection on the protein ubiquitination induced by 6 hr of Cd treatment in HK-2 cells. Mammalian ubiquitin-coding genes comprise four genes, and those are *UBB*, *UBC*, *UBA80,* and *UBA52* (Baker and Board, 1991). Transfection of *UBA80* siRNA into HK-2 cells specifically inhibited gene expression of *UBA80* (Fig. 2A). In agreement with our previous study, Cd promoted protein ubiquitination in control siRNA transfected cells (Fig. 2B). However, in contrast to the knockdown of *UBB*, *UBA80* knockdown did not diminish the Cd-induced protein ubiquitination (Fig. 2B). These results suggest that Cd increases protein ubiquitination in *UBA80* knockdown cells at least to the same degree as control cells.

The expression of not only *UBB* but also *UBA80* increases after Cd treatment in HK-2 cells (Lee *et al.*, 2015). In that study, knockdown of *UBB* suppressed the Cd-induced protein ubiquitination and diminished Cd

toxicity. However, knockdown of *UBA80* did not affect Cd toxicity (Lee *et al.*, 2015). In the present study, knockdown of *UBA80* did not suppress Cd-induced protein ubiquitination. As previously noted, *UBB* encodes polyubiquitin, whereas *UBA80* encodes monoubiquitin. Therefore, our results suggest that polyubiquitin is preferably engaged in Cd-induced accumulation of ubiquitinated proteins in HK-2 cells.

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

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