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The involvement of *GPRC5B* in cadmium toxicity in HK-2 cells

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ABSTRACT — Cadmium (Cd) is a nephrotoxic heavy metal. Several signal transduction pathways have been reported to be associated with Cd toxicity. GPRC5B is a member of the family of G-protein-coupled receptors, which recognize various ligands and can transmit signals from the cell surface into the cell interior. We examined the involvement of GPRC5B in Cd toxicity in HK-2 human proximal tubular cells. Herein, we found that Cd significantly reduced *GPRC5B* gene expression in HK-2 cells. Moreover, knockdown of *GPRC5B* by siRNA transfection strengthened Cd toxicity in HK-2 cells. Our findings suggest that Cd partially conferred its toxicity by suppressing *GPRC5B* gene expression in HK-2 cells.

Key words: Cadmium, HK-2 cells, *GPRC5B*

INTRODUCTION

Cadmium (Cd) is a toxic heavy metal present in the environment that causes adverse health effects by damaging various tissues, including the kidney (Järup and Akesson, 2009). Proximal tubular cells are the major targets of Cd-induced renal toxicity (Järup and Akesson, 2009). Our recent studies yielded some insights into the target genes involved in Cd toxicity in proximal tubular cells (Tokumoto *et al.*, 2011b; Lee *et al.*, 2013, 2014). Cd can affect the activities of signaling cascades (Inanobe *et al.*, 2011; Thévenod and Lee, 2013). Various receptor proteins are expressed on the cell surface that can relay extracellular signals received at the cell surface into the cell interior. G protein-coupled receptors (GPCRs) are not only the largest family of receptors found on the cell surface, but they are also important targets of many drugs (Lagerström and Schiöth, 2008). Recently, GPRC5B (G protein-coupled receptor, family C group 5 member B), a member of a subgroup of the C family of GPCRs, was found to be involved in apoptosis (Soni *et al*., 2013). Cd cytotoxicity is mediated by apoptosis signaling (Tokumoto *et al.*, 2011a; Fujiwara *et al.*, 2012). Therefore, in this study, we examined the involvement of GPRC5B in Cd toxicity in human proximal tubular cells (HK-2 cells).

MATERIALS AND METHODS

Cell culture

HK-2 cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (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 (Sigma-Aldrich), and 5 ng/mL hydrocortisone (Sigma-Aldrich) at 37°C in a humidified incubator containing 5% CO_2.

Real-time RT-PCR

HK-2 cells were transferred into 60-mm tissue culture dishes (Falcon, Franklin Lakes, NJ, USA) at a density of 2.0×10^4 cells/cm^2 and were cultured until confluent. Then, culture medium was discarded and the cells were treated with various concentrations of cadmium chloride ($CdCl_2$; Wako Pure Chemical Industries, Osaka, Japan) in serum-free medium for 6 hr. Cd-treated HK-2 cells were washed twice with ice-cold phosphate-buffered saline (Nissui, Tokyo, Japan). Total RNA was extracted using the Quick Gene RNA cultured cell kit S (Fujifilm, Tokyo, Japan), according to the manufactur-

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er's instructions. Total RNA was incubated with PrimeScript reverse transcription (RT) Reagent Kit (Perfect Real Time, Takara Bio, Shiga, Japan) to generate cDNA. Real-time PCR was performed with SYBR Premix Ex Taq II (Perfect Real Time, Takara Bio) using the 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 30 sec at 60°C. Gene expression was normalized to *GAPDH* mRNA levels. The primer oligonucleotide sequences were as follows: sense, 5′-GCACCGTCAAGGCTGAGAAC-3′, and antisense, 5′-TGGTGAAGACGCCAGTGGA-3′, for the human *GAPDH* gene; sense, 5′-CCCTCAGTACGTGTCCCTGT-3′, and antisense, 5′-TCAGGAGCAGTGTGATCAGG-3′, for the human *GPRC5B* gene.

siRNA transfection

Double-stranded siRNAs were purchased from Qiagen (Germantown, MD, USA). The siRNA product names were as follows: Hs_GPRC5B_2; Hs_GPRC5B_3; Hs_GPRC5B_4; and Hs_GPRC5B_5, for human *GPRC5B*. Control siRNA (Silencer® Select Negative Control #1 siRNA) was purchased from Ambion (Grand Island, NY, USA). The siRNA transfections were 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 for 48 hr.

Cell viability

HK-2 cells were transferred to a 96-well tissue culture plate (Falcon) at a density of 2.0×10^4 cells/cm^2 with the siRNA mixture [1 nM siRNA, 0.2% (v/v) Lipofectamine RNAiMAX, and 10% (v/v) Opti-MEM]. After 48 hr, cells were incubated with $CdCl_2$ in serum-free culture medium for 24 hr. After treatment, serum-free medium containing $CdCl_2$ was replaced with fresh growth medium containing 10% (v/v) Alamar Blue (Invitrogen) and incubated for 4 hr at 37°C. Fluorescence was measured at an excitation wavelength of 540 nm and an emission wavelength of 595 nm.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA). When the F-value was significant ($P < 0.05$), Bonferroni's multiple *t*-test was performed for post-hoc comparisons ($P < 0.05$).

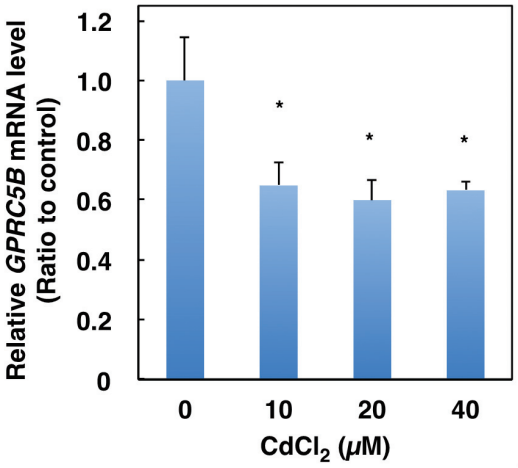

Fig. 1. The effects of Cd on *GPRC5B* gene expression in HK-2 cells. HK-2 cells were treated with various concentrations of cadmium chloride for 6 hr. The mRNA levels of *GPRC5B* were measured using real-time RT-PCR. *GPRC5B* mRNA levels were normalized to *GAPDH* mRNA levels. Values are the means ± S.D. ($n = 3$). *Significantly different from the control, $P < 0.05$.

RESULTS AND DISCUSSION

The cell viability of HK-2 cells exposed to Cd was examined using the Alamar Blue assay. When HK-2 cells were treated with 20 μM Cd, the cell viability after 24 hr incubation was decreased by 40%; however, 6 hr incubation did not show such toxic effect (data not shown). The cell viability of HK-2 cells after treatment with 40 μM Cd for 6 and 24 hr decreased to 50% and 70%, respectively (data not shown). Figure 1 shows the mRNA levels of *GPRC5B*, a gene encoding the GPRC5B protein, in HK-2 cells treated with 10, 20, or 40 μM Cd for 6 hr. The mRNA levels of *GPRC5B* were significantly reduced following Cd treatment (Fig. 1).

The susceptibility of *GPRC5B* knockdown cells to Cd toxicity is shown in Fig. 2. *GPRC5B* mRNA levels in cells transfected with *GPRC5B* siRNA were reduced by 20% compared with control cells (Fig. 2A). HK-2 cells transfected with *GPRC5B* siRNA were more susceptible to Cd toxicity than control cells (Fig. 2B). In Cd-untreated HK-2 cells, however, the cell viability was not different between *GPRC5B* knockdown cells and control cells (Fig. 2B). These results suggest that Cd may strengthen its toxicity *via* the suppression of *GPRC5B* gene expression in HK-2 cells. Several studies demonstrated that GPRC5B might be involved in neuropathic pain and insulin secretion (Soni *et al.*, 2013; Chung *et al.*, 2014). However, the

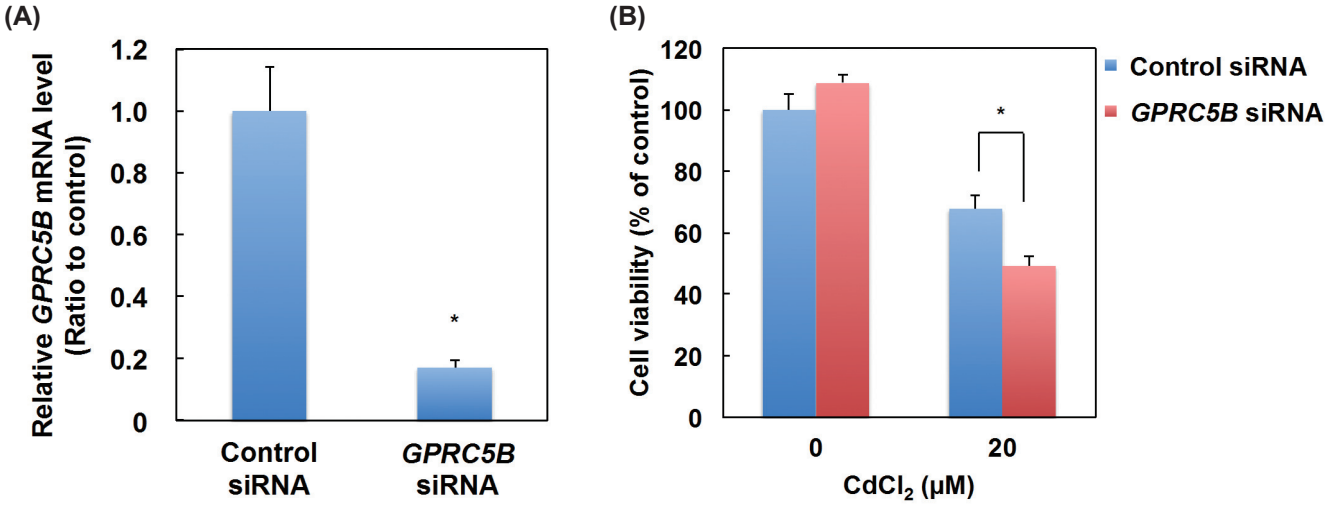

Fig. 2. The effects of *GPRC5B* knockdown on Cd toxicity in HK-2 cells. (A) The knockdown efficiency of *GPRC5B* in HK-2 cells following *GPRC5B* siRNA treatment. *GPRC5B* siRNA was added to HK-2 cells and then cells were incubated for 48 hr. The mRNA levels of *GPRC5B* were measured using real-time RT-PCR. *GPRC5B* mRNA levels were normalized to *GAPDH* mRNA levels. Values are the means ± S.D. (n = 3). (B) The cell viability of HK-2 cells transfected with *GPRC5B* or control siRNA after treatment with cadmium chloride for 24 hr using the Alamar Blue assay. Values are the means ± S.D. (n = 3). *Significantly different from the corresponding control siRNA group, $P < 0.05$.

role of GPRC5B in kidney function or Cd-induced renal toxicity has not been elucidated. The present study has found that *GPRC5B* represents a novel gene involved in Cd toxicity in HK-2 cells. Additionally, *GPRC5B* may be one of the factors that determine susceptibility to Cd.

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

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