Original Article

FBXO6 attenuates cadmium toxicity in HEK293 cells by inhibiting ER stress and JNK activation

Ke Du^{1,2}, Tsutomu Takahashi¹, Shusuke Kuge^{1,3}, Akira Naganuma¹ and Gi-Wook Hwang¹

¹Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Miyagi 980-8578, Japan

²Department of Pharmacology, China Medical University, Puhe Road 77, Shenyang North New Area, Shenyang 110013, Liaoning Province, PR China

³Department of Microbiology, Tohoku Pharmaceutical University, Komatsushima, Aoba-ku, Sendai, Miyagi 981-8558, Japan

[Contributed by Akira Naganuma]

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ABSTRACT — Cadmium-induced cell death is associated with endoplasmic reticulum (ER) stress. We previously found that inhibition of FBXO6 expression, which is a ubiquitin ligase involved in ER-associated protein degradation (ERAD), induces high sensitivity to cadmium in HEK293 cells. However, the precise role of FBXO6 in ER stress remains unexplored. In this study, we investigated the role of FBXO6 in cadmium-induced ER stress in HEK293 cells. Our results showed that the cadmium-induced increase in expression of the ER stress marker proteins, BiP and CHOP, was further enhanced by inhibiting FBXO6 expression. Cadmium-induced c-Jun phosphorylation was also markedly increased by inhibition of FBXO6 expression. However, this c-Jun phosphorylation was almost entirely abolished by inhibition of c-Jun N-terminal kinase 1 (JNK1) expression. The level of high cadmium sensitivity induced by inhibition of FBXO6 expression was markedly lower in the JNK1-ablated cells than in the control cells. In addition, cadmium elevated the cellular level of ERAD substrate proteins, and this elevation was further enhanced by inhibiting FBXO6 expression. These results suggest that FBXO6 might inhibit cadmium-induced ER stress by functioning as a ubiquitin ligase in the ERAD system, thereby attenuating the cell death induced by subsequent JNK1 activation.

Key words: FBXO6, Cadmium, Cytotoxicity, Endoplasmic reticulum-associated protein degradation

INTRODUCTION

Cadmium, an environmental pollutant, is known to cause the itai-itai disease (Baba *et al.*, 2014; Nogawa and Kido, 1993). Cadmium persists in the human body because it cannot be easily excreted and its concentration increases with age (Ikeda *et al.*, 2004). An epidemiological survey reported that higher intake of cadmium is associated with a higher incidence of renal impairment (Heilmaier *et al.*, 1987; Lee *et al.*, 2013; Miura *et al.*, 2013; Summer *et al.*, 1986; Tokumoto *et al.*, 2014); thus, the health problems caused by cadmium are a global concern.

Endoplasmic reticulum (ER) stress is known to play a role in cadmium toxicity. Cadmium increases the cellular concentrations of G protein-coupled receptor

94 (GPR94), binding immunoglobulin protein (BiP) and C/EBP homologous protein (CHOP); the expressions of which are known to be induced by ER stress (Kitamura and Hiramatsu, 2010; Kwon *et al.*, 1999; Liu and Rondinone, 2005; Yokouchi *et al.*, 2007). On the other hand, some studies reported that increased expression of ER molecular chaperons, such as BiP or ORP150, inhibition of ER stress-induced CHOP expression, or treatment of cells with inhibitor of c-Jun N-terminal kinase (JNK) prevents the cadmium-induced cell death (Yokouchi *et al.*, 2007, 2008).

We previously reported that inhibiting FBXO6 expression, which is involved in ER-associated protein degradation (ERAD), leads to increased cadmium sensitivity in human embryonic kidney-derived HEK293 cells (Hwang *et al.*, 2011). Many of the proteins synthesized

Correspondence: Gi-Wook Hwang (E-mail: gwhwang@m.tohoku.ac.jp)

in the ER undergo sugar chain modification and accurate folding. These proteins are then transported to Golgi bodies. ER stress is induced when misfolded proteins or proteins that cannot form multimers persist in the ER (Rao *et al.*, 2004). In the event of ER stress, the cells immediately activate their defense systems to counteract the stress. One of these defense systems is the ERAD, which transports misfolded proteins accumulated in the ER to the cytoplasm, and enhances ubiquitination to promote their proteasome-dependent degradation (Brodsky, 2007; Nishikawa *et al.*, 2005). Although FBXO6 has been reported to act as a ubiquitin ligase in the ERAD system (Yoshida *et al.*, 2003), its role in ER stress remains unexplored.

In this study, we investigated the role of FBXO6 in cadmium-induced ER stress in HEK293 cells.

MATERIALS AND METHODS

Cell culture and transfections

Human Embryonic Kidney (HEK) 293 cells were maintained in a humidified 5% CO₂ atmosphere at 37°C in complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. The following siRNAs were obtained from Qiagen (Germantown, MD, USA): AllStars® negative control siRNA (Cat. No. 1027280), FBXO6 siRNAs (5'-CUGGGUAGGUGGACAAUUGUA-3' and 5'-UACGCUGAUGCCUGCUGUCA-3') and CHOP siRNAs (5'-UGGGAGAACCAGGAAACGGAA-3' and 5'-AAGGAAGUGUAUCUUCACUACA-3'). JNK1 siRNAs (5'-GUUGCAAUCAAGAAGCUAA-3' and 5'-CAAAGAUCCCUGACAAGCA-3') were purchased from Ambion (Austin, TX, USA). Two double-stranded siRNAs targeting the relevant genes were introduced into the HEK293 cells by the reverse transfection technique using HiPerFect® Transfection Reagent (Qiagen) in serum-free Opti-MEM. The transfection complex (at a final siRNA concentration of 10 nM) was mixed with 5 × 10⁵ cells and then incubated for 15 min at room temperature. The HEK293 cells were then seeded at a density of 5 × 10³ cells per well in 96-well plates for 48 hr for the cell viability assay. Alternately, 5 × 10⁵ cells per well were plated in 6-well plates for 24 hr at 37°C with 5% CO₂ for immunoblotting. The plasmid DNA (pcDNA5-TCRα-FLAG and pREP9-NHK) and the double-stranded siRNA were co-transfected into the HEK293 cells using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Cell viability assay

After incubation for 48 hr, HEK293 cells were treated with various concentrations of cadmium chloride (CdCl₂) and then re-incubated for a further 48 hr. Cell viability was determined by treating the cells with Alamar Blue (Biosource, Camarillo, CA, USA), according to the manufacturer's instructions. The Alamar Blue reagent was added directly to the culture media at 10% of the total volume, and the cells were incubated at 37°C for 2 hr. The fluorescence was measured at an excitation of 544 nm and emission of 590 nm using the Gemini XPS® Microplate Spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA).

Immunoblotting

HEK293 cells were washed on ice with cold PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, 0.5% NP-40, and protease inhibitor cocktail (Roche, Mannheim, Germany). The cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA), and visualized using the relevant antibodies. The anti-BiP, anti-CHOP, anti-c-Jun, and anti-phospho-c-Jun (Ser63) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA); the anti-GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the anti-FLAG antibody was purchased from Sigma (St Louis, MO, USA); and the anti-α-1-antitrypsin antibody was from DakoCytomation (Glostrup, Denmark).

RESULTS AND DISCUSSION

When HEK293 cells were treated with cadmium, the levels of the ER stress marker proteins, BiP and CHOP, were markedly increased (Fig. 1). When FBXO6 expression was inhibited by siRNA, this increase in the levels of BiP and CHOP was observed at lower concentrations of cadmium than in the control cells (Fig. 1), suggesting the possibility that FBXO6 attenuates cadmium toxicity by inhibiting the cadmium-induced ER stress.

CHOP and JNK are known to be involved in the onset of cadmium toxicity via ER stress (Yokouchi *et al.*, 2007, 2008). CHOP, a transcription factor belonging to the CCAAT/enhancer-binding protein (C/EBP) family, induces the expression of BCL-2-interacting mediator of cell death (BIM), which promotes apoptosis by inhibiting BCL expression, which is an anti-apoptotic gene (Oyadomari and Mori, 2004). Therefore, we investigated the relationship between CHOP and FBXO6 in the cadmium sensitivity of HEK293 cells. We found that the

Fig. 1. Effect of FBXO6 knockdown on the induction of BiP and CHOP by cadmium. FBXO6-ablated cells (5×10^5 cells/well) were treated with cadmium chloride at the indicated concentrations and cultured for 18 hr. The expression of BiP, CHOP, and GAPDH (loading control) were monitored by immunoblotting.

Fig. 2. Effect of the knockdown of FBXO6 and/or CHOP on the sensitivity of HEK293 cells to cadmium toxicity. (A) HEK293 cells (5×10^3 cells/well) transfected with double-stranded FBXO6 siRNA and/or CHOP siRNA were plated on 96-well plates and cultured in 100 μL aliquots of medium. After incubation for 48 hr, cadmium chloride was added and the cells were cultured for a further 48 hr. Cell viability was determined by treating the cells with Alamar Blue (Biosource, Camarillo, CA, USA). Each point represents the mean value and standard deviation of the results from three cultures. (B) CHOP-ablated cells (2.5×10^5 cells/well) were treated with cadmium chloride at the indicated concentrations. The expression levels of CHOP and GAPDH (loading control) were monitored by immunoblotting.

Fig. 3. Effect of the knockdown of FBXO6 and/or JNK1 on c-Jun phosphorylation following cadmium treatment. (A and B) FBXO6- and JNK-ablated cells (5×10^5 cells/well) were treated with cadmium chloride at the indicated concentrations and cultured for 18 hr. The expression levels of phospho-c-Jun, c-Jun, and GAPDH (loading control) were monitored by immunoblotting.

cadmium sensitivity of the cells transfected with siRNA targeting CHOP was similar to that of the control cells (Fig. 2). Moreover, inhibition of CHOP expression had little or no effect on the high cadmium sensitivity caused by inhibition of FBXO6 expression (Fig. 2). Inhibition of CHOP expression has been reported to inhibit cadmium-induced cell death (Yokouchi *et al.*, 2007); however, the above result suggests that CHOP may not be involved in cadmium toxicity, at least in the HEK293 cells.

JNK, a kinase that phosphorylates c-Jun at Ser63 and Ser73, induces cell death following activation by stress, such as radiation, LPS, IL-1, osmotic pressure, and heat shock (Barr and Bogoyevitch, 2001). JNK has 3 isoforms, namely, JNK1, JNK2, and JNK3. JNK1 and JNK2 are widely distributed in the cells of the body, but JNK3 is mainly expressed in the nervous system and testicles (Bogoyevitch, 2006). JNK1 is associated with cell death, neurodegeneration, and production of inflammatory cytokines (Ham *et al.*, 2000; Liu and Rondinone, 2005). In order to investigate the role of JNK in the enhance-

ment of cadmium toxicity induced by inhibition of FBXO6 expression, we examined the effects of inhibition of FBXO6 expression and addition of cadmium on the phosphorylation level of c-Jun, a downstream factor of JNK. We found that c-Jun phosphorylation was enhanced by cadmium treatment and that the level of increase was further elevated by inhibition of FBXO6 expression (Fig. 3A). The c-Jun phosphorylation by cadmium observed in the control and FBXO6-ablated cells was almost entirely abolished by inhibition of JNK1 expression (Fig. 3B). Although JNK1 and JNK2 are expressed in HEK293 cells (McNeill *et al.*, 2004), the transfection of JNK1 siRNA in the HEK293 cells did not affect the mRNA levels of JNK2 (data not shown). This result suggests that c-Jun phosphorylation by cadmium in the HEK293 cells is mainly associated with JNK1. Next, we examined the relationship between the cadmium sensitivity of the HEK293 cells and JNK1, and found that the JNK1-ablated cells exhibited marked resistance to cadmium (Fig. 3). In addition, the level of high cadmium sensitivity

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Fig. 4. Effect of the knockdown of FBXO6 and/or JNK1 on the sensitivity of HEK293 cells to cadmium toxicity. HEK293 cells (5×10^3 cells/well) transfected with double-stranded FBXO6 siRNA and/or JNK1 siRNA were plated in 96-well plates and cultured in 100 μ L aliquots of medium. For further details, see the legend to Fig. 2A.

Fig. 5. FBXO6 knockdown increases the expression level of NHK and FLAG-TCR α after cadmium treatment. HEK293 cells (5×10^5 cells/well) transfected with FBXO6 or non-targeting siRNAs and plasmids encoding NHK and FLAG-TCR α were plated in 6-well plates. After incubation for 24 hr, cadmium chloride was added and the cells were cultured for a further 18 hr. The expression of NHK, FLAG-TCR α , CHOP, and GAPDH (loading control) was monitored by immunoblotting.

induced by inhibition of FBXO6 expression was markedly lower in the JNK1-ablated cells than in the control cells (Fig. 4). These results suggest that the ER stress-JNK1 pathway is involved in cadmium cytotoxicity and that FBXO6 can inhibit cadmium-induced ER stress, thereby alleviating cell death induced by the subsequent JNK1 activation.

Taken together, our results suggest that inhibition of FBXO6 expression decreases the function of the ERAD

system, thereby enhancing the induction of ER stress by cadmium. We therefore investigated the effect of cadmium on the cellular levels of Null Hong Kong variant α 1-antitrypsin (referred to herein as NHK) and the T-cell receptor α -subunit (TCR α), the substrate proteins degraded by the ERAD system, in FBXO6-ablated cells. Cadmium was found to increase expression of both the ERAD proteins in a dose-dependent manner (Fig. 5). Inhibition of FBXO6 expression did not affect the cellular lev-

els of NHK and TCR α , however, it facilitated cadmium-induced increase in the levels of these two proteins (Fig. 5). Therefore, it is possible that FBXO6 promotes ubiquitination of misfolded proteins induced by cadmium and promotes their rapid proteasome-dependent degradation, thereby inhibiting cadmium-induced ER stress.

In conclusion, the present study demonstrates that FBXO6 inhibits cadmium-induced ER stress and JNK1 activation. It is possible that cadmium induces ER stress by promoting the accumulation of misfolded proteins and that FBXO6, along with the ERAD system, inhibits the cadmium-induced ER stress by expediting the degradation of these misfolded proteins by the ubiquitin proteasome system.

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

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