Phosphorylation of eIF2α suppresses cisplatin-induced p53 activation and apoptosis by attenuating oxidative stress via ATF4-mediated HO-1 expression in human renal proximal tubular cells

SUNG-MIN JU1, YONG-SEOK JO1, YOO-MIN JEON1, HYUN-OCK PAE2, DAE-GILL KANG3, HO-SUB LEE3, JUN-SANG BAE4 and BYUNG-HUN JEON1,5

1Department of Pathology, College of Korean Medicine; 2Department of Microbiology and Immunology, School of Medicine; 3Hanbang Cardio-Renal Syndrome Research Center, Wonkwang University, Iksan, Jeonbuk 54538; 4Department of Pathology, Chonbuk National University Medical School, Research Institute of Clinical Medicine and Institute for Medical Sciences, Jeonju, Jeonbuk 54907; 5Research Center of Traditional Korean Medicine, Wonkwang University, Iksan, Jeonbuk 54538, Republic of Korea

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Abstract. Cisplatin is one of the most widely used chemotherapeutic agents for the treatment of human cancers. However, the nephrotoxicity of cisplatin limits its use as a therapeutic agent. It has been suggested that oxidative stress and p53 activation play important roles in cisplatin-induced nephrotoxicity. It has been demonstrated that the eukaryotic translation initiation factor 2α (eIF2α) may protect HK-2 human renal proximal tubular cells against cisplatin-induced apoptosis through inhibition of reactive oxygen species (ROS)-mediated p53 activation. The aim of the present study was to investigate the effects of siRNA-mediated knockdown of the PKR-like endoplasmic reticulum kinase (PERK) gene, which induces the phosphorylation of eIF2α, or Sal003, a selective inhibitor of eIF2α dephosphorylation, on cisplatin-induced apoptosis in HK-2 cells. Cisplatin induced eIF2α phosphorylation as well as p53 activation. In particular, inhibition of p53 by pifithrin-α, and upregulation of eIF2α phosphorylation by Sal003, reduced cisplatin-induced apoptosis. Of note, Sal003-mediated upregulation of eIF2α phosphorylation suppressed cisplatin-induced p53 activation. Furthermore, reduction of eIF2α phosphorylation by PERK knockdown enhanced cisplatin-induced p53 activation and apoptosis. In addition, the ROS scavenger N-acetyl-L-cysteine inhibited eIF2α phosphorylation as well as p53 activation in HK-2 cells treated with cisplatin, suggesting that oxidative stress induced by cisplatin may lead to apoptosis through p53 activation; furthermore, this stress may confer resistance to apoptosis via eIF2α phosphorylation, which was further supported by the finding that cisplatin-induced ROS generation was attenuated by Sal003, whereas it was enhanced by PERK knockdown. Furthermore, cisplatin induced the expression of activating transcription factor 4 (ATF4) and heme oxygenase-1 (HO-1) that were enhanced by Sal003 and reduced by PERK knockdown. Taken together, these results suggest that phosphorylation of eIF2α suppresses cisplatin-induced p53 activation and apoptosis by attenuating oxidative stress via ATF4-mediated HO-1 expression in HK-2 cells, as ATF4 expression is usually dependent on the phosphorylation of eIF2α and may also transcriptionally induce the expression of HO-1 in response to oxidative stress. Therefore, regulation of eIF2α phosphorylation may play an important role in alleviating cisplatin-induced nephrotoxicity.

Introduction

Cisplatin [cis-diaminedichloroplatinum(II)] is a potent chemotherapeutic agent, which is widely used for the treatment of human cancers (1,2). However, the clinical application of cisplatin is limited by nephrotoxicity, leading to acute renal failure (3-6). Cisplatin accumulates predominantly in the kidney, as this is the major route for its excretion (7). Therefore, cisplatin nephrotoxicity appears to be associated with its accumulation in the kidney.

Cisplatin nephrotoxicity is a complex multifactorial process, including oxidative stress, mitogen-activated protein kinases, p53 tumor suppressor and inflammation (8). In
particular, reactive oxygen species (ROS) and p53 have been shown to be important factors contributing to cisplatin nephrotoxicity. Cisplatin induces oxidative stress by generating ROS in renal cells, which leads to the activation of apoptotic pathways (9-11). p53 is activated during cisplatin nephrotoxicity and contributes to renal cell injury and death (12). Of note, p53 may be activated by ROS during cisplatin treatment of renal cells (13,14). Moreover, ROS scavengers and p53 inhibitors may prevent cisplatin-induced apoptosis by suppressing the activation of p53 in renal cells (13-15). This suggests that regulation of p53 activity may be an important target for alleviating cisplatin nephrotoxicity.

Cisplatin nephrotoxicity is also associated with endoplasmic reticulum (ER) stress (16). Pre-activation of ER stress alleviates cisplatin-induced nephrotoxicity in various renal cell lines (17). ER stress, which may be observed under physiological or pathological conditions (18), is caused by accumulation of unfolded proteins in the ER. This accumulation attenuates mRNA translation through activation of PKR-like ER kinase (PERK) and subsequent phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) (19). ER stress is involved in the stabilization of p53 through inhibiting ubiquitin-mediated degradation of p53. Qu et al have reported that ER stress prevents p53 stabilization and p53-mediated apoptosis upon DNA damage (20). Lee and Kim have also reported that ER stress-mediated eIF2α phosphorylation attenuates cell death by inhibiting p53 stabilization in statin-induced apoptosis (21). Thus, it is most likely that eIF2α phosphorylation plays an important role in the regulation of p53 expression or stability.

The aim of the present study was to investigate whether cisplatin induces p53-mediated apoptosis and ER stress-mediated eIF2α phosphorylation in human renal proximal tubular HK-2 cells. In addition, since eIF2α phosphorylation has been implicated in the stabilization of p53, which is due to p53 phosphorylation, the effect of ER stress-mediated eIF2α phosphorylation on p53-mediated apoptosis induced by cisplatin was investigated in HK-2 cells.

Materials and methods

Reagents and antibodies. Cisplatin, dimethyl sulfoxide (DMSO), 2,7'-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetyl-L-cysteine (NAC), propidium iodide (PI), protease inhibitor cocktail, ribonuclease A (RNase A) and anti-β-actin (sc-69879) antibody were all purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Control siRNA and PERK siRNA (h) were both purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Caspase-3 activity assay. The activity of caspase-3 was determined by the colorimetric MTT metabolic activity assay. HK-2 cells were seeded at a density of 5x10^4 cells/well in a 24-well plate. The cells were treated with different concentrations of cisplatin for 24 h. After treatment, 500 µl of MTT solution (0.5 mg/ml in serum-free medium) was added to each well. After incubation for 4 h at 37°C in the dark, the MTT-containing medium was removed by aspiration. The generated blue formazan product was dissolved by the addition of 200 µl 100% DMSO per well. The amount of formazan was determined at 570 nm using the SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage of viable cells was calculated as follows: Mean optical density (OD) of treated cells/mean OD of control cells x100.

Flow cytometric analysis. The percentage of apoptotic cells was quantitated by PI staining and flow cytometric analysis, where the sub-G1 peak represented apoptotic cells. The HK-2 cells exposed to cisplatin were harvested, washed with ice-cold phosphate-buffered saline (PBS; pH 7.4), and fixed in 1 ml of 70% ice-cold ethanol in PBS at 4°C for 1 h. After removing the ethanol by repeated washing in PBS, cells were resuspended in 1 ml of PI/RNase A solution (PBS with 10 µg/ml PI and 100 µg/ml RNase A) and incubated in the dark at 37°C for 1 h. The sub-G1 DNA contents were quantified using Fluorescence-Activated Cell Sorting Calibur and CellQuest Pro software (both from BD Biosciences, San Jose, CA, USA).

siRNA transfection. Transfection of siRNAs was performed using Lipofectamine 3000 reagent (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. HK-2 cells were seeded at a density of 2x10^5 cells in 60-mm dishes, cultured in complete
medium for 24 h, and transfected with control or PERK siRNAs. The siRNAs were diluted to 200 nM with 250 µl of Opti-MEM. Lipofectamine 3000 reagent was also diluted 1:50 in Opti-MEM. Diluted siRNAs and Lipofectamine 3000 reagent were mixed in equal volumes and incubated for 15 min at room temperature. The mixtures were added to culture dishes containing 2 ml fresh serum-free medium, and incubated at 37°C in a 5% CO₂ incubator for 24 h. Thereafter, complete medium was added, the cells were further incubated for 24 h and subjected to the experiments.

**ROS production assay.** The relative levels of ROS were determined by the DCFH-DA fluorescence assay. HK-2 cells were seeded into 96-well microplates (1x10⁴ cells/well) for 24 h and incubated in Hank's Balanced Salt Solution (HBSS; pH 7.4) containing 20 µM DCFH-DA at 37°C for 1 h. Following incubation, the cells were washed with HBSS and treated with 40 µM cisplatin under HBSS for the indicated time periods. The relative DCF fluorescence intensity was determined with an excitation wavelength of 485 nm and an emission wavelength of 538 nm using the SpectraMax M2 multi-mode microplate reader (Molecular Devices).

**Western blot analysis.** The cells were lysed in RIPA lysis buffer containing 1% halted protease and phosphatase inhibitor cocktails. Cell lysates were centrifuged at 20,000 x g for 15 min at 4°C, and the protein concentration was determined using a Bradford assay. Samples containing 50 µg of total protein were resolved by SDS-PAGE gel and transferred onto a nitrocellulose membrane for 3 h at 40 V. The membranes were blocked with Tris-buffered saline with Tween-20 (20 mM Tris-HCl, pH 7.6; 150 mM NaCl; and 0.05% Tween-20) containing 5% non-fat dry milk and probed with primary antibodies (all 1:1,000 in 3% BSA in Tris-buffered saline with 0.05% Tween-2) overnight at 4°C with gentle shaking. Protein spots were detected using horseradish peroxidase-conjugated secondary antibodies (all 1:2,000 in 3% BSA in Tris-buffered saline with 0.05% Tween-2). Immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then developed using the FluorChem E system (ProteinSimple, San Jose, CA, USA). The density of the bands was quantitated using Quantity One software, version 4.6.6 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Statistical analysis was performed using Microsoft Office Excel 2013 (Microsoft, Redmond, WA, USA) and data are expressed as means ± standard deviation. The statistically significant differences between two groups were calculated using the Student’s t-test. P-values <0.05 were considered to indicate statistically significant differences.

**Results**
Cisplatin induces apoptotic cell death in HK-2 cells. HK-2 cells were incubated with different concentrations of cisplatin for 24 h. Cell viability was assessed by the MTT assay. Apoptotic cells were determined using flow cytometry with PI staining. The viability of HK-2 cells markedly decreased with increasing concentrations of cisplatin (Fig. 1A). The percentage of apoptotic cells was significantly increased among cisplatin-treated cells (10, 20 and 40 µM cisplatin)
compared with the untreated cells (Fig. 1B). Caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage were determined by using caspase-3 colorimetric assay kit and western blot analysis. As shown in Fig. 1C and D, treatment of HK-2 cells with cisplatin significantly increased the activity of caspase-3 and the levels of cleaved active caspase-3. Similarly, cisplatin treatment increased proteolytic cleavage of PARP to form an 89-kDa fragment (Fig. 1D). These results indicate that cisplatin induced apoptotic cell death in HK-2 cells.

Cisplatin induces p53-mediated apoptosis in HK-2 cells. The tumor suppressor p53 plays an important role in regulating cisplatin-induced apoptosis of renal cells (13-15). Thus, we examined whether cisplatin could induce the activation of p53 via phosphorylation at serine 15 in HK-2 cells. HK-2 cells were treated with 40 µM cisplatin for different times, and the expression and phosphorylation status of p53 were then determined by western blot analysis. The levels of total and phosphorylated p53, which is directly associated with p53 activation, were increased in a time-dependent manner (Fig. 2A), whereas pifithrin-α, a specific p53 inhibitor, reduced p53 expression and phosphorylation in cells exposed to cisplatin (Fig. 2B). We next examined whether p53 activation was associated with cisplatin-induced apoptosis. HK-2 cells were treated with cisplatin in the absence or presence of pifithrin-α, and caspase-3 activation and PARP cleavage were then determined with the caspase-3 colorimetric assay kit and western blot analysis. As shown in Fig. 2C and D, cisplatin-induced activation of caspase-3 and cleavage of PARP were markedly inhibited by pifithrin-α. These results indicate that p53 activation is required for cisplatin-induced apoptosis in HK-2 cells.

Phosphorylation of eIF2α suppresses cisplatin-induced p53 activation and apoptosis in HK-2 cells. Lee and Kim reported that the stabilization of p53 is affected by ER stress-mediated eIF2α phosphorylation (21). p53 stability is associated with the phosphorylation of p53, which leads to stabilization of p53 by reducing its interaction with murine double minute 2, a negative regulator of p53 (22). It was first examined whether cisplatin was able to induce phosphorylation of eIF2α in HK-2 cells. The cells were treated with 40 µM cisplatin for the indicated times, and the phosphorylation status of eIF2α was then determined by western blot analysis. The levels of eIF2α phosphorylation were increased time-dependently (Fig. 3A). Changes in the expression and phosphorylation status of p53 according to phosphorylation or dephosphorylation of eIF2α were next examined in HK-2 cells exposed to cisplatin. The cells were pretreated with Sal003 to inhibit dephosphorylation of eIF2α, or transfected with siRNA against PERK to block PERK-eIF2α phosphorylation prior to cisplatin treatment. As shown in Fig. 3B, Sal003 enhanced cisplatin-induced phosphorylation of eIF2α, and reduced expression and phosphorylation of p53 in cisplatin-treated cells. Furthermore, the siRNA-mediated knockdown of PERK completely blocked cisplatin-induced phosphorylation of eIF2α, and promoted expression of p53 and its phosphorylation in cisplatin-treated cells. Finally, it was investigated whether eIF2α-regulated p53 activation could be associated with cisplatin-induced apoptosis in HK-2 cells. As shown in Fig. 4, cisplatin-induced activation...
Oxidative stress is involved in cisplatin-induced eIF2α p53 activation in HK-2 cells. 

of caspase-3 and cleavage of PARP were inhibited by Sal003, whereas they were promoted by PERK knockdown. These results indicate that phosphorylation of eIF2α may confer resistance to cisplatin-induced apoptosis, at least in part, via p53 activation in HK-2 cells.

Oxidative stress is involved in cisplatin-induced eIF2α phosphorylation and p53 activation in HK-2 cells. Similar to p53, ROS is also known to play an important role in cisplatin-induced nephrotoxicity (13-15). Oxidative stress induced by ROS production is involved in p53 activation in cisplatin-induced renal cell injury. Therefore, it was examined whether cisplatin induced the production of ROS in HK-2 cells and, if so, whether it contributed to p53 activation. First, HK-2 cells were treated with 40 µM cisplatin for the indicated times, and intercellular ROS production was then determined by measuring the fluorescence intensity of DCF. As shown in Fig. 5A, the levels of ROS production were significantly increased after 1 h of cisplatin treatment, and were maintained during prolonged cisplatin treatment. Previous studies have reported that N-acetylcysteine (NAC), a ROS scavenger, suppresses cisplatin-induced p53 activation in renal cells (13,15). Therefore, the effect of ROS scavenging on cisplatin-induced p53 activation was examined in HK-2 cells. The cells were treated with cisplatin in the absence or presence of NAC, a general antioxidant, and the levels of total and phosphorylated p53 were then determined by western blot analysis. The levels of p53 expression and phosphorylation were almost completely blocked by NAC in cisplatin-treated cells (Fig. 5B). Since eIF2α is phosphorylated mainly by PERK in response to oxidative stress, it was examined whether cisplatin-induced oxidative stress affects the phosphorylation of eIF2α in HK-2 cells. As shown in Fig. 5B, the blockade of ROS production by NAC completely abolished the phosphorylation of eIF2α in cells treated with cisplatin. Finally, changes in intracellular levels of ROS according to phosphorylation or dephosphorylation of eIF2α were examined in HK-2 cells exposed to cisplatin. As shown in Fig. 5C, cisplatin-induced ROS production was suppressed by Sal003, whereas it was promoted by PERK knockdown. These results indicate that oxidative stress may mediate eIF2α phosphorylation as well as p53 activation induced by cisplatin in HK-2 cells. In particular, phosphorylation of eIF2α has been shown to exert a scavenging effect on cisplatin-induced ROS production in HK-2 cells.

Phosphorylation of eIF2α induces ATF4 and HO-1 expression in cisplatin-treated HK-2 cells. Phosphorylation of eIF2α by PERK selectively enhances the translation of ATF4 that induces the expression of genes involved in antioxidant response and apoptosis regulation (23). HO-1, which is a redox-sensitive microsomal enzyme, is known to be cytoprotective through its potent antioxidant effect in cisplatin-induced acute renal injury (8). Of note, ATF4 is able to transcriptionally induce the expression of the antioxidant gene HO-1 in response to oxidative stress (24,25). Accordingly, it was investigated whether cisplatin was able to induce the expression of ATF4 and HO-1 in HK-2 cells. The cells were treated with 40 µM cisplatin for the indicated times, and the expression of ATF4 and HO-1 was then determined by western blot analysis. Cisplatin time-dependently induced the expression of both ATF4 and HO-1 (Fig. 6A). It was next examined whether differences in the phosphorylation status of eIF2α affected the expression levels of ATF4 and HO-1 in HK-2 cells exposed to cisplatin. As shown in Fig. 6B and C, cisplatin-induced ATF4 and HO-1 expression were elevated by Sal003 (Fig. 6B), while their expression was inhibited by PERK knockdown (Fig. 6C). These results indicate that phosphorylation of eIF2α attenuates cisplatin-induced oxidative stress through ATF4-mediated HO-1 expression in HK-2 cells.
Figure 4. Effects of eukaryotic translation initiation factor 2α (eIF2α) phosphorylation on cisplatin-induced apoptosis in HK-2 cells. (A) The cells were pretreated with or without 5 µM Sal003 for 1 h and then exposed to 40 µM cisplatin for 24 h. (B) Cells transfected with 200 nM control-siRNA or PKR-like endoplasmic reticulum kinase (PERK)-siRNA were incubated with or without 40 µM cisplatin for 24 h. Caspase-3 activity was measured using a caspase-3 colorimetric assay kit. Values are presented as means ± standard deviation, n=3. *P<0.05 vs. cells treated with cisplatin alone. The expression levels of cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP) were examined by western blot analysis using anti-cleaved caspase-3 (p20) and anti-PARP antibodies. The numbers below the bands indicate the relative density ratio of the respective protein normalized to the internal control (β-actin).

Figure 5. Effects of oxidative stress on cisplatin-induced eukaryotic translation initiation factor 2α (eIF2α) phosphorylation and p53 activation in HK-2 cells. (A) The cells were incubated with 40 µM cisplatin for the indicated times. Reactive oxygen species (ROS) production was measured with the DCFH-DA fluorescence assay. Values are presented as means ± standard deviation (SD), n=3. *P<0.05 vs. the 0 h timepoint. (B) The cells were pretreated with or without 5 mM N-acetyl-L-cysteine (NAC) for 1 h and then exposed to 40 µM cisplatin for 8 h. The phosphorylation and expression levels of eIF2α and p53 were examined by western blot analysis using anti-phospho-eIF2α (Ser51), anti-eIF2α, anti-phospho-p53 (Ser15) and anti-p53 antibodies. The numbers below the bands indicate the relative density ratio of the respective protein normalized to the internal control (β-actin). (C) The cells were pretreated with or without 5 µM Sal003 for 1 h and then exposed to 40 µM cisplatin for 4 h. (D) Cells transfected with 200 nM control-siRNA or PKR-like endoplasmic reticulum kinase (PERK)-siRNA were incubated with or without 40 µM cisplatin for 4 h. ROS production was measured with the DCFH-DA fluorescence assay. Values are presented as means ± SD, n=3. *P<0.05 vs. cells treated with cisplatin alone.
involved in eIF2α stress-mediated eIF2α phosphorylation in cisplatin-protected human renal proximal epithelial cells. Furthermore, cisplatin-induced ROS production was shown to activate p53-mediated apoptosis of HK-2 cells, resulting in protection against apoptosis.

**Discussion**

Nephrotoxicity is one of the main side effects that limit the therapeutic efficacy of the antineoplastic drug cisplatin (1-6). ROS and p53 have been found to be leading causes of cisplatin nephrotoxicity (8-15). Previous reported studies have demonstrated that cisplatin induces apoptotic cell death in HK-2 cells through ROS-mediated p53 activation. Oxidative stress may also lead to phosphorylation of eIF2α via PERK activation, which enhances the translation of ATF4 (31). The phosphorylation of eIF2α has been shown to be crucial for the selective translational induction of ATF4 during ER stress (32). In accordance with these findings, our results demonstrate that cisplatin-induced oxidative stress was involved in eIF2α phosphorylation, thus leading to increased ATF4 expression in HK-2 cells. It is noteworthy that ATF4 regulates the expression of several antioxidant genes in response to oxidative stress, which plays a central role in antioxidant responses (24). In particular, ATF4 may induce expression of the antioxidant gene HO-1 in response to oxidative stress (24,25). Dey et al. reported that ATF4 induces expression of HO-1 in response to matrix detachment-activated oxidative stress in human fibrosarcoma cells (24). He et al. also reported that ATF4 regulates basal and CdCl₂-induced expression of the HO-1 gene in a cell-specific manner (25). In the present study, cisplatin induced HO-1 expression, which was partially regulated by the eIF2α-ATF4 pathway in HK-2 cells. HO-1 has been shown to exert renoprotective effects through a potent antioxidant pathway in cisplatin-induced nephrotoxicity (8). Agarwal et al. reported that the early induction of HO-1 plays an important role in cytoprotection against cisplatin-induced acute kidney injury (33). Thus, it is most likely that the phosphorylation of eIF2α suppresses cisplatin-induced p53 activation by attenuating oxidative stress via the ATF4-mediated HO-1 expression in HK-2 cells, thereby resulting in protection against apoptosis.

In conclusion, cisplatin-induced oxidative stress leads to accumulation and activation of p53, leading to induction of apoptosis in HK-2 cells, while interfering with p53 activation through induction of eIF2α phosphorylation. Interference in p53 activation by eIF2α phosphorylation may be associated with expression of ATF4 and HO-1 in HK-2 cells exposed to cisplatin. In other words, the phosphorylation of eIF2α may inhibit cisplatin-induced p53 activation and apoptosis.
by attenuating oxidative stress via the ATF4-mediated HO-1 expression in HK-2 cells. Therefore, the phosphorylation of eIF2α may play an important role in protective strategies to alleviate cisplatin nephrotoxicity.

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