Deletion of p18^{INK4c} aggravates cisplatin-induced acute kidney injury

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Abstract. Protection of cyclin-dependent kinase inhibitors (CDKIs) has been demonstrated in acute kidney injury (AKI). However, previous studies on CDKIs have mainly focused on CIP/KIP family members, with INK4 family members rarely being investigated. This study investigated the behaviors of p18^{INK4c} (p18) in cisplatin-induced AKI using p18 gene knockout mice (p18-/-). AKI was induced in p18-/- and wild-type (p18+/+) mice after a single cisplatin (12.5 mg/kg) intraperitoneal injection. Protection by p18 was identified by a comparison of survival, renal function and morphological injuries between p18-/- and p18+/+ mice. Further investigation of endoplasmic reticulum stress (ERS) was performed by western blot analysis in p18-/- and p18+/+ kidneys at day 3 after cisplatin injection. The results revealed that after cisplatin injection, the survival of p18-/- mice was significantly shorter than that of p18+/+ mice, accompanied by aggravated renal function and more severe morphological injuries. Deletion of p18 also significantly aggravated ERS in cisplatin-induced AKI. In conclusion, p18 exerts protective effects on cisplatin-induced AKI, which may be associated with the effect of p18 on cell death pathways, such as the ERS pathway.

Introduction

Acute kidney injury (AKI) is a life-threatening condition with high morbidity and mortality, even in patients who have received medical intervention. As there is a lack of effective remedies for AKI other than dialysis, experimental efforts are being made to explore the pathogenesis of AKI and to seek materials with therapeutic potency. Cell cycle arrest is known to be beneficial for repairing of damaged DNA, thereby reducing the severity and teratogenicity of the injury (1). For this reason, cyclin-dependent kinase inhibitors (CDKIs) have been much investigated and have proven to possess cell protective properties in AKI (2-10). It is therefore believed that cell cycle regulation is a potential remedy for the treatment of AKI.

Based on sequence and the inhibitory effects they exert on cyclin-dependent kinase (CDK), the seven CDKIs are divided into two families. The CIP/KIP family includes p21, p27 and p57, which act with multiple CDK and extensively inhibit the cell cycle (11). The INK4 family includes p16, p15, p18 and p19, which only interact with CDK4/6 and specifically arrest the cell cycle in early G1 phase (12). CDKIs studies in AKI have mainly focused on the CIP/KIP family, especially p21, which has been identified as the protective factor in AKI (5-10). Compared to CIP/KIP family members, the role of INK4 members in AKI has yet to be determined. However, previous studies have reported that some novel additional biological functions are present in INK4 family members, such as p16 and p19. P16 controlled apoptosis induced by ultraviolet light and cisplatin through the intrinsic mitochondrial cell death pathway (13-14). Overexpression of p19 conferred resistance to cells exposed to UV irradiation (15-16).

Therefore, we hypothesized the beneficial behaviors of p18 and investigated its role in cisplatin-induced AKI using p18-/- mice. As oxidative stress is important factor in cisplatin-induced AKI, we also investigated the effect of p18 on cisplatin-induced endoplasmic reticulum stress (ERS) in an attempt to elucidate the possible mechanism involved in p18 actions.

Materials and methods

Animals. P18+/-mice in a C57BL/6 and 129/Sv background were kind gifts from Professor Tao Cheng of the laboratory of Cancer Research Center at Pittsburgh University. P18-/- or p18+/+ mice were generated from p18+/- breeding pairs. The mice were genotyped by a PCR approach, using tail DNA as previously described (17).

The primer sequences for genotype identification included: p18 WT forward, 5'-AGCCATCAAATTTATTCATGTTGC AGG-3'; p18 MG-47 reverse, 5'-CCTCCATCAGGCTAATG ACC-3'; and PGKNEO reverse, 5'-CCAGCCTCTGAGCCCAG AAAGCGAAGG-3'.

The detailed characteristics of the p18-/- mice were previously described by Franklin *et al* (18). Briefly, p18-/- mice grew

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and developed to become larger in body size than their p18+/+ littermates. Accordingly, the heart, liver and kidneys of the p18-/- mice exhibited proportional organomegaly; however, no abnormal structures, such as hepatic hypertrophy, glomerular sclerosis, diffuse kidney tubular atrophy, or dermal abnormalities, were detected in p18-/- mice.

Littermates or age-matched male mice (8-12 weeks) were used in our experiments. The animals were housed in a specific pathogen-free facility with access to water and food *ad libitum* at the Second Military Medical University Animal Center. All procedures were approved by the Ethics Committee of the Experimental Animals Center of the University.

Animal experiments. AKI was induced by a single intraperitoneal injection of cisplatin (Sigma, St. Louis, MO, USA) at a dose of 12.5 mg/kg in p18-/- (n=35) and p18+/+ (n=35) mice, while the controls (n=15) were injected with isovolumic saline.

After cisplatin injection, the 28-day survival of p18-/- (n=15) and p18+/+ (n=15) mice was determined, while the other animals were dealt with at day 3 after the injection. Blood samples were collected using the method of eye enucleation. Kidneys were collected after the animals were sacrificed by cervical dislocation. Kidneys and blood were collected at day 3 after cisplatin injection for morphological and renal function analysis. Serum creatinine (SCr) and urea nitrogen were determined by enzymatic colorimetric assay. Kidney tissues were stained with hematoxylin and eosin (H&E) and morphological assessment was determined under light microscopy by the same experienced histologist. Tubular necrosis, brush border loss and cast formation were used as the main damage parameters. Scoring was performed according to the percentage of damaged tubuli in the kidney as follows: I, 0-25%; II, 25-50%; III, 50-75%; IV, >75%.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) and analysis of ERS signal proteins by quantitative PCR (qPCR) and western blot analysis were performed at day 3 after cisplatin injection for the p18-/- and p18+/+ kidneys.

TUNEL. A commercial kit (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) was used to detect apoptotic cells for *in situ* kidneys. Briefly, paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. After being washed with PBS, the sections were treated with 0.5% pepsin at 37˚C for 8 min, and 0.3% Triton X-100 for 10 min at room temperature. To inactivate endogenous peroxidase, the sections were incubated in 3% H_2O_2 at 37˚C for 15 min and then incubated with terminal deoxynucleotidyl transferase (TdT) in a humid chamber at 37˚C for 1 h. The signals were detected with a horseradish peroxidase-conjugated sheep anti-alkaline phosphatase antibody. Quantitative measurement of apoptotic cells was performed by examining 10 randomly selected fields under a light microscope (magnification, x400) in the cortex. Twelve sections from at least six animals of each group were counted, and the data were presented as the mean number of apoptotic cells in each HPF field. Differences were considered statistically significant if P<0.05.

qPCR. Total RNA was extracted from kidney tissues (renal cortex) by means of the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and an RT kit (Takara Bio, Inc., Shiga, Japan) was used to synthesize cDNA. The expression of signal proteins in ERS was determined by qPCR using the ABI PRISM 7000 Sequence Detection System, and PCR reactions were performed using the SYBR-Green real-time PCR Master mix (Toyobo Co., Ltd., Osaka, Japan). The ribosomal gene 18S (18S rRNA) was selected as an endogenous reference and the samples were assayed in triplicate. Based on the analysis by the ΔΔCt method, the expression of the target genes was determined. The primer sequences used for qPCR were: 18S rRNA forward, 5'-AGGAGTGGGCCTGCGGC TTA-3' and reverse, 5'-GCCGGGTGAGGTTTCCCGTG-3'; Grp78 forward, 5'-AGACATTTGCCCCAGAAGAA-3' and reverse, 5'-ATCTTTGGTTGCTTGTCGCT-3'; Grp94 forward, 5'-TGAAGGAGAAGCAGGACAAAA-3' and reverse, 5'-AGTCGCTCAACAAAGGGAGA-3'; and CCAAT/enhancer-binding protein-homologous protein (CHOP) forward, 5'-TATCTCATCCCCAGGAAACG-3' and reverse, 5'-GGACGCAGGGTCAAGAGTAG-3'.

Western blot analysis. Protein was extracted from the renal cortex using a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50 mM sodium fluoride, 0.1% Nonidet P-40, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 μg/ml aprotinin. After a 30-min incubation on ice, the lysates were heated at 100˚C for 15 min and centrifuged at 12,000 x g for 15 min at 4˚C. Lysates containing equal amounts of proteins (100 μg) were dissolved in an SDS sample buffer, separated on 12% SDS slab gels and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes. Equal protein loading and protein transfer was confirmed by Ponceau S staining. After blocking with 5% non-fat dry milk in TBST, the membrane was incubated at 4˚C overnight with the following primary antibodies: mouse anti-GAPDH (1:5,000 dilution; Kangcheng Biotechnology, Shanghai, China), rabbit anti-p18 (1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-grp78 (1:1,000 dilution), rabbit anti-phosphorylation of pancreatic endoplasmic reticulum (ER) eukaryotic translation initiation factor 2α (eIF2α) kinase (PERK) (1:1,000 dilution), rabbit anti-phospho-PERK (1:1,000 dilution), rabbit anti-eIF2α (1:1,000 dilution) and rabbit anti-phospho-eIF2α (1:1,000 dilution) (all from Cell Signaling Technology, Beverly, MA, USA). After washing, a horseradish peroxidase-conjugated secondary antibody was applied. Proteins that bound to the secondary antibody were visualized using ECL (Amersham Pharmacia Biotech, Amersham, UK).

Statistical analysis. Data are presented as mean ± SD and were analyzed for significance using an ANOVA model. Comparisons between the two groups were made using the t-test or Wilcoxon-Mann-Whitney test. Differences were considered tatistically significant if P<0.05.

Results

Deletion of p18 aggravated cisplatin-induced AKI. As shown in Fig. 1, the 28-day survival of p18-/- mice was significantly worse than that of their p18+/+ counterparts. All 15 p18-/-

Table I. Histological assessment of p18-/- and p18+/+ mice at day 3 after cisplatin (12.5 mg/kg, i.p) injection.

Groups	No. of mice in each grade			
	I	II	III	IV
p18-/- (n=15)	0	2	6	7
p18+/+ (n=15)	0	4	8	3

For this assessment, 15 mice in each group were analyzed. The scoring was according to the proportion of damaged tubuli in the renal cortex, which was graded as follows: I, 0-25%; II, 25-50%; III, 50-75%; IV, >75%. Two slides for each mouse were randomly selected to determine the grade and all the slides were assessed by the same experienced histologist.

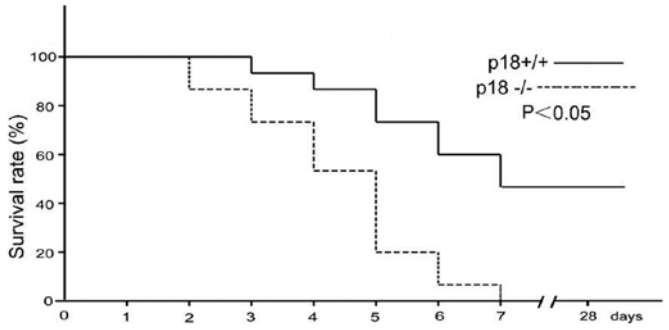

Figure 1. Deletion of p18 aggravated animals' survival after cisplatin injection. All 15 p18-/- mice died at day 7 after cisplatin injection, while 7-day survival rate for the p18+/+ mice was 53.3%, with no deaths occurring in the p18+/+ mice from day 8 to day 28. The 28-day survival of the p18-/- mice was significantly worse than that of the p18+/+ mice after the cisplatin injection (P<0.05 for the log-rank test).

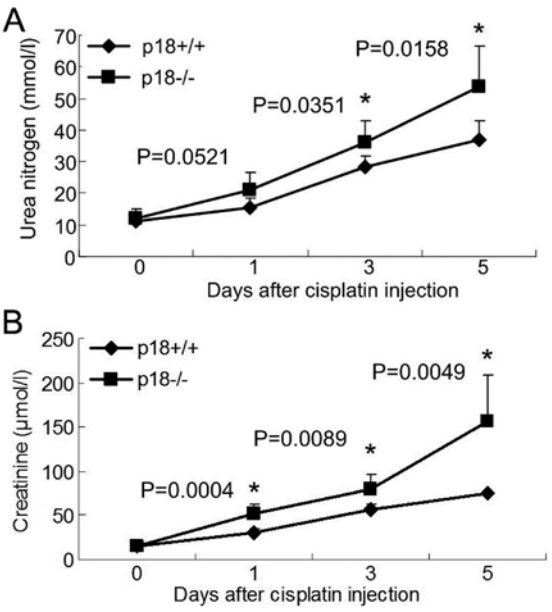

Figure 2. Deletion of p18 aggravated animal renal function after cisplatin injection. Compared to p18+/+ mice, (A) urea nitrogen and (B) creatinine were increased significantly in p18-/- mice after cisplatin injection. Six animals were sacrificed at each time point for renal function analysis. *P<0.05 for p18-/- mice vs. p18+/+ mice at various time points.

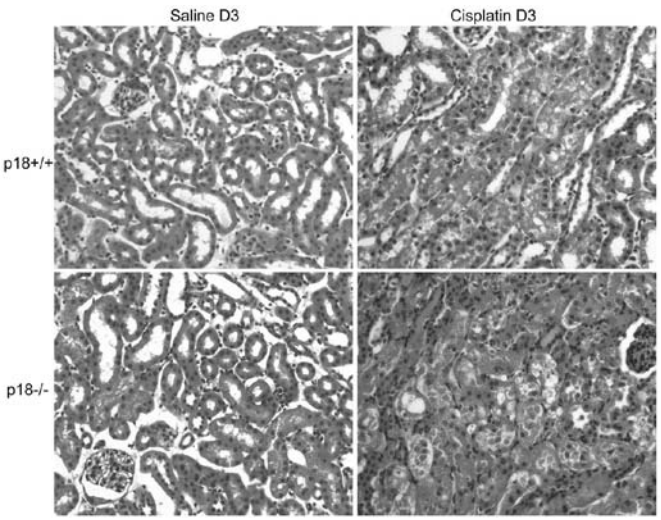

Figure 3. More severe lesions were present in p18-/- mice after cisplatin injection. No apparent difference existed in the kidney morphology between p18-/- and p18+/+ mice at day 3 after saline injection. However, more severe lesions and loss of tubular integrity were present in p18-/- mice at day 3 after cisplatin injection. Representative morphological images are shown [hematoxylin and eosin (H&E); magnification, x400].

mice died at day 7 after cisplatin injection, while the 7-day survival rate for the p18+/+ mice was 53.3%, with no deaths occurring in p18+/+ mice from day 8 to 28. A significant difference was observed between the survival curves of p18-/- and p18+/+ mice after cisplatin injection (P<0.05 for the log-rank test).

Compared to p18+/+ mice, aggravated urea nitrogen (Fig. 2A) and creatinine (Fig. 2B) of p18-/- mice was demonstrated at day 3 after cisplatin injection.

Aggravated morphological changes were present in p18-/- mice at day 3 after cisplatin injection as demonstrated by H&E staining (Fig. 3 and Table I) and TUNEL assessment (Fig. 4). A higher degree of kidney damage and a higher percentage of apoptotic cells were present in p18-/- kidneys as compared to p18+/+ kidneys at day 3 after cisplatin injection.

Deletion of p18 aggravated cisplatin-induced ERS. As shown in Fig. 5, the expression of molecular chaperones grp78 and grp94 mRNAs was upregulated in kidneys of animals with AKI at day 3 after cisplatin injection. However, compared to p18+/+ mice, the basal and inducible expression of grp78 and grp94 mRNAs was significantly higher in the p18-/- mice. Similar results were observed in the analysis of CHOP mRNA, a particular transcription factor activated by ERS.

Results were confirmed by western blot analysis (Fig. 6). The renal expression of grp78 protein was upregulated after cisplatin injection in p18-/- and p18+/+ mice. Compared to p18+/+ mice, the basal and inducible renal expression of grp78 protein was significantly higher in p18-/- mice.

As a rapid response to ERS, PERK was also analyzed by western blot analysis. The degree of PERK/eIF2α phosphory-

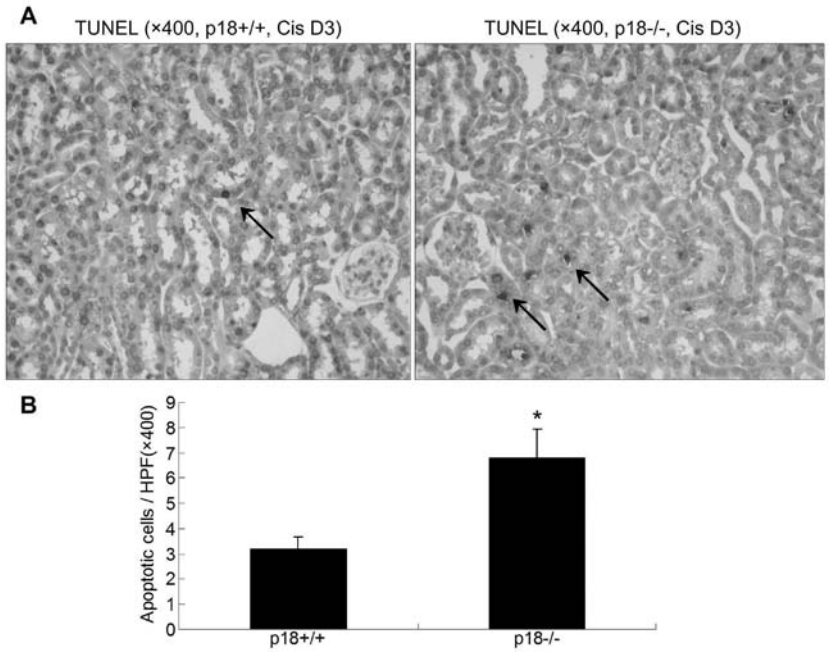

Figure 4. A higher percentage of apoptotic cells was present in p18-/- mice after cisplatin injection. (A) Compared to p18+/+ mice, more apoptotic cells were observed in kidneys of p18-/- mice at day 3 after cisplatin injection. Apoptotic cells are shown by arrows. (B) For statistical analysis, apoptotic cells were counted at 10 unrepeated cortical fields under light microscopy (HPF; magnification, x400), 12 sections were counted, which were obtained from at least six animals. Data are shown as the mean number of apoptotic cells in each HPF field. *P<0.05 for p18+/+ vs. p18-/- mice.

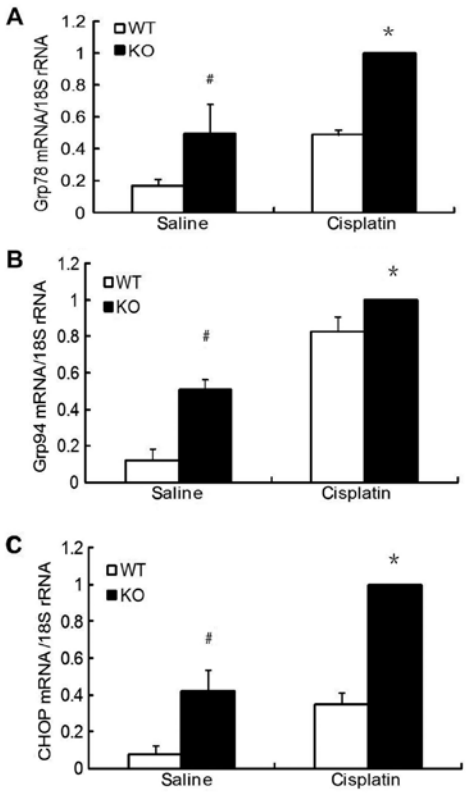

Figure 5. Deletion of p18 aggravated cisplatin-induced endoplasmic reticulum stress (ERS) demonstrated by the basal and inducible expression of grp78, grp94 and CCAAT/enhancer-binding protein-homologous protein (CHOP) mRNAs. Compared with saline injection, (A) grp78, (B) grp94 and (C) CHOP mRNAs were upregulated after cisplatin injection in p18+/+ (WT) and p18-/- (KO) mice. However, the basal and inducible expression of these target genes was significantly higher in p18-/- (KO) mice. Data are shown as mean ± SD. At least three independent experiments were performed for identification and statistical analysis. #P<0.05 for p18+/+ vs. p18-/- mice after saline injection; *P<0.05 for p18+/+ vs. p18-/- mice after cisplatin injection.

lation was higher in p18-/- mice as compared to that of p18+/+ mice after cisplatin injection (Fig. 7).

Discussion

Clinical use of cisplatin is largely limited due to drug resistance and nephrotoxicity (19-20). Since the mechanism by which cisplatin produces its nephrotoxic effect is similar to human AKI (21-22), AKI animals were administered cisplatin.

As the manifestation of cisplatin nephrotoxicity is apoptosis and/or necrosis, cell death pathways were involved in the mechanism of cisplatin nephrotoxicity. Previous studies have confirmed that in addition to the classical death-receptor and mitochondrial pathways (23-26), the ERS pathway is activated in cisplatin-induced kidney injury *in vitro* and *in vivo* (27-30). Therefore, the effect of p18 deletion on the ERS pathway was investigated to elucidate the actions of p18 in cisplatin-induced AKI. In ERS, the unfolded protein response (UPR) was identified and considered to interpret the mechanism of ERS-induced apoptosis (31-35). Three transmembrane proteins are activated in UPR: inositol-requiring enzyme-1 (IRE1), PERK and activating translation factor-6 (ATF6). In this study, upregulation of the molecular chaperones and CHOP and activation of the PERK/eIF2α pathway were analyzed to evaluate ERS severity in cisplatin-induced AKI.

It was found that p18 exerted protective actions in cisplatin-induced AKI. Compared to p18+/+ mice, p18-/- mice exhibited a higher degree of kidney damage, accompanied with aggravated renal function and worse survival after cisplatin injection. Deletion of p18 also aggravated cisplatin-induced ERS. Compared to p18+/+ mice, the basal and inducible expression of the molecular chaperones (grp78 and grp94) and transcription factor (CHOP) in kidney were significantly

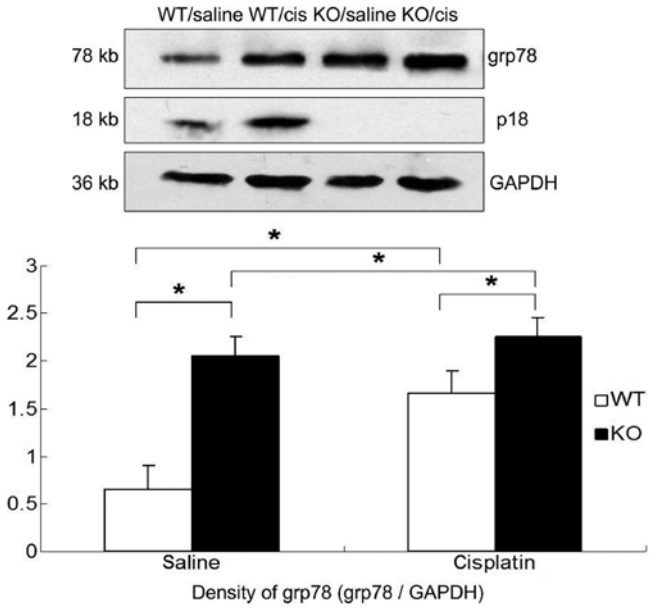

Figure 6. Deletion of p18 aggravated cisplatin-induced endoplasmic reticulum stress (ERS) demonstrated by the expression of grp78 protein. Compared with saline injection, grp78 protein was upregulated after cisplatin injection in the p18+/+ (WT) and p18-/- (KO) mice. However, the basal and inducible expressions of grp78 protein was significantly higher in p18-/- (KO) mice. Data are shown as mean ± SD. At least three independent experiments were performed for identification and statistical analysis. *P<0.05.

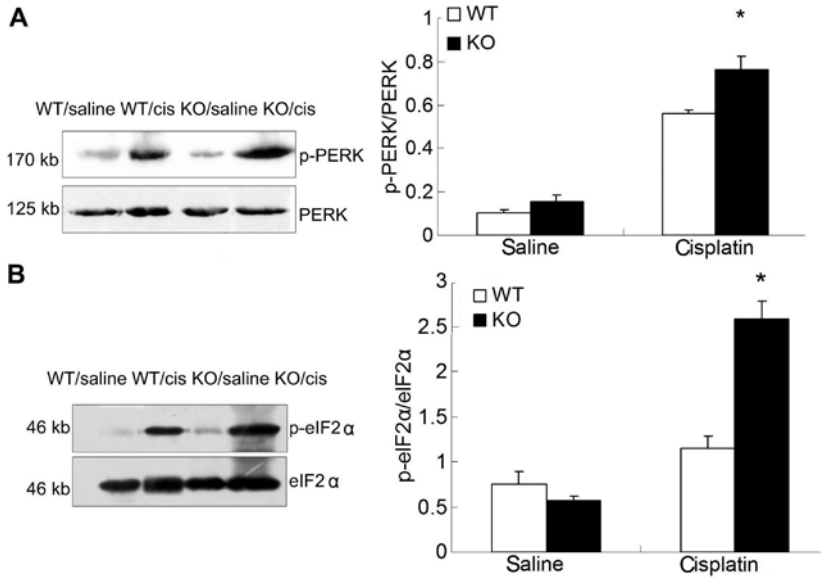

Figure 7. Deletion of p18 aggravated cisplatin-induced endoplasmic reticulum stress (ERS) demonstrated by the phosphorylation of phosphorylation of pancreatic endoplasmic reticulum (ER) eukaryotic translation initiation factor 2α kinase (eIF2α) (PERK)/eIF2α pathway. Compared with the saline injection, the phosphorylation of (A) PERK and (B) eIF2α was observed in p18+/+ (WT) and p18-/- (KO) mice after cisplatin injection. However, the degree of the phosphorylation was much higher in p18-/- (KO) kidneys compared to the p18+/+ (WT) kidneys. Data are shown as mean ± SD. At least three independent experiments were performed. *P<0.05 for p18+/+ vs. p18-/- mice after cisplatin injection.

higher in the p18-/- mice. The degree of PERK/eIF2α phosphorylation was also higher in p18-/- mice kidneys compared to p18+/+ mice after cisplatin injection. These results indicate that the effect of p18 on cell death pathways, such as the ERS pathway, may be the facet of its protective mechanism in cisplatin-induced AKI. However, other classical death pathways affected by p18 cannot be excluded as they were not the focus of our investigation.

P18, as a member of the INK4 family, is different from p21, whose protection in cisplatin-induced AKI has been demonstrated in previous studies (2-10). Protection of p21 occurs mainly due to the inhibitory effect on CDK2 activity, which has been demonstrated as an important factor in the promotion of apoptosis in cisplatin-induced AKI (36-37). However, the INK4 family members only interact with CDK4/6 and arrest the cell cycle in the early G1 phase, with no direct interaction with CDK2 (12,38). INK4 family members are also considered to be involved more in cell differentiation than CIP/KIP family members as they are often mutant or deleted in a number of tumors (39). No abnormality or defect exists

in p21 gene knockout mice, whereas p18 gene knockout mice acquire pituitary tumors with age (18,40). These limitations may be the possible reasons for INK4 members rarely being investigated in AKI. However, some studies have reported the involvement of INK4 family members in the cell response to genotoxic agents, such as p16 and p19 (13-16), as well as p18. These observations suggest that INK4 family members also exert protection and are involved in organ injury, despite the differences between the INK4 and CIP/KIP family members.

In conclusion, protection of p18 was demonstrated in cisplatin-induced AKI by using p18 gene knockout mice in this study. The main results of this study are the finding that p18 regulates cell death pathways, such as the ERS pathway, against cisplatin-induced AKI, although the exact signal transduction pathways connecting p18 to cell death pathways remain to be investigated in detail.

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