Cyclin-dependent kinase inhibitor p18^{INK4c} is involved in protective roles of heme oxygenase-1 in cisplatin-induced acute kidney injury

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Abstract. Experimental studies have demonstrated the protective effect of heme oxygenase (HO)-1 and cyclin-dependent kinase inhibitors (CDKIs) in acute kidney injury (AKI), and it has been documented that some of the protective effect of HO-1 is mediated by CDKIs. However, the role of p18^{INK4c} (p18), an inhibitor of CDK4 (INK4), which is a family member of CDKIs, has not been well characterized in kidney diseases. The aim of the present study was to demonstrate p18 protection from the relationship between p18 and HO-1 in cisplatin-induced AKI. Upregulation of p18 and HO-1 was demonstrated by quantitative polymerase chain reaction (qPCR) and western blotting in cisplatin-induced AKI *in vitro* and *in vivo*. The effect of HO-1 on p18 was determined by western blotting using the inducer and inhibitor of HO-1 *in vitro*. The potential effect of p18 on HO-1 in cisplatin-induced AKI was examined by p18 gene knockout mice *in vivo*. The results showed that p18 and HO-1 were upregulated in cisplatin-induced AKI *in vitro* and *in vivo*. Deletion of the p18 gene did not affect the basal and inducible expression of HO-1 in the AKI animals, while hemin (10 μM) and znpp (10 μM), the inducer and inhibitor, respectively, of HO-1, regulated p18 expression when incubated with the cells. The results indicated that p18 may play protective roles and may be associated with or partially account for the cytoprotective effects of HO-1 in cisplatin-induced AKI.

Introduction

Acute kidney injury (AKI) is a life-threatening condition with high morbidity and mortality, even in patients who have

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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 identify materials with therapeutic potency. It is known that the induction of heme oxygenase (HO)-1 potentially alters deleterious tissue damage (1,2), including kidney injury, and has therefore become a potential therapeutic target in the process of AKI.

HO catalyzes the initial and rate-limiting step of heme metabolism (3). This reaction consumes molecular oxygen and NADPH, requires cytochrome P-450 reductase activity (4) and generates carbon monoxide, Fe^{2+}, and biliverdin as products. Three HO isoforms have been identified in mammals: HO-1, -2 and -3. Without stimulation, HO-1 expression is extremely low or undetectable, while HO-2 is constitutively expressed, and HO-3 is considered to be a pseudogene. HO-1 gene expression is known to be highly induced as a protective response to a wide variety of stimuli (5-7) and involves many signaling pathways (8). The induction of HO-1 and its products has been shown to exert protective effects in AKI animal models, such as in rhabdomyolysis, ischemia-reperfusion injury, cisplatin-induced nephrotoxicity and radiographic contrast nephropathy (9-13). Nevertheless, the precise protective mechanism of HO-1 in AKI has not been completely defined. Results of previous studies have shown that the overexpression of HO-1 upregulates p21, a cell cycle inhibitor, and confers resistance to apoptosis in renal cells (14,15). As p21 has also been proven to be a mediator of the renal-protective effect in AKI animals (16-24), the upregulation of cell cycle inhibitors may serve as a partial interpretation of the protective mechanisms of HO-1 in AKI.

Previous studies examining cyclin-dependent kinase inhibitors (CDKIs) in AKI have focused mainly on the members of the cyclin inhibition protein/kinase inhibition protein (CIP/KIP) family (p21, p27 and p57), which restrain all phases of cell cycle progression (25). Another family of CDKIs, known as inhibitors of CDK4 (INK4) and comprising p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}, which arrest the cell cycle at the early phase of G1, have seldom been discussed (26). INK4 members were previously defined as tumor suppressors on the basis of their frequent mutations in many tumors. Novel functions of members of the INK4 family have gradually been

identified, including the results of a previous study showing that the overexpression of p18 in LLC-PK1 cells increased resistance to cisplatin-induced apoptosis by affecting the cell death pathway (27). Concerning the effect of HO-1 on p21, we hypothesized that the protective actions of p18 may be associated with HO-1 in cisplatin-induced AKI.

Materials and methods

Cell culture and experiments in vitro. Murine renal epithelial cell lines (TCMK-1, CCL139) were purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2.

TCMK-1 cell injury was induced by incubation of cisplatin at a final concentration of 60 μM in a standard medium as demonstrated by flow cytometry and western blot analysis. Cells were collected at 8, 16 and 24 h following exposure to cisplatin. To examine the effect of HO-1 on the expression of p18 in TCMK-1 cells, hemin (10 μM), an HO-1 inducer, was incubated with the cells in serum-free culture medium. Subsequently, zinc protoporphyrin (iron protoporphyrin IX, znpp, 10 μM), an HO-1 inhibitor, was added to the medium with cisplatin in a standard medium. The final concentration of hemin or znpp used in the present study was the same as that previously reported (14,15,28).

Animals and experiments in vivo. 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 (Pittsburgh, PA, USA). p18^-/- or p18^+/+ mice were generated from p18^+/- breeding pairs. The mice were genotyped by a quantitative polymerase chain reaction (qPCR) approach (primers described below), using tail DNA as previously described (29). The detailed characteristics of the p18^-/- mice were described in a study by Franklin *et al* (30). Briefly, p18^-/- mice grew 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, but 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. All the animals were housed in a specific pathogen-free facility with free access to water and food in the Second Military Medical University Animal Center (Shanghai, China). All procedures were approved by the Ethics Committee of Experimental Animals Cencer of the University.

The primers included p18 wild-type, forward: 5'-AGC CATCAAATTTATTCATGTTGCAGG-3' and, p18 MG-47, reverse: 5'-CCTCCATCAGGCTAATGACC-3', and PGKNEO, reverse: 5'-CCAGCCTCTGAGCCCAGAAAGCGAAGG-3'.

AKI was induced by a single intraperitoneal injection of cisplatin at a dose of 12.5 mg/kg in p18^-/- and the p18^+/+ mice. The kidneys were collected at 1, 3, 5 and 7 days following cisplatin injection. Kidney tissues were stained with hematoxylin and eosin (H&E), and the degree of morphological assessment was determined by light microscopy.

Flow cytometric analysis. Analysis was assayed using a Vybrant Apoptosis Assay kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. Briefly, floating and adherent cells were collected following cisplatin incubation, washed with PBS, and resuspended in binding buffer and Annexin V for 15 min in the dark. Then, 5 μg/ml propidium iodide (PI) was added to each tube, and the suspensions were analyzed by flow cytometry on the FACSCalibur. The staining allowed the distinction among living (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+).

Western blot analysis. Protein was extracted from the renal cortex or cultured cells 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^-1 leupeptin, 2 μg·ml^-1 pepstatin, and 1 μg·ml^-1 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 primary antibodies: rabbit anti-β-actin (1:1,000 dilution; CST, Beverly, MA, USA), rabbit anti-p18 (1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) rabbit anti-PARP (1:1,000 dilution; CST) and rabbit anti-HO-1 (1:500 dilution; Abcam, Cambridge, MA, USA). After washing, horseradish peroxidase-conjugated secondary antibody was applied. Proteins that bound to the secondary antibody were visualized using ECL (Amersham Biosciences, Piscataway, NJ, USA).

Real-time PCR. Total RNA was extracted from kidney tissues (renal cortex) or cultured cells by means of the TRIzol reagent (Invitrogen), and an RT kit (Takara Bio Inc., Otsu, Japan) was used to synthesize cDNA. p18 expression was determined by qPCR using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed using the SYBR-Green real-time PCR Master mix (QPK-201, Toyobo Co., Ltd., Osaka, Japan). The ribosomal gene 18S (18S rRNA) was selected as an endogenous reference and all the samples were assayed in triplicate. Based on the analysis by the $\Delta\Delta$Ct method, expression of the target gene at different time points was determined. The sequences of the primers used for the qPCR were: 18S rRNA forward: 5'-AGG AGTGGGCCTGCGGCTTA-3' and reverse: 5'-GCCGGGGTG AGGTTTCCCGTG-3'; p18 forward: 5'-AAGATGGAACTG GTTTTGCTGTCA-3' and reverse: 5'-GGCGGTGTCCCC CTTATGGTTC-3'; and HO-1 forward: 5'-CCCCTGCAGAGA CACCCCGA-3' and reverse: 5'-CAGCCCCTGGGGGGCC AGTAT-3'.

Statistical analysis. Data were presented as the mean ± SD and analyzed for significance using ANOVA. Comparisons between two groups were made using the t-test. $P<0.05$ was considered statistically significant.
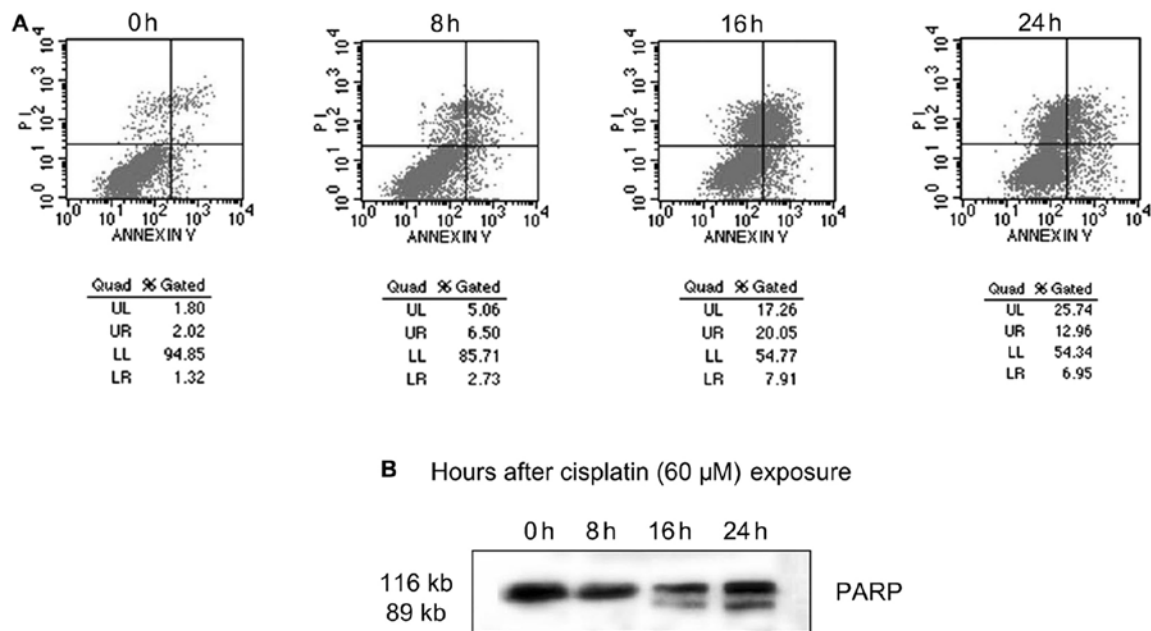
Figure 1. TCMK-1 cell injury was induced by cisplatin (60 μM) incubation as demonstrated by flow cytometry. (A) Apoptosis and necrosis in TCMK-1 cells were increased with extended incubation period, which was demonstrated by flow cytometry. The relative distribution (in percentage) within each population of early (LR), late apoptotic (UR) and necrotic (UL) cells is shown. (B) Apoptosis was confirmed by western blotting of PARP. PARP was cleaved with the extended incubation period, showing that TCMK-1 cell injury was induced by cisplatin (60 μM) incubation.

Results

TCMK-1 cell injury was induced by cisplatin (60 μM) incubation. TCMK-1 cell injury was induced by cisplatin (60 μM) incubation (Fig. 1). Results of the flow cytometric analysis revealed that the incubation induced apoptosis and necrosis, while an increase in apoptotic or necrotic cells was observed with the extended incubation period (Fig. 1A).

PARP, a 116-kDa nuclear poly (ADP-ribose) polymerase, is a highly conserved nuclear enzyme involved in DNA repair and in the apoptotic response of cells. It can be cleaved by many caspases *in vitro* and is one of the main cleavage targets of caspase-3 *in vivo*. As shown in Fig. 1B, PARP in TCMK-1 cells was cleaved by cisplatin (60 μM) incubation, resulting in the induction of apoptosis by the incubation.

AKI was induced by a single intraperitoneal administration of cisplatin at a dose of 12.5 mg/kg. AKI was induced by a single intraperitoneal injection of cisplatin at a dose of 12.5 mg/kg weight (Fig. 2). Tubular necrosis, brush border loss, cast formation, edema, tubular dilatation, and tubular degeneration were detected subsequent to cisplatin administration. It was evident that kidney damage was more severe on the third day as compared to the other time-points following cisplatin injection.

p18 and HO-1 were upregulated by cisplatin administration. In cisplatin-induced TCMK-1 cell or kidney injury, p18 and HO-1 were upregulated at the mRNA (Fig. 3) and protein levels (Figs. 4 and 5).

p18 mRNA expression was increased in a time-dependent manner, time to peak was 24 h *in vitro* (Fig. 3A) and 7 days *in vivo* (Fig. 3B), respectively, following cisplatin administration. The expression of p18 protein was also increased in a similar manner (Figs. 4A and 5A).

HO-1 expression was rapidly induced as is evident by the significant increase in the mRNA (Fig. 3C and D) and protein (Figs. 4B and 5B) expression at 8 h after cisplatin treatment *in vitro* and 3 days after cisplatin injection *in vivo*. Time to peak of HO-1 expression was earlier than that of p18. This result was similar to that reported previously whereby HO-1 expression was rapidly induced in AKI animals (13,31).

p18 was regulated by HO-1 in cisplatin-induced TCMK-1 cell injury. Hemin, the inducer of HO-1, upregulated the expression of HO-1 and p18 at 8 and 16 h, respectively, after its addition into the serum-free medium (Fig. 6A). Conversely, following the co-addition of cisplatin and Znpp to the cultured cells, the expression of HO-1 and p18 was significantly attenuated at 8 and 16 h, respectively, compared to cisplatin incubation alone (Fig. 6B). These data indicated that the expression of p18 was regulated by the inducible HO-1 in cisplatin-induced TCMK-1 cell injury.

Deletion of the p18 gene did not affect the basal and inducible expression of HO-1 in cisplatin-induced AKI. As mentioned above, p18 and HO-1 were upregulated by cisplatin injection compared to saline injection in p18^{+/+} (WT) kidneys. Additionally, HO-1 was upregulated as normal by cisplatin injection although p18 was not expressed in p18^{-/-} (KO) kidneys. There was no difference in the basal and inducible expression of HO-1 between p18^{+/+} and p18^{-/-} kidneys. Thus, deletion of the p18 gene did not affect the expression of HO-1 in cisplatin-induced AKI.

Discussion

Cell cycle regulation is the final result of and is involved in the processes of cell proliferation, hypertrophy and apoptosis
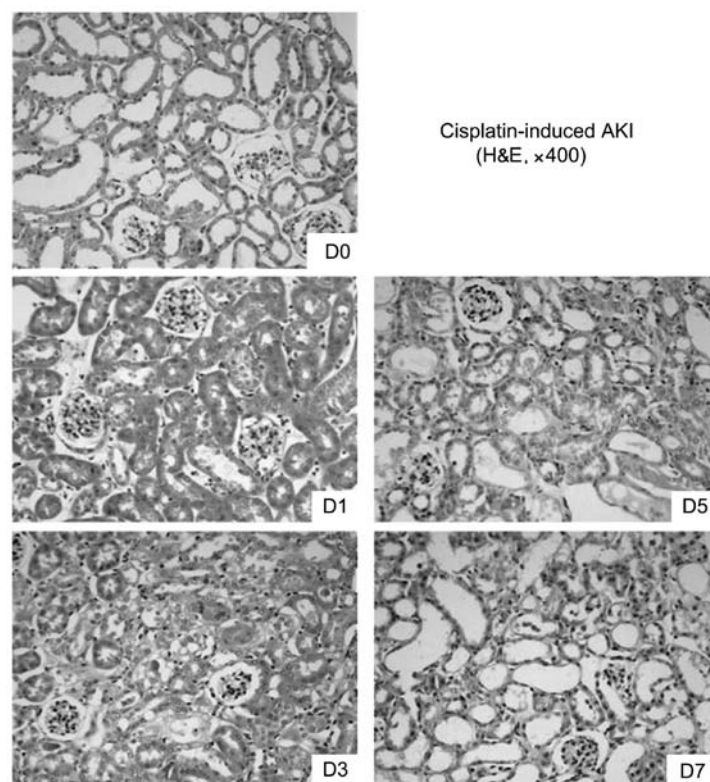
Figure 2. Acute kidney injury (AKI) was induced by a single intraperitoneal injection of cisplatin at a dose of 12.5 mg/kg weight as demonstrated by hematoxylin and eosin (H&E) staining. Tubular necrosis, brush border loss, cast formation, edema, tubular dilatation, and tubular degeneration were detected in kidney tissues following cisplatin injection (12.5 mg/kg, IP). Kidney damage was more severe on the third day than the other time-points following the injection (H&E; magnification, x400).
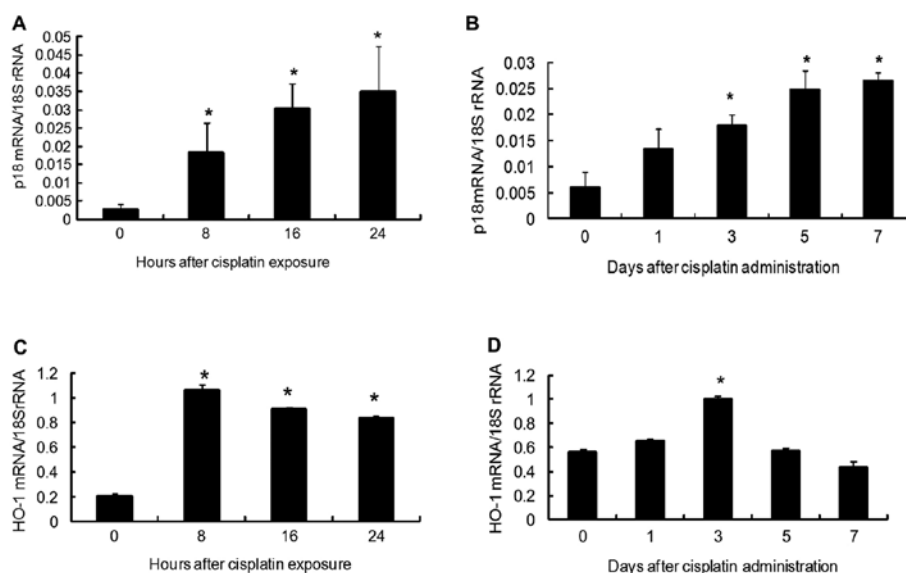

Figure 3. Expression of p18 and heme oxygenase (HO)-1 was upregulated at the mRNA level in cisplatin-induced renal cell injury or acute kidney injury (AKI). (A) In cisplatin-induced TCMK-1 cell injury, p18 mRNA expression was upregulated in a time-dependent manner, reaching to the peak at 24 h after cisplatin incubation; (C) HO-1 mRNA expression was rapidly induced, peaking at 8 h after the incubation. (B) In AKI mice, renal p18 mRNA expression was upregulated in a time-dependent manner, reaching to the peak at day 7 following cisplatin injection; (D) HO-1 mRNA expression was rapidly induced and peaking earlier than that of p18 on day 3 following the injection. Panels were shown as the mean ± SD, and at least three independent experiments were performed for each target gene. $^{*}P<0.05$, compared to t=0 h/day 0.

during the course of organ injury (32). In all of these processes, cell cycle arrest is beneficial for the repair of damaged DNA and thus reduces the severity and teratogenicity of the injury (33). For this reason, CDKIs have been much investi-gated and have proven to be cell protective in many kidney diseases, including in the setting of AKI. Of all seven CDKIs, p21 has been discussed and identified as the protective factor in AKI (16-24). Compared to CIP/KIP family members, INK4
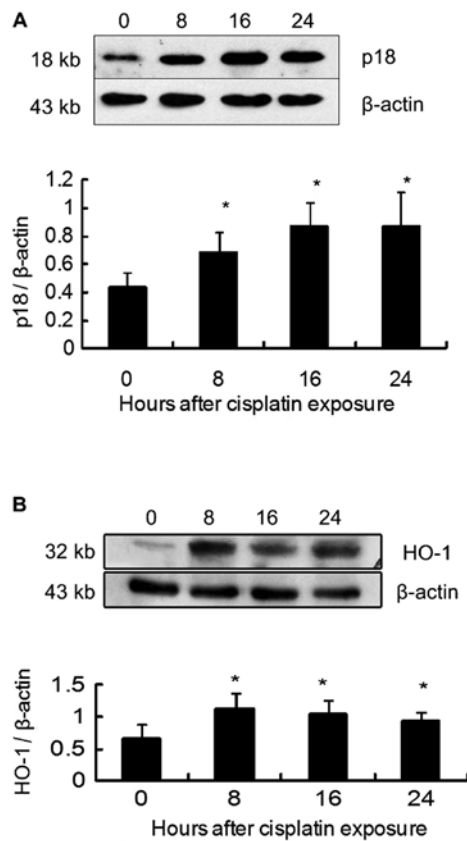
Figure 4. Expression of p18 and heme oxygenase (HO)-1 was upregulated at the protein level in cisplatin-induced renal cell injury. (A) Expression of p18 protein was upregulated in a time-dependent manner and reached to the peak at 24 h after cisplatin incubation with TCMK-1 cells, while (B) the expression of HO-1 protein was induced and peaked earlier than that of p18 at 8 h after the incubation. Data are shown as the mean ± SD. The data are from at least three independent experiments. *P<0.05, compared to t=0 h.
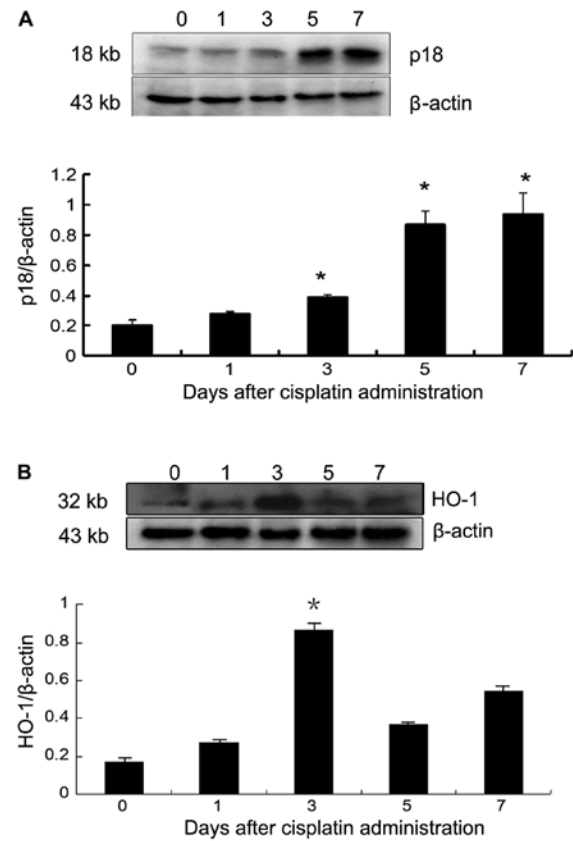
Figure 5. Expression of p18 and heme oxygenase (HO)-1 was upregulated at the protein level in cisplatin-induced acute kidney injury (AKI). (A) Expression of p18 protein was upregulated in a time-dependent manner and reached to the peak at day 7 following cisplatin injection, while (B) the expression of HO-1 protein was induced and reached the peak earlier than that of p18 at day 3 following the injection. Data are shown as the mean ± SD. The data are from at least three independent experiments. *P<0.05, compared to the t=0 day group.
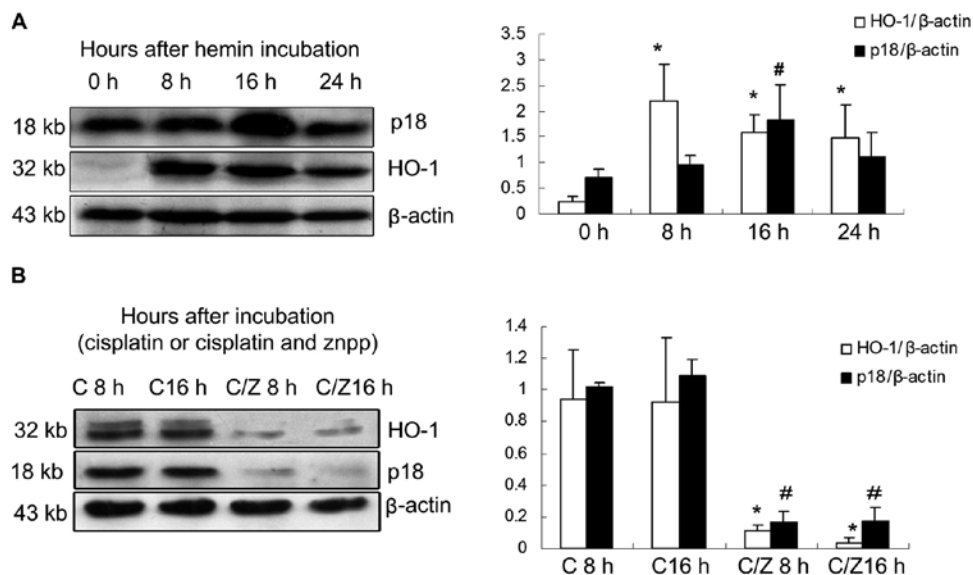

Figure 6. p18 expression was regulated by heme oxygenase (HO)-1 in cisplatin-induced renal cell injury. (A) HO-1 expression was rapidly induced at 8 h after hemin (10 μM) was incubated with the cultured cells in a serum-free medium, while p18 expression was significantly upregulated in cultured cells at 16 h after the addition of hemin to the medium. Data are shown as the mean ± SD. The data are from at least three independent experiments. *P<0.05 for expression of HO-1 at 8, 16 and 24 h vs. 0 h. #P<0.05 for expression of p18 at 16 vs. 0 h. (B) Cisplatin-induced p18 upregulation was almost completely inhibited when znpp (10 μM, an inhibitor of HO-1) and cisplatin (60 μM) were co-incubated in cultured cells. Data are shown as the mean ± SD. The data are from at least three independent experiments. *P<0.05 for expression of HO-1 in cisplatin and znpp co-incubation (C/Z) 8 and 16 h groups vs. incubation with cisplatin alone C8 and C16 h groups. #P<0.05 for expression of p18 in C/Z 8 and 16 h groups vs. C8 and C16 h groups.
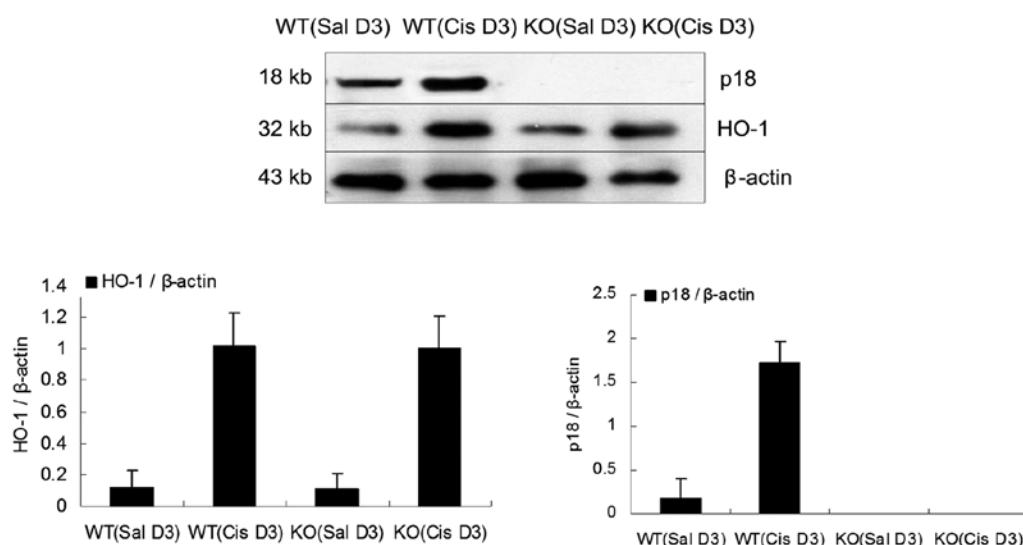
Figure 7. Deletion of the p18 gene did not affect the basal and inducible heme oxygenase (HO)-1 expression in cisplatin-induced acute kidney injury (AKI). p18 and HO-1 were upregulated by cisplatin injection compared to saline injection in p18$^{+/+}$ (WT) kidneys, and HO-1 was also upregulated as normal by cisplatin injection although the expression of p18 was lacking in p18$^{-/-}$ (KO) kidneys. There was no difference in the basal and inducible expression of HO-1 between p18$^{+/+}$ and p18$^{-/-}$ kidneys. Data are shown as the mean ± SD. The data are from at least three independent experiments.

members only inhibit the activity of cyclin D/CDK complexes and arrest the cell cycle at G1 early phase. As regulators of the G1- to S-phase transition, INK4 family members should exert a protective role in AKI. Results of a recent study have confirmed the hypothesis that some novel additional biological functions are also present in INK4 family members, such as the effect on DNA damage and apoptosis (34).

HO-1, another protective factor in AKI, has been demonstrated to exert a protective role in several previous studies (9-13,35). The renoprotective mechanism in AKI of several drugs or compounds, such as sulforaphane, isoflavone and the statins, has been identified as HO-1 induction (36-39). As an important enzyme in degrading heme to attenuate the overall production of reactive oxygen species (ROS) (40-43), the protective effect of HO-1 in AKI animals is possibly connected with heme degradation products, which have been demonstrated to possess cytoprotective properties of vaso-dilatation and attenuation of oxidative stress, apoptosis and inflammation in several disease events (44-46). However, the precise protective mechanism of HO-1 remains unclear.

In the present study, we investigated the relationship of p18 and HO-1 in cisplatin-induced renal cell injury and AKI. p18 expression was apparently upregulated by cisplatin adminis-tration, and as mentioned above, HO-1 was also induced in cisplatin-induced AKI and TCMK-1 cell injury. In accor-dance with the effect of HO-1 on p21 reported by a previous study (14), the expression of p18 was also regulated by HO-1 in cisplatin-induced TCMK-1 cell injury. In serum-free cultured cells, hemin, a chemical HO-1 inducer, upregulated the expression of p18, while znpp, an inhibitor of HO-1, almost completely inhibited p18 upregulation when co-incubated with cisplatin. On the other hand, we observed the possible effects of p18 on the expression of HO-1. These effects showed that deletion of the p18 gene did not affect the basal and induc-ible expression of HO-1 in the animals when either cisplatin or saline treatment was used. Results of this study therefore

confirm the hypothesis that p18 exerts some protective actions in cisplatin-induced AKI, which is associated with HO-1 or may partially account for the cytoprotective effects of HO-1 in cisplatin-induced AKI.

Notably, the results showed that p18, a member of the INK4 family, may be crucial in cisplatin-induced AKI, since it was regulated by HO-1, an important protective factor in AKI. Thus, it would be more appropriate to demonstrate the rela-tionship between HO-1 and p18 by regulating HO-1 expression by means of transfection or RNA interference. However, the transfection efficiency of TCMK-1 cells was not sufficient in our preliminary experiments, analyzed by β-gal staining. Therefore, as an alternative approach, we used the chemical inducer and inhibitor to manipulate the expression levels of HO-1, which proved to be feasible in this study.

The limitations of this study include that we did not inves-tigate further how HO-1 regulates the expression of p18 in cisplatin-induced AKI and observe the effect of HO-1 on p18 in cisplatin-induced AKI animals. Another limitation was that we had to use the murine kidney epithelial cell line (TCMK-1) as an alternative option as we did not have a sufficient amount of the murine tubular cell line.

In conclusion, this study reported a novel role of p18 in cisplatin-induced AKI, and found that the expression of p18 was regulated by HO-1 through undefined signaling pathways. However, the role played by the protective mechanisms of p18 in cisplatin-induced AKI and how HO-1 regulates p18 in cispl-atin-induced TCMK-1 cells injury remain to be determined. We believe that findings of this study have broadened the roles of cell cycle regulatory proteins and the results are beneficial for current investigations on AKI.

Acknowledgements

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