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MEK inhibitor, U0126, attenuates cisplatin-induced renal injury by decreasing inflammation and apoptosis

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MEK inhibitor, U0126, attenuates cisplatin-induced renal injury by decreasing inflammation and apoptosis.

Background. Although inflammation and apoptosis are known to play important roles in cisplatin nephrotoxicity, the exact intracellular signaling mechanisms are not well understood. Recent reports that extracellular signal-regulated kinase (ERK1/2) pathway mediates cisplatin-induced caspase activation and apoptosis in cultured renal tubular cells led us to investigate the effect of MAPK/ERK kinase (MEK) inhibitor, an immediate upstream of ERK1/2 in cisplatin-induced acute renal failure (ARF) in mice.

Methods. The effect of MEK/ERK1/2 inhibition on kidney tumor necrosis factor-α (TNF-α(gene expression, inflammation, the activation of tissue caspases, and apoptosis were examined in addition to its effects on renal function and histology in cisplatin-induced ARF in mice.

Results. Pretreatment of MEK inhibitor, U0126, decreased ERK1/2 phosphorylation following cisplatin administration with significant functional and histologic protection. This beneficial effect was accompanied by decrease in TNF-α gene expression level and inflammation, as well as in caspase 3 activity and apoptosis.

Conclusion. These data provide evidence that ERK1/2 pathway functions as an upstream signal for TNF-α–mediated inflammation and caspase 3–mediated apoptosis in cisplatin-induced ARF in mice and suggest that ERK1/2 can be a novel therapeutic target in cisplatin nephrotoxicity.

Cisplatin is one of the most effective chemotherapeutic agents used in the treatment of a variety of human solid tumors. However, dose-related nephrotoxicity frequently limits the use of optimal dose of this drug [1, 2]. Primary targets of cisplatin in kidney are proximal straight and distal convoluted tubules, where it accumulates and promotes cellular damage, and multiple mechanisms, including oxidative stress, DNA damage, apoptosis, and recently inflammation have been implicated to contribute to the pathogenesis of cisplatin-induced renal injury [3–12].

In particular, recent observations that tumor necrosis factor-α (TNF-α) increased following cisplatin administration and inhibition of its production markedly attenuated renal damage indicate that TNF-α, an inflammatory cytokine, plays an important role in cisplatin-induced renal injury, but precise intracellular signaling mechanisms leading to increased TNF-α production still remain unknown [6].

Caspases are a family of cell death proteases involved in the initiation and execution phase of apoptosis and several in vitro studies have demonstrated that cisplatin activated multiple caspases, including caspase 8, 9, and 3, leading to apoptotic cell death in cultured renal tubular cell [13]. However, detailed pattern of activation in vivo nor intracellular signaling mechanisms of caspase activation by cisplatin have not been fully examined.

Proteins comprising the mitogen-activated protein kinase (MAPK) family are important mediators of signal transduction processes that serve to regulate diverse cellular responses to extracellular stimuli. Of the three major mammalian MAPK superfamily, the extracelluar signal-regulated kinases (ERK) pathway has been known to play a role in cell growth and differentiation, giving survival advantage to cells, but there is growing evidence suggesting that the activation of ERK1/2 also contributes to cell death [14–20]. Cisplatin can activate multiple signaling pathways, including c-JUN N-terminal kinase (JNK), ERK, and p38 in cultured tubular cells but there is no in vivo data and their specific role in mediating renal dysfunction remains unknown.

Recent several reports demonstrating that the inhibition of ERK1/2 pathway reduces the infarct volume together with down-regulation of interleukin-1β (IL-1β) expression in focal cerebral ischemia animal model, and also reduces cisplatin-induced caspase 3 activation with resultant decrease in apoptosis in cultured renal tubular cells prompted us to investigate the effect of ERK1/2 inhibition on cisplatin-induced renal injury in vivo [18, 20]. Because the increase of TNF-α production and caspase

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activation are thought to be important in the pathogenesis of cisplatin-induced renal injury, we examined the effect of ERK1/2 inhibition on their tissue expression, as well as on inflammation and apoptosis in cisplatin-induced acute renal failure (ARF) in mice. We found that inhibiting ERK1/2 pathway reduced TNF-α expression and caspase activation in kidney tissue, as well as providing functional and histologic protection in cisplatin-induced renal injury in mice. Phosphorylated ERK1/2 were mainly localized in distal tubules and collecting ducts. These results indicate that ERK1/2 pathway is an upstream signal for TNF-α production and caspase activation in cisplatin-induced renal injury and targeting ERK1/2 pathway can be a novel therapeutic strategy for the prevention of cisplatin nephrotoxicity.

METHODS

Animals and drugs

Male balb/c mice (20 to 25 g) were purchased from Orient (Charles River Korea, Seoul, Korea) and had free access to water and chow before manipulation. Animal care followed the criteria of animal care committee of Korea University for the care and the use of laboratory animal in research. Cisplatin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in normal saline at a concentration of 2 mg/mL and mice were given a single intraperitoneal injection of 20 mg/kg cisplatin and control animals received same volume of normal saline. One hour before cisplatin administration, either vehicle [0.1% dimethyl sulfoxide (DMSO) in normal saline] or U0126 (A.G. Scientific, Inc., San Diego, CA, USA) (10 mg/kg, in 0.1% DMSO) was injected intravenously via tail vein. Mice were sacrificed at 24, 48, and 72 hours after cisplatin administration. Blood was collected by intracardiac puncture and both kidneys were processed for histology, RNA, and protein isolation, respectively.

Biochemical analysis

Blood urea nitrogen (BUN) and creatinine levels were measured using Hitachi 747 automatic analyzer.

Histologic examination

Paraformaldehyde (4%) fixed and paraffin-embedded kidney tissues were stained with periodic acid-Schiff (PAS) or naphthol AS-D chloroacetate esterase (Sigma Aldrich). Histologic changes in outer medulla were evaluated semiquantitatively. Briefly, tubular damage was estimated in 8 to 10 high power fields (HPF) (×200) per section by using scoring system based on the percentage of damaged tubules per field (1, < 25%; 2, 25% to 50%; 3, 50% to 75%; and 4, > 75%). The mean score of each animal was compared. Esterase-positive leukocyte infiltration was measured by counting 8 to 10 HPF (×200) per section. For immunohistochemical detection of phospho-ERK1/2, kidney tissue sections were treated with 1:100 rabbit antiphospho ERK1/2 polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA) and then incubated with goat antirabbit HRP for 1 hour at room temperature. The sections were developed in stable diaminobenzidine (DAB) and counterstained with Mayer hematoxylin.

Detection of apoptosis

Detection of apoptotic cells in kidney was performed on paraffin-embedded kidney tissue sections using a ApopTag Plus (Intergen, Purchase, NY, USA), following the manufacturer's protocol. The number of apoptotic cells in outer medulla was semiquantitatively measured by counting 8 to 10 HPF (× 200) per section.

Western blot analysis

Kidney tissues were homogenized in phosphate-buffered saline (PBS) with protease inhibitor cocktail (Calbiochem, San Diego, CA, USA) and the protein concentration was quantitated (BCA protein assay reagent) (Pierce, Rockford, IL, USA). Samples of protein (30 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Western blot analysis was performed using antibody for phopho-ERK1/2, phospho-JNK, phospho-p38 (New England Biolabs, Beverly, MA, USA), and blots were striped and reprobed with antibody for total ERK1/2, total JNK, and total p38 (New England Biolabs).

Quantitation of TNF-α mRNA by real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated by using TRIzol reagent (Life Technology, Rockville, MD, USA) according to the manufacturer's protocol. After precipitation by isopropyl alcohol, total RNA was subjected to further purification using RNeasy minikit (Qiagen, Valencia, CA, USA). One microgram of total RNA was reverse transcribed in a reaction volume of 50 μL using Superscript II reverse transcriptase and random primers. The real-time PCR was run in triplicate on the iCycler System (Bio-Rad, Hercules, CA, USA) with using gene specific primer and probe sets provided as a Pre-Developed TaqMan® Assay Reagents (PDAR) (Applied Biosystem Inc., Foster City, CA, USA), following the manufacturer's protocol. Amplification condition was 40 cycles of 95°C, 15 seconds, and 60°C, 1 minute. The abundance of TNF-α gene expression was normalized to the expression of 18S (Taqman® Ribosomal Control Reagent) (Applied Biosystem Inc.) and was expressed as fold differences relative to saline-treated control animals.

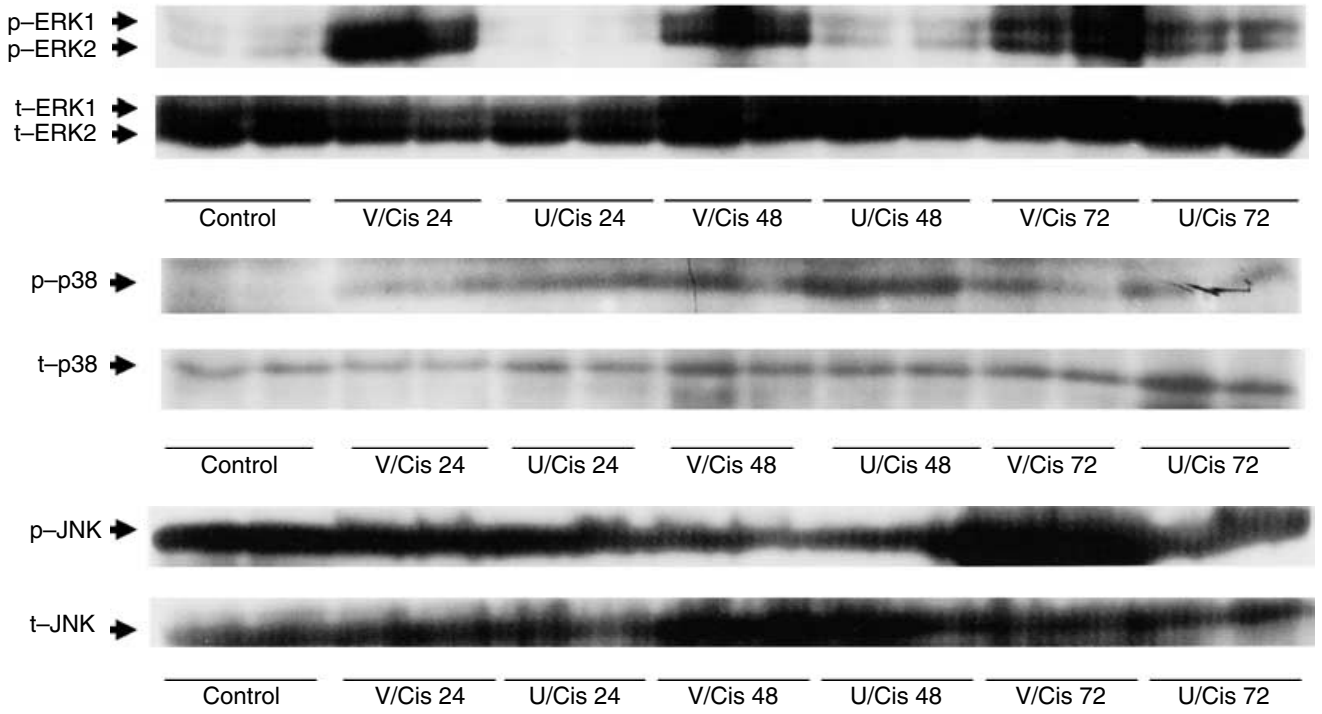

Fig. 1. Effect of U0126 on phosphorylation of ERK1/2, p38, and JNK in cisplatin-induced ARF in mice. Mice were either injected with saline (control), or 20 mg/kg cisplatin (Cis) intraperitoneally. Vehicle (V) [0.1% dimethyl sulfoxide (DMSO) in normal saline] or U0126 (U) (10 mg/kg in 0.1% DMSO in normal saline) were injected 1 hour before cisplatin administration and sacrified at 24, 48, and 72 hours. Western blots for phospho-ERK1/2, p38, and JNK were performed and then the blots were striped and reprobed with antibody for total ERK1/2, p38, and JNK.

Measurement of caspase activity

The activities of caspase 3, 8, and 9 in kidney tissue were determined by fluorometric detection of free 7-amino-4-trifluoromethylcoumarin (AFC) (caspase 3 and 8) or 7-amino-4-methylcoumarin (AMC) (caspase 9), following the manufacturer's protocol (ApoAlert^{TM} Caspase Fluorescent Assay Kits) (BD Bioscience, Palo Alto, CA, USA) by using Synergy^{TM} HT Multi-Detection Microplate Reader (Biotek, Woburn, MA, USA). Briefly, kidney tissues were homogenized in 1 mL of lysis buffer, incubated on ice for 10 minutes and centrifuged at 15,000 $\times$ g for 10 minutes at 4°C. Supernatant containing 500 μg of protein was incubated for 1 hour at 37°C in the presence of reaction buffer, 1 mmol/L dithiothreitol (DTT) and 50 μmol/L AMC or AFC substrate conjugates. The fluorescence was read at 400/505 (excitation/emission) nm for caspase 3 and 8 and 380/460 nm for caspase 9 and the samples were run in triplicates. The activity of caspases was expressed as percent increase compared to saline-treated control group and samples which has caspase inhibitors served as negative controls.

Statistical analysis

All data presented as mean $\pm$ SE and were analyzed by Student t test. P value less than 0.05 was considered statistically significant.

RESULTS

ERK1/2 phosphorylation increased in kidney tissue following cisplatin administration

First, we examined the changes in phosphorylation of ERK1/2 in cisplatin-induced ARF. ERK1/2 phosphorylation increased from 24 hours after cisplatin administration and persisted until 72 hours (Fig. 1). To make sure the specificity of U0126 in inhibiting ERK1/2, we also did Western blot analysis of phospho-JNK and p38 and found that there were no inhibitory effects of U0126 on phosphorylation of JNK, p38 despite increased phosphorylation by cisplatin. In immunohistochemistry, phosphoylated ERK was detected in occasional collecting duct cells in control kidneys, but not in glomeruli or proximal tubules. Following cisplatin administration, phospho-ERK expression markedly increased mainly in distal tubules and collecting ducts (Fig. 2). The staining was mostly in cytosol, but a few cells showed strong positive nuclear staining.

Effect of U0126 on renal damage following cisplatin administration

To clarify the role of ERK1/2 pathway in cisplatin-induced ARF, we injected a specific MEK1/2 inhibitor, U0126, 1 hour before cisplatin administration. Renal

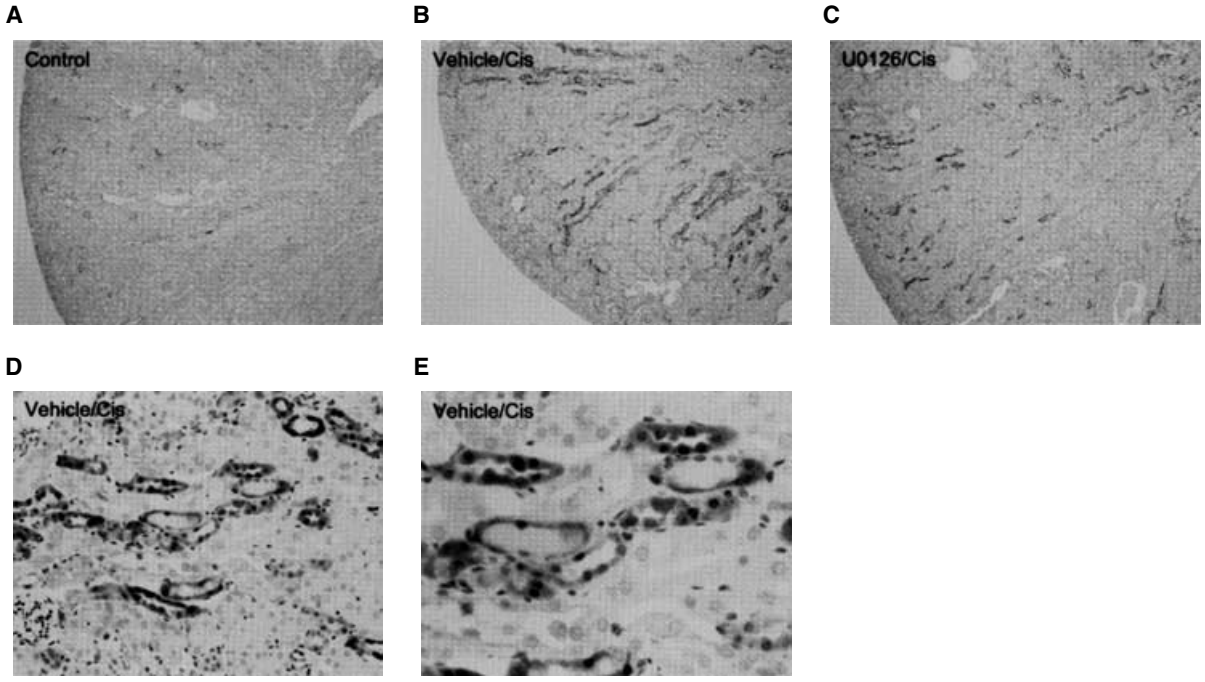

Fig. 2. Immunohistochemical localization of phospho-ERK1/2 in kidneys from control, vehicle + cisplatin, and U0126 + cisplatin-treated mice. Mice were either injected with saline (control), or 20 mg/kg cisplatin (Cis) intraperitoneally. Vehicle [0.1% dimethyl sulfoxide (DMSO) in normal saline] or U0126 (10 mg/kg in 0.1% DMSO in normal saline) were administered 1 hour before cisplatin administration. Immunohistochemistry for phospho-ERK1/2 was done at 72 hours. (*A*) Control. (*B*, *D*, and *E*) Vehicle + cisplatin. (*C*) U0126 + cisplatin [magnification (A to C) ×40, (D) ×100, (E) ×200].

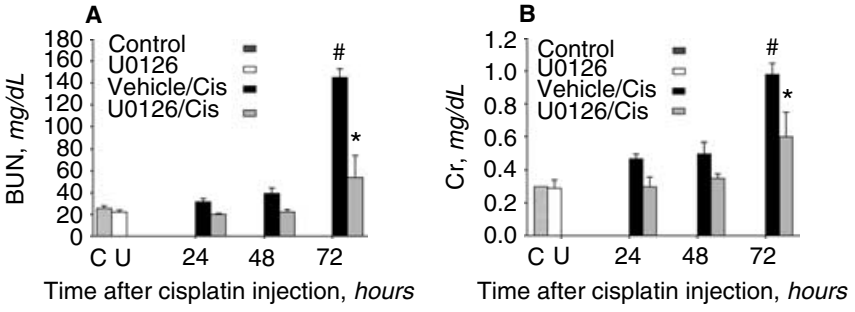

Fig. 3. Effect of U0126 on renal function in cisplatin-induced ARF in mice. Mice were either injected with saline (control), or 20 mg/kg cisplatin (Cis) intraperitoneally. Vehicle [0.1% dimethyl sulfoxide (DMSO) in normal saline] or U0126 (10 mg/kg in 0.1% DMSO in normal saline) were intravenously injected 1 hour before cisplatin administration and sacrificed at 24, 48, and 72 hours. Animals injected intravenously with U0126 (U) alone served as U0126 control (C). Data were presented as mean ± SE ($N = 5$ animals per group). #$P < 0.05$ compared to control; *$P < 0.05$ compared to vehicle + cisplatin.

function was measured at 24, 48, and 72 hours and histologic changes were compared at 72 hours. U0126 itself had no effect on renal function (Fig. 1). U0126 pretreatment reduced the phosphorylation of ERK1/2 at 24, 48, and 72 hours (Fig. 1), and these changes were accompanied by marked functional protection (Fig. 3). The improvement in renal function was also reflected in less severe histologic damage at 72 hours (Fig. 4). Cisplatin caused loss of brush border, necrosis of tubular cells, cast formation, and these changes were significantly attenuated in U0126 pretreated animals. Semiquantitative assessment of histologic damage also showed a significant beneficial effect of U0126 in cisplatin-induced ARF (Fig. 4).

Effect of U0126 on TNF-α mRNA expression and leukocytes infiltration

Because there have been several reports suggesting that TNF-α play an important pathogenetic role in cisplatin nephrotoxicity, we examined the effect of U0126 on TNF-α gene expression by real-time quantitative RT-PCR. Like other studies, TNF-α mRNA expression markedly increased following cisplatin administration. The up-regulation was evident from 24 hours and persisted until 72 hours and U0126 almost completely abolished TNF-α abundance at each time point (Fig. 5). Since TNF-α has been known to incite an inflammatory reaction, we performed naphthol-AS-D chloroacetate staining and counted the number of esterase positive

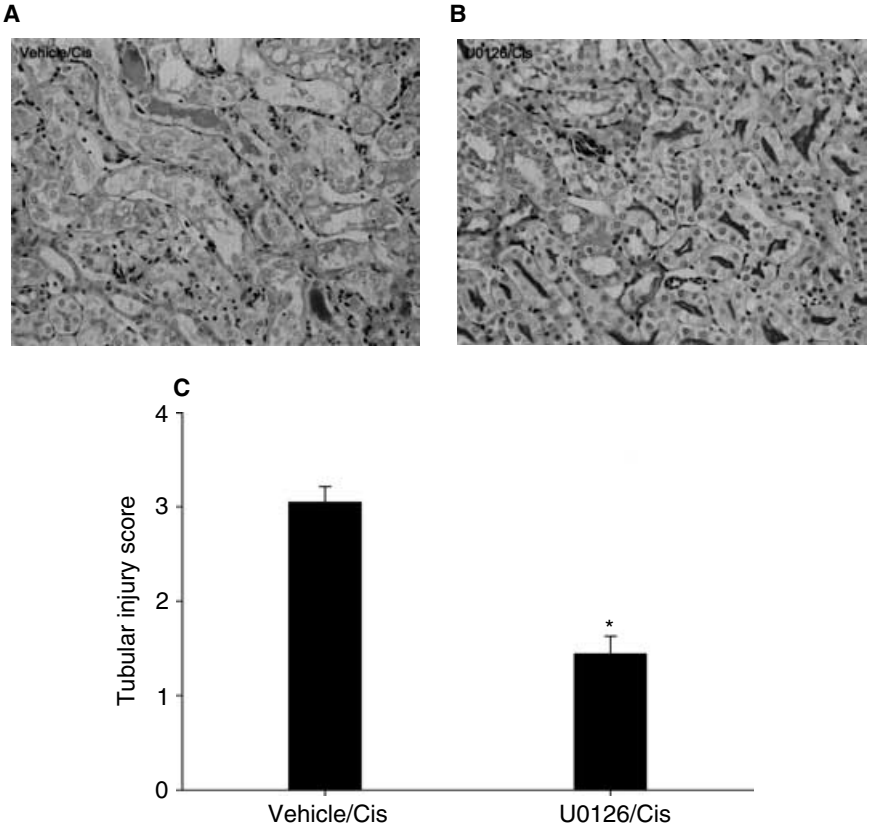

Fig. 4. Effect of U0126 on renal histology in cisplatin-induced ARF in mice. Mice were injected intravenously with either vehicle or U0126 (10 mg/kg) 1 hour before cisplatin (Cis) administration intraperitoneally. Renal histology was examined at 72 hours. Semiquantitative assessment of renal damage was scored as described in the **Methods** section. (*A*) Vehicle + cisplatin, (*B*) U0126 + cisplatin, (*C*) Semiquantitative histologic injury score. (A and B) Periodic acid-Schiff (PAS) × 200 ($N = 4$ animals per group). $^*P < 0.05$ compared to vehicle + cisplatin.

leukocytes in kidney tissue. Esterase positive leukocytes infiltration increased markedly at 48 and 72 hours in outer medulla and U0126 pretreatment also significantly reduced the leukocytes infiltration at each time point (Fig. 6).

Effect of U0126 on caspase activation and apoptosis

Caspases are cysteine proteases responsible for apoptotic cell death and can be activated by many different stimuli. Caspases can be divided into an initiator and executioner caspases. Generally, the activation of initiator caspases, like caspase 8 or 9 lead to the activation of executioner caspases like caspase 3 via different cell death pathway. Although renal tubular cell apoptosis through caspase activation by cisplatin has been demonstrated in several in vitro studies, there has been no in vivo data showing the activation of caspase following cisplatin administration. Therefore, we examined various caspase activations following cisplatin administration and also the effect of U0126 pretreatment on their activities. The activation of caspase 3, an executioner caspase increased markedly at 24 hours after cisplatin, peaked at 48 hours and started to decrease at 72 hours. U0126 pretreatment significantly decreased the activation at each time point (Fig. 7). The activities of caspase 8 and 9 also increased slightly, but much lesser degree than that of caspase 3

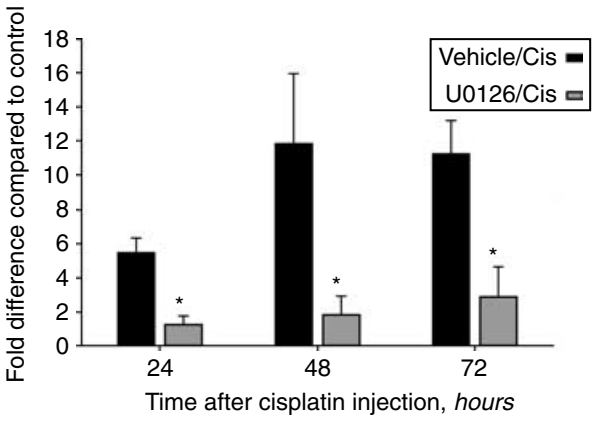

Fig. 5. Effect of U0126 on tumor necrosis factor-α (TNF-α) mRNA expression in cisplatin-induced acute renal failure (ARF) in mice. TNF-α mRNA expression was measured at 24, 48, and 72 hours after injection of vehicle + cisplatin or U0126 + cisplatin by real-time reverse transcription-polymerase chain reaction (RT-PCR). In each experiment, the level was normalized to the expression of 18 S and were expressed as fold differences relative to saline-treated control animals. Data are presented as mean ± SE ($N = 4$ animals per group). $^*P < 0.05$ compared to vehicle + cisplatin.

and did not precede the activation of caspase 3, indicating that the activation of caspase 3 is independent of caspase 8 or 9 in this model (Fig. 7). Morphologically, terminal deoxynucleotidyl transferase-mediated uridine triphosphate (dUTP) nick-end labeling (TUNEL)-positive cells

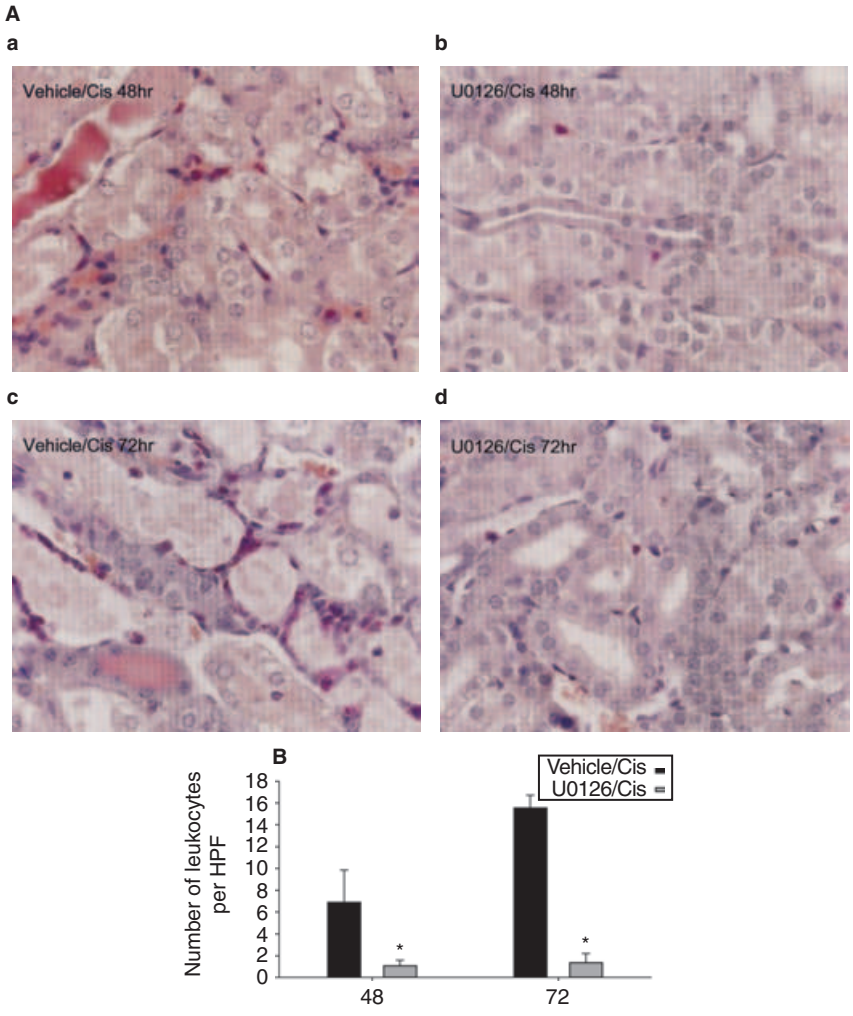

Fig. 6. Effect of U0126 on leukocycytes infilatration in cisplatin-induced ARF in mice. Eight to ten × 200 fields were counted and mean numbers of esterase-positivie leukoyctes were compared. (*A*) Panels A and C are vehicle + cisplatin. Panels B and D are U0126 + cisplatin, Panels A and B are at 48 hours and panels C and D at 72 hours (naphthol AS-D chloroacetate esterase staining ×400). (*B*) Mean numbers of leukocytes ($N = 4$ animals per group). $^*P < 0.05$ compared to vehicle + cisplatin.

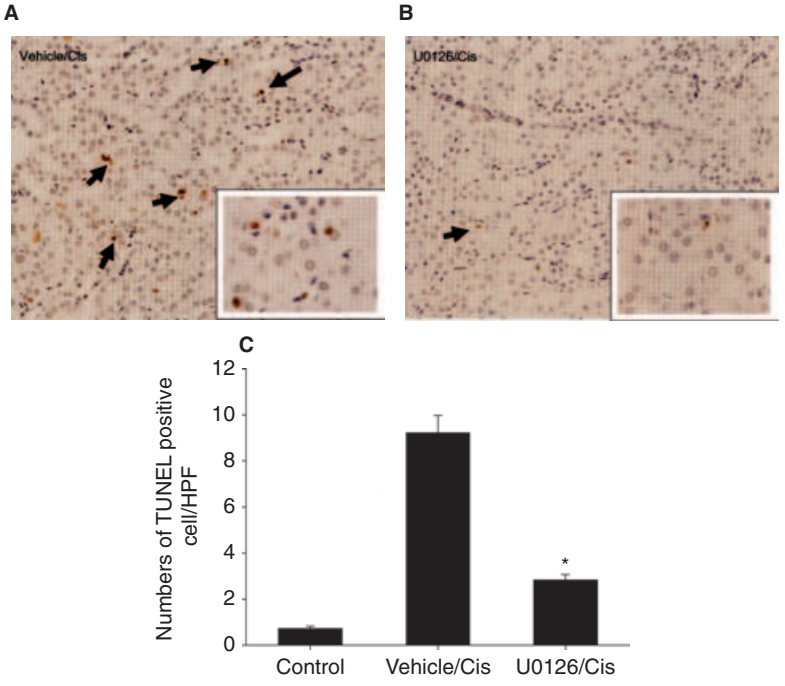

Fig. 8. Effect of U0126 on apoptosis in cisplatin-induced ARF in mice. Mice were injected intravenously with either vehicle or U0126 (10 mg/kg) 1 hour before cisplatin administration intraperitoneally. Detection of apoptosis was done at 48 hours. (*A*) Vehicle + cisplatin, (*B*) U0126 + cisplatin, (*C*) Mean numbers of terminal deoxynucleotidyl transferase-mediated uridine triphosphate (dUTP) nick-end labeling (TUNEL)-positive cells were counted in eight to ten high power fields (A and B) TUNEL stain ×100, insets ×400, arrows TUNEL-positive apoptotic cells ($N = 4$ animals per group). $^*P < 0.05$ compared to vehicle + cisplatin.

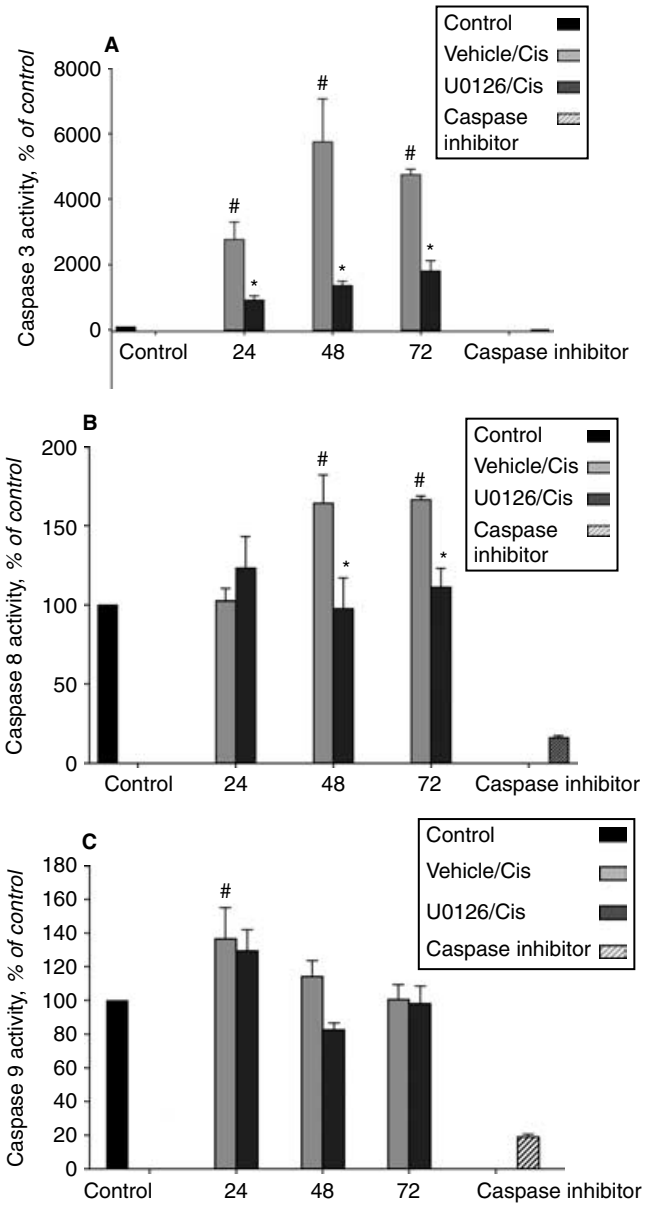

Fig. 7. Effect of U0126 on the activation of caspases in cisplatin-induced acute renal failure (ARF) in mice. Tissue caspase activities were measured at 24, 48, and 72 hours and were expressed as percent increase compared to saline-treated control group. (*A*) Caspase 3 activity. (*B*) Caspase 8 activity. (*C*) Caspase 9 activity ($N = 4$ animals per group). #$P < 0.05$ compared to control; *$P < 0.05$ compared to vehicle + cisplatin (Cis).

increased in ciplatin treated animals and U0126 pretreatment also significantly decreased apoptosis (Fig. 8).

DISCUSSION

In the present study, we demonstrated that (*1*) systemic administration of a selective MEK inhibitor, U0126, significantly attenuated cisplatin-induced renal injury in mice; and (*2*) inhibiting ERK1/2 phosphorylation dramatically reduced both inflammatory, apoptotic pathways of renal injury in cisplatin-induced ARF in mice.

Members of MAPK family proteins play important roles in regulating diverse cellular responses to a variety of extracellular stimuli. One of the most extensively characterized MAPK families are ERK1/2 and has been known to be associated with cell growth and differentiation, but recent several reports suggest that ERK1/2 pathway can also be related to inflammation, apoptosis, and cell injury [14, 15, 17, 18, 21, 22]. In particular, Nowak [20] demonstrated that inhibiting ERK1/2 phosphorylation reduced cisplatin-induced caspase 3 activation and apoptosis in cultured renal proximal tubular cells, suggesting ERK1/2 pathway can be a therapeutic target in cisplatin-induced renal dysfunction. However, because most of our understanding of how MAPK signaling promotes cell injury is based on cell culture studies, we have little knowledge of how ERK1/2 pathway regulates cellular responses to extracellular stimuli in vivo. We therefore first examined the ERK1/2 phosphorylation following cisplatin administration in mice. ERK1/2 phosphorylation increased from 24 hours and continued to increase until 72 hours after cisplatin administration. U0126 pretreatment 1 hour before cisplatin reduced the phosphorylation of ERK1/2 and this inhibition was accompanied by marked functionional and histologic protection at 72 hours. This novel finding is in contrast with previous report by di Mari, Davis, and Safirstein [21] suggesting that ERK is critical in renal tubular cell survival during oxidative injury. The fact that ERK1/2 activation can provide cell death or survival signal suggests that ERK1/2 can activate different intracellular pathways that regulate cell fate in response to different kinds of cell stresses.

The exclusive localization of phospho-ERK in distal nephron is somewhat unexpected, because the primary target of cisplatin is known to be proximal straight tubule and most of in vitro experiments have used proximal tubular cells for the study of cisplatin nephrotoxicity. But distal nephron injury-like mitochondrial swelling, apoptosis, and frank necrosis also have been described and magnesium wasting commonly seen in human cisplatin nephrotoxicity also reflects distal nephron injury. Constitutive expression of phospho-ERK1/2 in distal nephron was also observed by Fujita et al [23] in diabetic rats. Whether ERK1/2 activation in distal nephron mediates distal nephron injury only or also contributes to proximal tubule injury in this model is not clear but certain genetic responses of distal nephrons to injuries may be maladaptive enough to induce not only distal nephron injury, but also adjacent proximal tubule injury by aurocrine and paracrine fashion.

Since the molecular mechanisms responsible for the beneficial effect of ERK1/2 inhibition have to be elucidated, we examined the effect of ERK1/2 inhibition on several key components involved in cisplatin-induced renal injury. We first examined TNF-α, a potent proinflammatory cytokine mRNA expression. Like in other studies, TNF-α mRNA expression markedly

increased at 24 hours, and persisted until 72 hours after cisplatin and U0126 pretreatment significantly reduced TNF-α abundance in kidney tissue. This indicates that ERK1/2 phosphorylation is an upstream signal in cisplatin-induced TNF-α mRNA expression. Recent several reports demonstrating the beneficial effect of ERK1/2 inhibition on various inflammatory parameters and on the production of inflammatory cytokine, like IL-1β in cerebral ischemia or acute pancreatitis model also support our finding that ERK pathway functions as an upstream signal mediating TNF-α production, but additional studies that can provide evidences that ERK pathway can lead to up-regulation of several transcription factors that can mediate TNF-α expression should also be performed [18, 22].

The role of TNF-α in cisplatin induced renal injury has been extensively studied by using inhibitors of TNF-α or blocking antibody, producing less severe renal damage with reduced TNF-α level [6]. This finding is further supported by additional studies by Deng et al [5] that α-melanocyte stimulating hormone (α-MSH) or IL-10, which suppress the production of TNF-α also ameliorated cisplatin-induced renal injury. Several different mechanisms seem to be involved in TNF-α–mediated renal injury, like apoptosis, production of reactive oxygen species, or provocation of inflammatory response [5, 24–28]. Of those mechanisms, inflammation seems to be important because administration of a potent anti-inflammatory cytokine, IL-10, or blocking TNF-α decreased the number of infiltrating leukocytes with functional and histologic protection [6]. In our study, esterase-positive leukocytes infiltration also significantly decreased in U0126 pretreated animals, suggesting that ERK1/2 phosphorylation mediates the inflammatory pathway in cisplatin-induced renal injury. Although whether increased TNF-α mRNA expression through ERK pathway directly mediates inflammation is not clear in this study, a recent study by Ramesh and Reeves [28], demonstrating TNF-α dependence of leukocyte infiltration, supports that TNF-α dependent inflammation mediated through ERK1/2 activation plays an important role in the pathogenesis of cisplatin-induced renal injury.

Apoptosis is now recognized as an important mode of cell death in normal or pathologic status and characterized by cell shrinkage, nuclear chromatin condensation, and activation of a family of cyteine protease called caspase. The activation of caspase is thought to be a key step in the generation of apoptosis and several in vitro studies demonstrated that cisplatin activated multiple caspases, including caspase 8, 9, and 3, in renal tubular cells, indicating that caspase activation and subsequent tubular cell apoptosis is also important in the pathogenesis of cisplatin-induced renal dysfunction [13, 29, 30]. Recently, Nowak [20] showed that the activation of caspase 3 and apoptosis by cisplatin were decreased by inhibition of ERK1/2, suggesting ERK1/2 pathway is an upstream signal for caspase 3 activation and subsequent apoptosis in renal tubular cell. Because caspase 3 is one of the executioner caspases directly responsible for the cleavage of diverse physiologic substrates in apoptosis, we measured the activity of caspase 3 in kidney tissue. Like other in vitro studies, caspase 3 activity increased markedly at 24 hours, increased further to over 50-fold, peaking at 48 hours and U0126 pretreatment significantly decreased the activation of caspase 3 at each time point. This is consistent with the in vitro finding by Nowak et al [20] and to our knowledge, it is the first time to measure kidney tissue caspase activity and to show that ERK1/2 is an upstream signal for caspase 3 activation in vivo. Because caspase 8, an initiator caspase from membrane death receptor-dependent pathway, or caspase 9 from mitochondria-dependent pathway can also result in the activation of caspase 3, we also measured caspase 8 and 9 activities. The activities of caspase 8 or 9 increased slightly, but neither of them showed exact temporal relationship with that of caspase 3, indicating that the huge increase of caspase 3 activity by cisplatin is independent of caspase 8 or 9 activation. This result is consistent with the finding by Kaushal et al [13] demonstrating that the activation of caspase 3 in porcine proximal tubular cells (LLC-PK1) far preceded those of caspase 8 or 9, suggesting an independent activation of caspase 3 by cisplatin. The role of caspase 8 or 9 in cisplatin-induced tubular cell apoptosis has been controversial. Tsuruya et al [31] reported that Fas and TNFR1-mediated apoptosis is directly involved in renal tubular cell death in murine model of cisplatin-induced ARF, suggesting the important role of caspase 8, but they did not determine the tissue caspase activity. Park, De Leon, and Devarajan [32] demonstrated the role of cytochrome c and caspase 9 dependent apoptotic pathway, but it was not an in vivo study. In our study, however, we observed the significant activation of caspase 3, but not caspase 8 or 9, suggesting the activation of caspase 3 is contributed by the other pathways. Because recent several studies have demonstrated that cisplatin-induced ERK activation is an upstream regulator of p53 which can subsequently induce cell cycle arrest and apoptosis, p53 may partially be responsible for huge increase in caspase 3 activity independent of caspase 8 or 9 in this study [33, 34]. The p53-dependent activation of caspase 3 and apoptosis in renal tubular cells have been also demonstrated by Cummings and Schnellmann [10] who showed that adding p53 inhibitor significantly reduced caspase 3 activation and apoptosis by cisplatin. The number of TUNEL-positive apoptotic cells significantly increased in cisplatin-treated animals and U0126 pretreatment decreased the morphologic evidence of apoptosis. Although many other factors, like proinflammatory cytokine, reactive oxygen species can induce apoptosis, the activation of caspase 3 is thought to be a key step in apoptosis in this model because direct causal relationship between caspase 3 activation and apoptosis has been demonstrated

from many other in vitro and in vivo studies using caspase inhibitor [32, 35–37].

CONCLUSION

The present study demonstrates that the inhibition of ERK1/2 pathway provided marked functional, histologic protection in cisplatin-induced ARF in mice. The mechanism of this beneficial effect is thought to be mediated by inhibition of TNF-α–mediated inflammation and caspase 3–mediated apoptosis of tubular cells. Elucidating the signaling mechanism can lead to a better understanding of pathogenetic mechanism of renal dysfunction and targeting ERK1/2 can be a novel therapeutic strategy to inhibit or reduce cisplatin nephrotoxicity.

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