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AMP-activated protein kinase contributes to ROS-mediated p53 activation in cisplatin-induced nephrotoxicity

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Abstract. – OBJECTIVE: Cisplatin is a widely used anticancer drug that provokes various side effects. Nephrotoxicity is one of the well-known major side effects in the chemotherapeutic use of cisplatin. Reactive oxygen species (ROS) and p53 play important roles in cisplatin-induced nephrotoxicity. AMP-activated protein kinase (AMPK) is known to be sensitively activated by ROS and can directly activate p53. The present study investigated the role of AMPK on cisplatin-induced apoptosis in rat renal epithelial NRK-52E cells.

MATERIALS AND METHODS: NRK-52E cells were treated with cisplatin in the absence or presence of specific ROS scavenger and AMPK inhibitor for indicated times under the serum-free condition. The expression and phosphorylation levels of proteins were evaluated by Western blot and densitometry analysis.

RESULTS: Cisplatin-induced apoptotic cell death through ROS-mediated p53 activation, which is associated with AMPK activation. AMPK inhibitor suppressed cisplatin-induced p53 activation, as well as AMPK activation. Interestingly, ROS scavenger also diminished cisplatin-induced p53 activation and AMPK activation. Furthermore, cisplatin induced phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which attenuated p53 activation, but did not affect the expression levels of total p53, cleaved caspase-3 and PARP. Meanwhile, inhibition of AMPK induced premature phosphorylation of eIF2α in cisplatin-treated cells.

CONCLUSIONS: Taken together, these suggest that AMPK may be required for activation of p53 by oxidative stress in cisplatin-induced nephrotoxicity. Moreover, eIF2α phosphorylation may interrupt the AMPK-activated p53 in NRK-52E cells exposed to cisplatin, but does not critically affect cisplatin-induced nephrotoxicity because AMPK activation can be disrupted eIF2α phosphorylation.

Key Words:
AMPK, Cisplatin, p53, ROS, eIF2α, NRK-52E cells, Nephrotoxicity.

Introduction

Cisplatin is one of the most effective and widely used chemotherapeutic agents for human cancer treatment[1,2]. However, its therapeutic use is severely limited because of nephrotoxicity and acute renal injury which develop due to renal accumulation of cisplatin in kidney[3,4]. Cisplatin nephrotoxicity involves a complex multifactorial process, of which reactive oxygen species (ROS) and p53 tumor suppressor protein are critical factors in the cellular response to cisplatin-induced nephrotoxicity[5]. Cisplatin nephrotoxicity involves the induction of oxidative stress by generating ROS, among which hydroxyl radicals can lead to apoptotic cell death through activation of p53[5-10]. However, it is still unclear how ROS contribute to p53 activation during cisplatin treatment in renal cells. ROS can activate various protein kinase signaling pathways, which may lead to activation of p53 through its phosphorylation[11,12].

The adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric protein kinase that consists of a catalytic α subunit and regulatory β and γ subunits, and operates as a sensor intracellular energy by monitoring the cellular AMP/ATP ratio[13]. It is activated sensitively in response to a variety of physiological and pathological stresses that depletes cellular adenosine triphosphate (ATP) caused by metabolic poisoning, hypoxia, nutrition deprivation, and oxidative stress[14]. Particularly, AMPK is known

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to be sensitively activated by ROS, which directly activate AMPK through S-glutathionylation of cysteines on the AMPK α and β subunits[15]. Moreover, AMPK can phosphorylate p53 directly on serine 15, which is required for activation of p53, and in turn induces cell cycle arrest and apoptosis[16]. Thus, it is most likely that AMPK plays an important role in activation of p53 triggered by ROS, and therefore, it would contribute to cisplatin-induced nephrotoxic actions on renal cells.

Endoplasmic reticulum (ER) stress is a cellular stress response related to the unfolded protein response (UPR), which usually results from the accumulation of misfolded or unfolded proteins in ER lumen[17]. ER stress is closely associated with cisplatin-induced nephrotoxicity which alleviates cisplatin-induced apoptosis in renal cells[18,19]. Previously, it has been reported that ER stress-mediated eIF2α phosphorylation suppresses cisplatin-induced apoptosis in human renal cells[18]. In addition, preconditioning with ER stress ameliorates cisplatin-induced nephrotoxicity[19]. Interestingly, AMPK has been reported to regulate ER stress in several diseases and acts as an upstream inhibitor of ER stress[20]. Despite these findings, the correlation between AMPK and ER stress in cisplatin-induced nephrotoxicity remains unknown.

In this study, we investigated whether AMPK is activated in response to cisplatin-induced oxidative stress and involved in subsequent p53 activation in normal rat kidney epithelial (NRK-52E) cells. Also, since AMPK activation has been implicated in regulation of ER stress, we investigated the influence of AMPK activation on eIF2α phosphorylation induced by cisplatin in NRK-52E cells.

Materials and Methods

Reagents and Antibodies

Cis-diammineplatinum (II) dichloride (cisplatin), dimethyl sulfoxide (DMSO), 2',7'-dichlorofluorescin diacetate (DCFH-DA), and 1,3-dimethylurea (DMTU) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Compound C was purchased from Calbiochem (La Jolla, CA, USA). Caspase-3 assay kit and Sal003 were purchased from Abcam (Cambridge, MA, USA). DMEM, fetal bovine serum (FBS) and antibiotic-antimycotic solution were purchased from GIBCO (Grand Island, NY, USA). Anti-cleaved-caspase-3 (Asp175), anti-PARP, anti-phospho-p53 (Ser15), anti-p53, anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-phospho-eIF2α (Ser51), and anti-eIF2α antibodies were purchased from Cell Signaling Technology, INC. (Beverly, MA, USA).

Cell Culture

NRK-52E cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in DMEM supplemented with 10% FBS and 1:100 dilutions of an antibiotic-antimycotic solution at 37°C in a 5% CO_2 incubator. Exponentially growing cells (1×10^5 cells/ml) were seeded for 24 h, followed by treating with cisplatin indicated times under serum-free medium.

Cell Viability Assay

Cell viability was determined by MTT assay. After treatment, the cells were incubated with 0.5 mg/ml of MTT in serum-free medium for 4 h at 37°C. The purple formazan crystals were dissolved in 100% DMSO and measured the quantity of formazan were measured at 570 nm using a SpectraMAX 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The percent of cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells) × 100.

Flow Cytometry Analysis

Apoptotic cells were monitored by quantitating cellular DNA content with propidium iodide (PI) staining. The cells were fixed with ice-cold 70% ethanol for 1 h, washed with phosphate buffered saline (PBS) (pH 7.4), and then, incubated with PI staining solution [10 μg/ml PI and 100 μg/ml RNase A in PBS (pH 7.4)] at 37°C for 1 h in the dark condition. DNA contents were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Caspase-3 Activity Assay

The activity of caspase-3 was determined by colorimetric caspase-3 assay kit according to the manufacturer's instructions. Briefly, the cells were lysed in cell lysis buffer and centrifuged at 10,000 × g for 1 min. The supernatants were incubated at 37°C in the dark for 1h with reaction buffer and DEVD-p-NA substrate (200 μM final concentration). The colorimetric release of p-nitroaniline from DEVD-p-NA substrate was measured at 405 nm using a SpectraMAX M2 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage

of caspase-3 activity was calculated using the equation: (mean OD of treated cells/mean OD of control cells) × 100.

Reactive Oxygen Species Production Assay

For determining the relative levels of reactive oxygen species (ROS) in DCFH-DA fluorescence assay, the cells were seeded into 96-well microplates (1×10^4 cells/well) for 24 h and incubated in HBSS (pH 7.4) solution containing 20 μM DCFH-DA at 37ºC for 1 h. After removing the excess DCFH-DA solution, cells were washed with HBSS (pH 7.4) and treated with 50 μM cisplatin in HBSS (pH 7.4) for the indicated time periods. Relative DCF fluorescence intensity was determined with excitation wavelength of 485 nm and emission wavelength of 538 nm using SpectraMAX M2 multi-mode microplate reader (Molecular Devices).

Western Blot Analysis

The cells were lysed in RIPA lysis buffer containing 1% halt protease and phosphatase inhibitor cocktails. Cell lysates were centrifuged at 14,000 rpm for 15 min in a refrigerated microcentrifuge, and protein concentrations were determined using a Bradford assay. An equal amount of total protein samples was separated by SDS-PAGE, and then, transferred onto a nitrocellulose membrane for 3 h at 40V. The blots were immersed in blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, 5% BSA) 4ºC for 1 h, followed by probed with primary antibodies (1:1000) at 4°C overnight with gentle shaking. Subsequently, the blot was washed three times and incubated HRP-conjugated secondary antibodies (1:2000) at 4ºC for 1 h. The blots were washed three times, and the bands were detected with a FluorChem E System (ProteinSimple, San Jose, CA, USA). The density of bands was quantitated using Quantity One 4.6.6 software (Bio-Rad, Hercules, CA, USA). The bands indicate the relative density ratio of respective protein normalized to the internal control (β-actin).

Statistical Analysis

Statistical analysis was performed using Microsoft Office Excel 2019 (Microsoft, Redmond, WA, USA). The data were expressed as means ± standard deviation (SD). The unpaired one-tailed Student's *t*-test was performed to check the significance. *p*-value <0.05 was considered to indicate statistically-significant differences.

Results

Cisplatin Induces Apoptotic Cell Death in NRK-52E Cells

Cisplatin reduces cell viability and induces apoptotic cell death in both cancer and renal cells. The viability of NRK-52E cells was markedly decreased by increasing concentration of cisplatin (Figure 1A). As the concentration of cisplatin in NRK-52E cells increased, the proportion of apoptotic cells increased significantly (Figure 1B). To further evaluate the cisplatin-induced apoptosis, we examined for caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage, which are the hallmarks of apoptosis. As shown in Figure 1C and 1D, treatment of NRK-52E cells with cisplatin significantly increased the enzymatic activity of caspase-3 and the active cleaved form of caspase-3. Similar to caspase-3, cisplatin treatment increased the 89-kDa proteolytic fragments of PARP resulting from caspase cleavage (Figure 1D). These results indicate that cisplatin induced apoptotic cell death in NRK-52E cells.

Cisplatin Induces Apoptosis Through ROS-Mediated p53 Activation in NRK-52E Cells

The ROS and p53 tumor suppressor play a pivotal role in regulating cisplatin-induced apoptosis of renal cells[5]. Treatment of renal cells with cisplatin generates excessive ROS that leads to the phosphorylation of p53 at serine 15, which is essential for the stabilization of the p53 protein, inducing p53-triggered apoptosis[10-12]. We examined whether cisplatin could induce the production of ROS and activation of p53 in NRK-52E cells. The intracellular contents of ROS began to increase at 30 min after treatment of NRK-52E cells with cisplatin, and remained constant during prolonged cisplatin treatment (Figure 2A). The levels of total and serine 15 phosphorylation of p53 also began to increase at 2 h after cisplatin treatment and further increased in a time-dependent manner (Figure 2B). We next examined whether the ROS, in particular the hydroxyl radicals could contribute to the activation of p53 via phosphorylation at serine 15 in cisplatin-treated NRK-52 E cells. Treatment of DMTU, a potent scavenger of hydroxyl radicals, almost completely reduced levels of both total and serine 15 phosphorylation of p53 in NRK-52E cells exposed to cisplatin (Figure 2C). Additionally, to confirm p53 activation associated

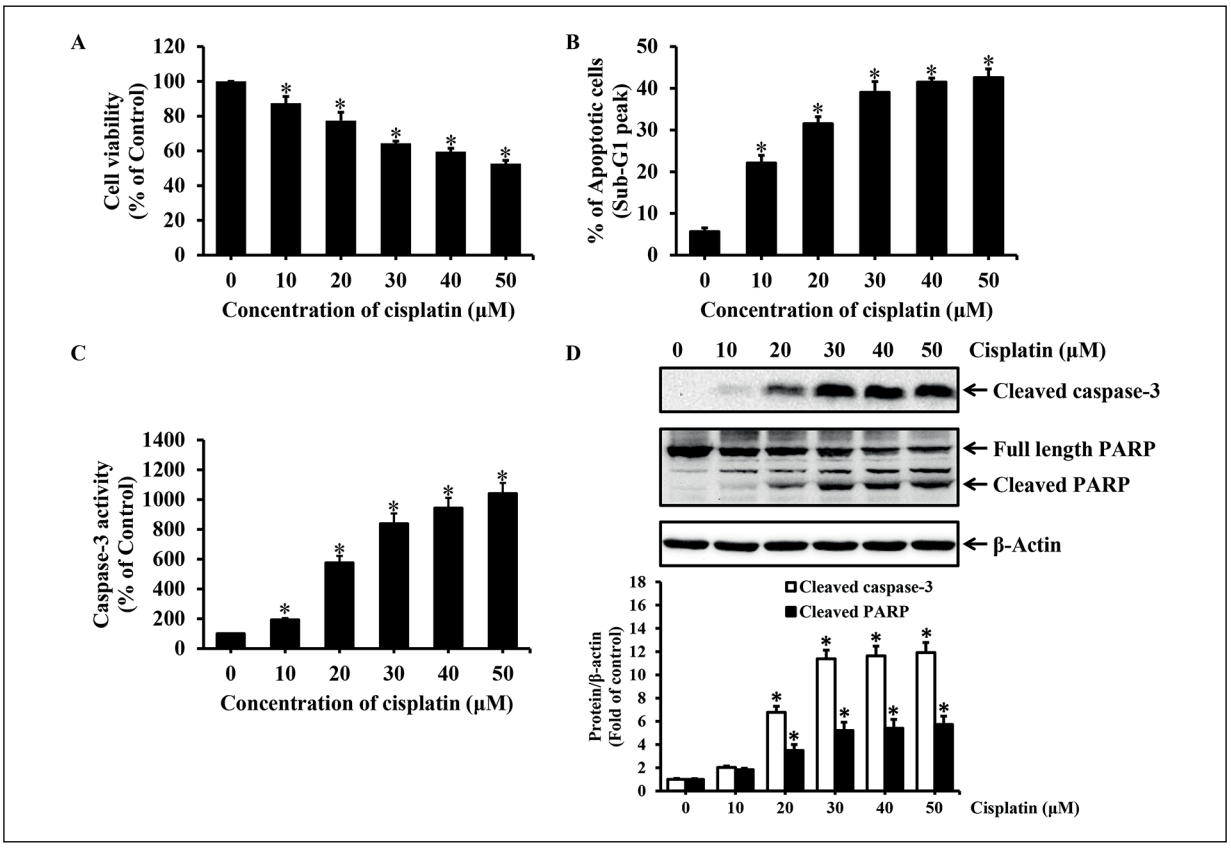

Figure 1. Effects of cisplatin in NRK-52E cells. The cells were treated with various concentrations of cisplatin for 24 h. **A,** Cell viability was measured using MTT assay. **B,** Apoptotic cells were quantified by flow cytometry after staining with PI. **C,** Caspase-3 activity was measured using a caspase-3 colorimetric assay kit. **D,** The expression levels of cleaved caspase-3 and PARP were examined by Western blot analysis using anti-cleaved caspase-3 (p20) and anti-PARP antibodies. The western bands were quantified using Quantity One 4.6.6 software. Value are means ± SD, $N = 3$. *$p < 0.05$ *vs.* untreated cells.

with cisplatin-induced apoptosis in NRK-52E cells, we used p53-specific inhibitor pifithrin-α. The pifithrin-α markedly inhibited the levels of cleaved caspase-3 and PARP induced by cisplatin treatment (Figure 2D). These results indicate that cisplatin-generated ROS contribute to the activation of p53, which induces p53-dependent apoptosis of NRK-52E cells.

Cisplatin Induces p53 Phosphorylation at Serine 15 Through ROS-Mediated Activation of AMPK in NRK-52E Cells

AMPK activation induces phosphorylation of p53 at serine 15, a residue known to be important for the activation of p53[16]. First, we examined whether cisplatin could induce the activation of AMPK in NRK-52E cells. The active levels of AMPKα phosphorylated at tyrosine 172 began to increase at 2 h after treatment of NRK-52E cells with cisplatin, peaked at 8 h, and returned to the control level at 24 h (Figure 3A). To determine whether AMPK is required to activate p53 in cisplatin nephrotoxicity, we next examined the effect of compound C a small molecule AMPK inhibitor, on cisplatin-induced p53 phosphorylation and expression in NRK-52E cells. Compound C remarkably diminished the levels of cleaved caspase-3 and PARP in cisplatin-exposed cells by inhibited cisplatin-induced phosphorylation levels of p53 at serine 15, as well as p53 expression (Figure 3B). Since AMPK is activated by ROS[15], we examined whether it affects the activation of AMPK in cisplatin-induced oxidative stress in NRK-52E cells. The scavenging of intracellular ROS by DMTU significantly abolished the phosphorylation levels of AMPKα at tyrosine 172 in cells treated with cisplatin (Figure 3C). These results indicate that activation of AMPK may be required for phosphorylation of p53 on serine 15 by cisplatin-induced ROS production in NRK-52E cells.

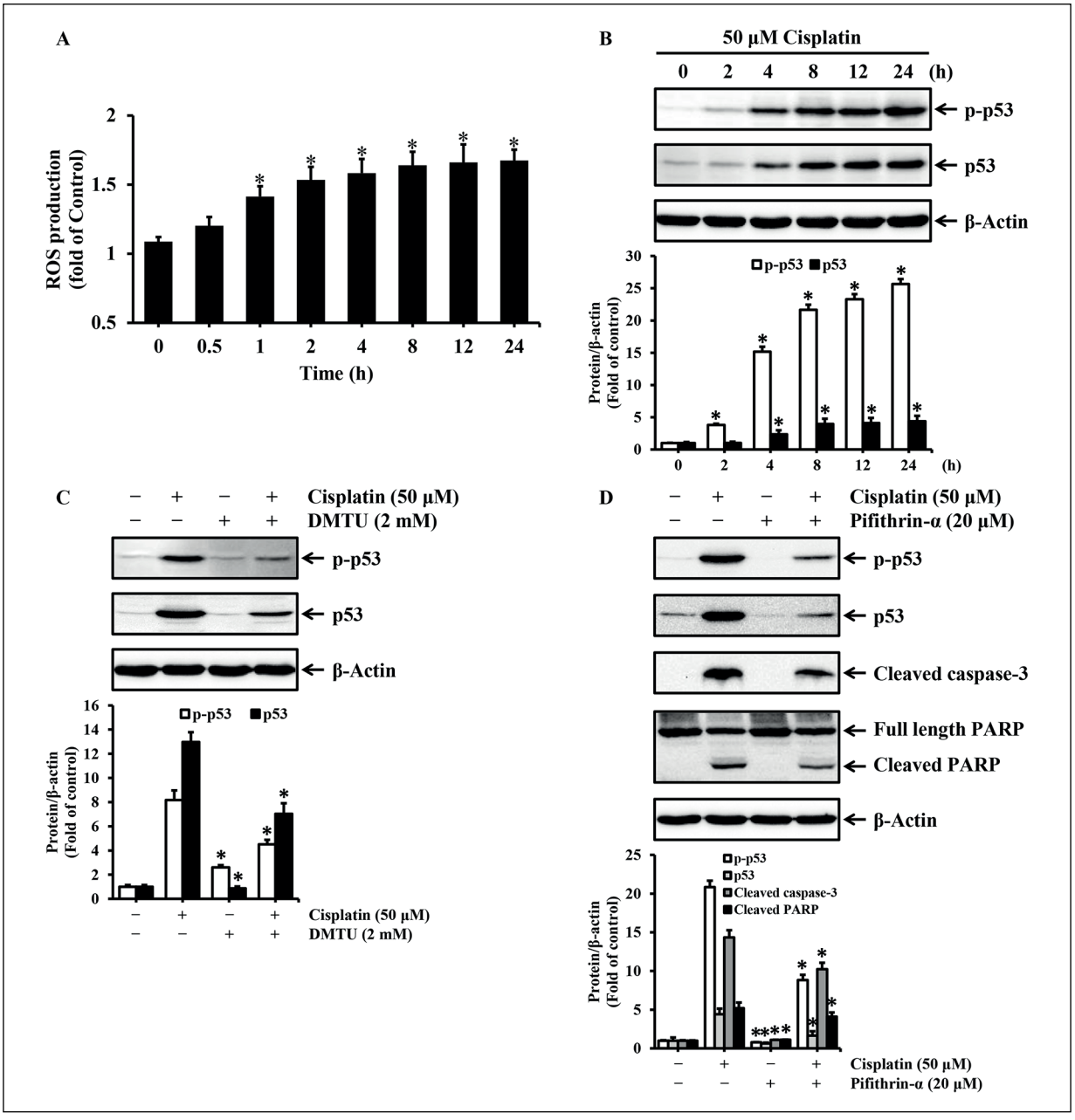

Figure 2. Effects of ROS production and p53 activation on cisplatin-induced apoptosis in NRK-52E cells. **A, B,** The cells were incubated with 50 μM cisplatin for indicated times. ROS production was measured by DCFH-DA fluorescence assay. The expression of p53 were examined by Western blot. **C, D,** The cells were pretreated with or without 2 mM DMTU or 20 μM pifithrin-α for 1 h and then exposed to 50 μM cisplatin for 12 h or 24 h. The expression of phosphorylated p53, p53, cleaved caspase-3, and PARP levels were examined by Western blot analysis. The western bands were quantified using Quantity One 4.6.6 software. Value are means ± SD, $N = 3$. *$p < 0.05$ versus 0 h time point cells or cisplatin-alone treated cells.

Phosphorylation of eIF2α Interrupts Cisplatin-Induced p53 Activation in NRK-52E Cells

The eIF2α phosphorylation is a component of the UPR, a regulatory program responsible for the promotion of cell survival under ER stress[21]. Lee and Kim[22] have reported that the phosphorylation of eIF2α counteracts the stabilization of p53. The p53 becomes stabilized by post-translational modifications, such as phosphorylation of p53 at serine 15[23]. A previous study[18] reported that eIF2α phosphorylation suppresses cispla-

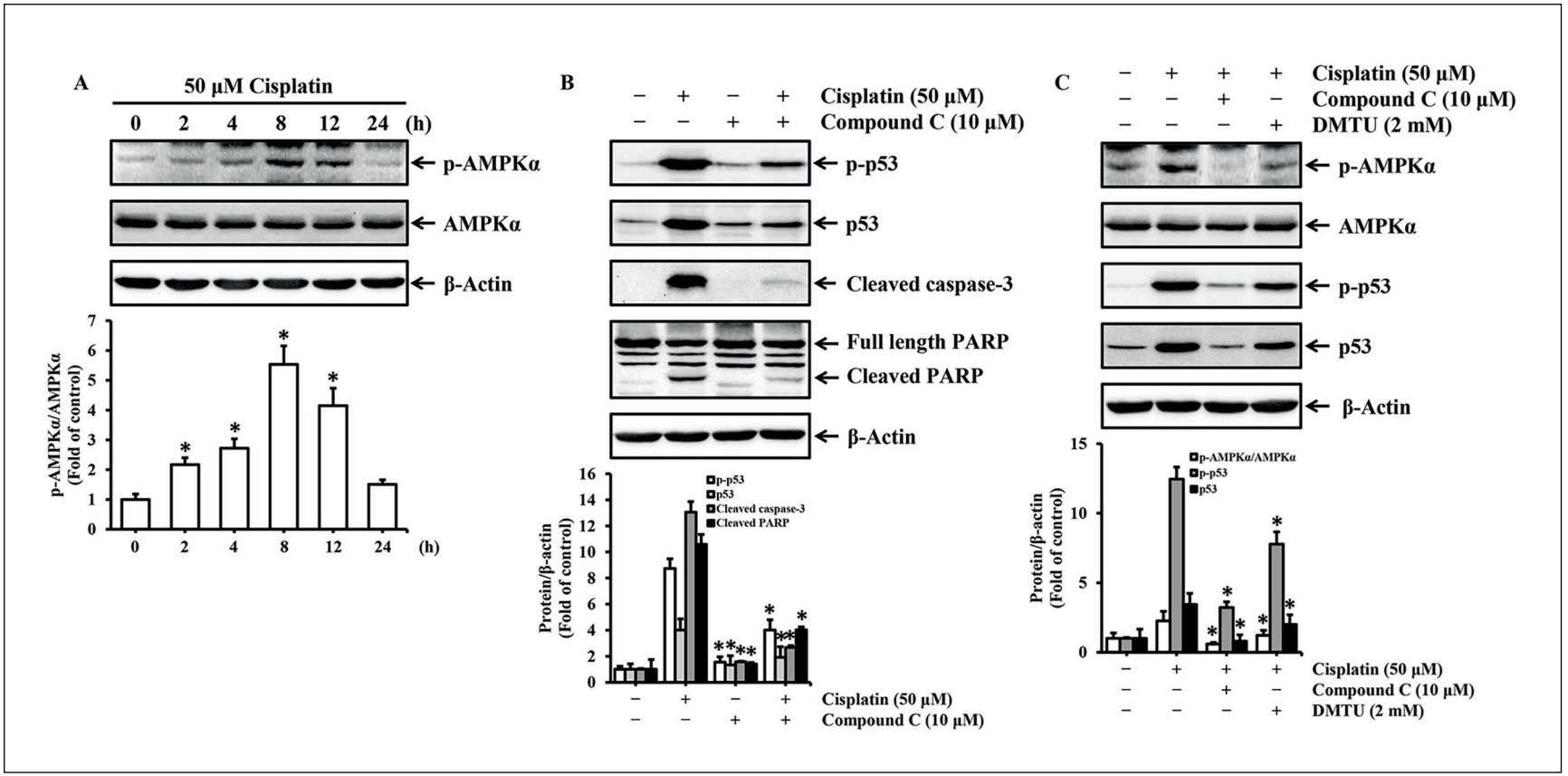

Figure 3. Effects of AMPK activation on cisplatin-induced p53 activation in NRK-52E cells. **A,** The cells were incubated with 50 μM cisplatin for indicated times and checked for the western blot expression of p-AMPKα and AMPkα. **B,** The cells were pretreated with or without 10 μM compound C for 1 h and then exposed to 50 μM cisplatin for 24 h and checked for the western blot expression. **C,** The cells were pretreated with or without 2 mM DMTU for 1 h and then exposed to 50 μM cisplatin for 8 h and checked for the western blot expressions. The western bands were quantified using Quantity One 4.6.6 software. Value are means ± SD, $N = 3$. *$p < 0.05$ versus 0 h time point cells or cisplatin-alone treated cells.

tin-induced phosphorylation of p53 at serine 15 in human renal cells. First, we examined whether cisplatin could increases eIF2α phosphorylation in NRK-52E cells, and if so, whether Sal003, an inhibitor of eIF2α dephosphorylation that has been show to inhibit cisplatin-induced phosphorylation and expression of p53. The phosphorylation of eIF2α at serine 51 were clearly observed at 12 and 24 h post cisplatin treatment in NRK52-E cells (Figure 4A). Sal003 enhanced cisplatin-induced phosphorylation of eIF2α, but attenuated phosphorylation of p53 induced by cisplatin in NRK-52E cells (Figure 4B). The total levels of p53 were not reduced by Sal003 in NRK-52E cells exposed to cisplatin and neither did the levels of cleaved caspase-3 and PARP. The phosphorylation of eIF2α is associated with activation of AMPK, and previous studies[24,25] have revealed that activation of AMPK can inhibit or induce phosphorylation of eIF2α. Therefore, we examined AMPK activation could have any influence on the phosphorylation of eIF2α in cisplatin-treated NRK-52E cells. Inhibition of AMPK phosphorylation with compound C early induced phosphorylation of eIF2α less than 8 h after cisplatin treatment (Figure 4C). These results indicate that enhanced phosphorylation of eIF2α may interrupt AMPK-activated p53 in cisplatin-treated NRK-52E cells, but does not affect cisplatin-induced apoptosis. Interestingly, phosphorylation of eIF2α has shown to be disrupted by AMPK activation in cisplatin-treated NRK-52E cells.

Discussion

Nephrotoxicity is one of the major side effect and main challenge in the chemotherapeutic use of cisplatin[1-4]. ROS and p53 are most important factors contributing to cisplatin nephrotoxicity[5]. Previous studies[8-10] have reported that cisplatin nephrotoxicity have been implicated in ROS-mediated p53 activation, which induces apoptotic cell death in renal cells. Similar to previous studies, our results demonstrated that cisplatin induced apoptotic cell death through ROS-mediated p53 activation in NRK-52E cells. However, it is still unclear how ROS can cause the activation of p53 in the process of cisplatin-induced nephrotoxicity. It is most likely that oxidative stress caused by ROS can activate various protein kinase signaling pathways, which may lead to p53 phosphorylation as the activation[11,12].

AMPK is known to be directly activated by oxidative stress and involved in p53 phosphorylation[15,16]. Our results demonstrated that AMPK included ROS-mediated p53 activation in cisplatin-induced nephrotoxicity. Cisplatin-induced ROS production was involved in the phosphorylation of AMPKα, which lead to activation of p53 in NRK-52E cells. Similar to our results, Jin et al[26] have reported that AMPK is involved in cisplatin-induced apoptosis through p53 activation in mouse renal epithelial cells. However, it is not yet certain that AMPK may alleviate cisplatin-induced nephrotoxicity. Rashtchizadeh et al[27] have reported that AMPK activation augments cisplatin-induced apoptosis in cancer cells, whereas it ameliorates cisplatin cytotoxicity in renal cells. Emodin, metformin and neferine, known as phytochemicals, have been shown to ameliorate cisplatin-induced apoptosis *via* AMPK signaling pathway in renal epithelial cells[28-30]. In our study, pretreatment with AICAR, an AMPK activator, diminished cisplatin-induced apoptosis in NRK-52E cells (data not shown). It is unclear why both inhibition or activation of AMPK alleviates cisplatin-induced nephrotoxicity. However, our results show that AMPK activation is increased up to 8 h during treatment with cisplatin in NRK-52E cells, and then, dropped out. Thus, it is likely that the enhancement or prolongation of AMPK activation may have alleviated cisplatin-induced nephrotoxicity, but this requires further study.

The eIF2α phosphorylation has been shown to play a cytoprotective role against stress by reducing global protein translation[31]. Previous scholars[18] reported that phosphorylation of eIF2α is associated with protection against cisplatin-induced nephrotoxicity in human renal epithelial cells. However, our results show that phosphorylation of eIF2α does not protect NRK-52E cells from cisplatin-induced nephrotoxicity, as it did not diminish the expression levels of total p53 despite p53 activation reduction. Furthermore, AMPK activation is associated with phosphorylation of eIF2α and can inhibit or induce phosphorylation of eIF2α. Xiong et al[25] reported that AMPK activation inhibits ox-LDL-induced eIF2α phosphorylation in human endothelial cells. On the contrary, Dagon et al[24] have reported that AMPK activation can phosphorylate eIF2α in adipocyte. Our results show that inhibition of AMPK induced premature phosphorylation of eIF2α in cisplatin-treated NRK-52E cells. Interestingly, cisplatin-induced eIF2α phosphorylation was observed after drop in AMPK activation in

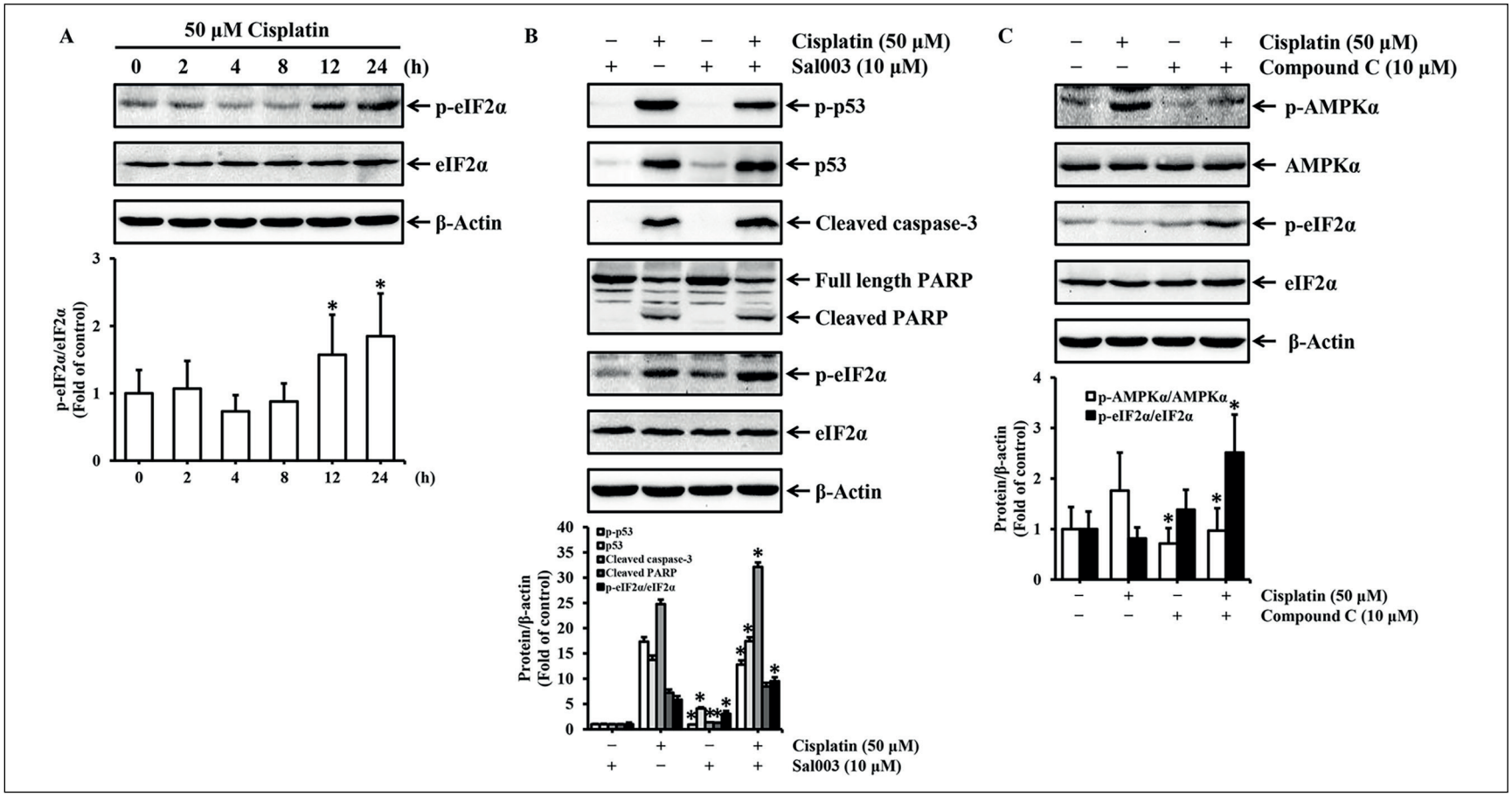

Figure 4. Effects of eIF2α phosphorylation on cisplatin-induced apoptosis in NRK-52E cells. **A,** The cells were incubated with 50 μM cisplatin for indicated times. The phosphorylation and expression of eIF2α were examined by Western blot analysis using anti-eIF2α (Ser 51) and anti-eIF2α antibodies. **B,** The cells were pretreated with or without 10 μM Sal003 for 1 h, and then, exposed to 50 μM cisplatin for 24 h. The phosphorylation and expression of p53, eIF2α, cleaved caspase-3 and PARP levels were examined by Western blot analysis using anti-phospho-p53 (Ser15), anti-p53, anti-eIF2α (Ser 51), anti-eIF2α, anti-cleaved caspase-3 (p20), and anti-PARP antibodies. The expression levels of cleaved caspase-3 and PARP were examined by Western blot analysis using anti-cleaved caspase-3 (p20) and anti-PARP antibodies. **C,** The cells were pretreated with or without 10 μM compound C for 1 h and then exposed to 50 μM cisplatin for 8 h. The phosphorylation and expression of AMPKα and eIF2α levels were examined by Western blot analysis using anti-phospho-AMPKα (Thr 172), anti-AMPKα, anti-phospho-eIF2α (Ser 51) and anti-eIF2α antibodies. The Western bands were quantified using Quantity One 4.6.6 software. Value are means ± SD, $N = 3$. *$p < 0.05$ versus 0 h time point cells or cisplatin-alone treated cells.

NRK-52E cells. Thus, it is likely that phosphorylation of eIF2α can interrupt cisplatin-induced p53 activation, but may not have effect on cisplatin-induced apoptosis in NRK-52E cells because eIF2α phosphorylation is blocked by AMPK activation and did not affect total p53 expression. Therefore, further study is needed to clarify the role of AMPK on cisplatin-induced nephrotoxicity and its therapeutic effect in cancer. Although we did not perform a pre-clinical test, in vivo experiment might be helpful in understanding the role of AMPK that may play an important role to improve cisplatin-induced nephrotoxicity.

In conclusion, cisplatin-induced oxidative stress causes activation of AMPK which regulates p53 activity in NRK-52E cells. On the other hand, phosphorylation of eIF2α interferes with cisplatin-induced p53 activation in NRK-52E cell, but has no significant effect on cisplatin-induced nephrotoxicity because AMPK activation can be disrupted eIF2α phosphorylation. Therefore, AMPK may play an important role in protective strategies to alleviate cisplatin-induced nephrotoxicity, but the role of eIF2α phosphorylation remains unclear.

Conclusions

Taken together, this study suggest that AMPK may be required for activation of p53 by oxidative stress in cisplatin-induced nephrotoxicity. In addition, eIF2α phosphorylation may interrupt to AMPK-activated p53 in NRK-52E cells exposed to cisplatin but does not critically affect cisplatin-induced nephrotoxicity because AMPK activation can be disrupted by eIF2α phosphorylation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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