

Original Article

Activation of unfolded protein response protects osteosarcoma cells from cisplatin-induced apoptosis through NF- κ B pathway

Mingming Yan, Jiangdong Ni, Deye Song, Muliang Ding, Jun Huang

Department of Orthopedic Surgery, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

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Abstract: The aim of this study was to uncover that unfolded protein response (UPR) contributed to the development of cisplatin resistance in osteosarcoma. MG-63 cells and SaOS-2 cells were exposed to cisplatin at presence or absence of 4-phenylbutyrate (4-pba) and then analyzed by MTT assay and flow cytometry to determine the cell survival rates and apoptosis. Levels of glucose regulated protein 78KD (GRP78), C/EBP homologous protein (CHOP), cytoplasmic and nuclear NF- κ B were detected by Western blot. Further, MG-63 cells and SaOS-2 cells were subjected to cisplatin with or without Bay 11-7082, a well-known inhibitor of NF- κ B. After that, MTT assay and flow cytometry were used to determine the cell survival rates and apoptosis. Cisplatin and 4-PBA co-treatment significantly enhanced the cell apoptosis. Administration of cisplatin substantially increased the levels of GRP78 and CHOP. Moreover, mechanistic investigation uncovered that cisplatin promoted the levels of nuclear NF- κ B whereas 4-PBA administration suppressed the cisplatin-induced accumulation of nuclear NF- κ B level in osteosarcoma cells. Cisplatin combined with Bay 11-7082 obviously augmented MG-63 cells and SaOS-2 cells apoptosis when compared to that in osteosarcoma cells treated by cisplatin alone. Taken together, our data show that UPR protects osteosarcoma from cisplatin-mediated apoptosis through activation of NF- κ B pathway. Therefore, targeting UPR may be a potential strategy to improve the osteosarcoma therapy.

Keywords: Osteosarcoma, UPR, cisplatin, chemoresistance, NF- κ B

Introduction

Osteosarcoma (OS) is the most common type of primary malignant bone tumor in children and adolescents [1]. It remains the second cause responsible for tumor-related death in young adults. Although therapeutic strategies such as local tumor excision combined with neo-adjuvant chemotherapy have greatly raised the 5-year survival rate in patient with localized OS in last 3 decades, OS patients who have a poor response to chemotherapy usually suffer from a deadly outcome [2, 3]. OS can develop into the acquired drug resistance phenotypes associated with chemotherapy, which is the main challenge to overcome. Therefore, it is of great importance to understand the mechanism underlying the OS drug resistance.

Endoplasmic reticulum (ER) is a critical organelle of eukaryotic cells that plays an essential role in correct folding of nascent secreted or

membrane-bound proteins, lipid biosynthesis and Ca²⁺ homeostasis [4]. However, when the balance of protein folding is disrupted, accumulation of unfolded or misfolded protein in ER lumen triggers ER stress. Cells initiate the unfolded protein response (UPR), an adaptive process to transmit signal from ER to nucleus, restoring the ER homeostasis [5, 6]. Activation of UPR leads to not only the general translation suppression but also an increase in capacity of ER protein folding. Recently, activation of UPR has been suggested to play a cytoprotective role in a range of human malignancies such as breast cancer, gastric cancer, hepatocellular carcinoma cells, pancreatic cancer, lung cancer and multiple myeloma [7-10]. However, the influence of UPR in osteosarcoma remains poorly understood.

The nuclear factor κ B (NF- κ B) has been shown to be a key regulator in immune response, inflammation as well as survival, proliferation,

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metastasis and chemoresistance in cancer [11-13]. NF- κ B is a dimer of the Rel family including RelA (p65), p50, p52, RelB and c-Rel. Each of them binds to their cognate response element as homo- or hetero-dimers with p65/p50 being the canonical heterodimers. Recently, activation of NF- κ B leads to increase cell proliferation in osteosarcoma and hinder osteoblastic differentiation. In addition, blockage of NF- κ B activation sensitizes osteosarcoma cell lines to chemotherapeutic agents [14, 15].

Accordingly, we raise the hypothesis that initiation of UPR may contribute to the drug resistance of osteosarcoma to cisplatin by activation of NF- κ B signal. In order to testify our hypothesis, we investigated the roles and mechanisms of UPR in chemoresistance of osteosarcoma to cisplatin. Attenuation of UPR activation sensitized osteosarcoma to cisplatin. Furthermore, we also unveiled that administration of cisplatin activated NF- κ B and 4-PBA suppressed cisplatin-induced NF- κ B activation. Blockage of NF- κ B significantly promotes cisplatin-induced apoptosis in human osteosarcoma cells.

Materials and methods

Cell culture

Human osteosarcoma cell line MG-63 and SaOS-2 cells were obtained from the American Type Culture Collection (ATCC). The osteosarcoma cell lines were cultured in DMEM medium supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml), containing 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Life Technologies, Carlsbad, CA). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Antibodies and chemicals

Thapsigargin (TG), dimethyl sulfoxide (DMSO), 4-phenylbutyrate (4-PBA), Bay 11-7082 and cisplatin were obtained from Sigma (Sigma, St. Louis, MO). TG, Bay 11-7082 and 4-phenylbutyrate were dissolved in DMSO. Cisplatin was dissolved in PBS. The antibody to GRP78 was purchased from Santa Cruz (Santa Cruz, CA). The antibodies to β -actin, PCNA, CHOP and NF- κ B p65 were obtained from Cell signaling (Cell signaling, Beverly, MA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sigma (Sigma, St. Louis, MO).

Cell viability assays

The MTT assay was used to test cell viability. We seeded growing MG-63 cells into 96-well culture plates in 200 μ l of media at a density of 1×10^4 cells/well. The following day, 200 μ l media with indicated chemicals were added to each well and incubated for indicated hours. Following the incubation, 20 μ l MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml in PBS; Sigma, St. Louis, MO) was added to each well for 4 h. Subsequently, the supernatants were discarded and 150 μ l DMSO was added to each well. After plates were shaken for 10 min in dark, absorbance was measured at a wavelength of 490 nm using a microplate reader (Multiskan GO, Thermo scientific, Waltham, MA). MG-63 and SaOS-2 cells were treated with indicated cisplatin concentrations for 24 hours. Then cells viability and IC50 of cisplatin were calculated. As 4-PBA has been used in various cells with different concentrations from 1 to 20 mmol/L, we first assessed the MG-63 viability by MTT assay after incubated with indicated concentration of 4-PBA for 24 hours. Then, we preferred to select 2.5 mmol/L, 5 mmol/L and 10 mmol/L 4-PBA to co-treat MG-63 cells with cisplatin.

Extraction of nuclear fractions

Nuclear protein extracts were prepared according to the previous protocol. Briefly, cells (1×10^6) were harvested after trypsinization, washed twice with cold PBS, and then lysed with cold buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L phenyl methane sulfonyl fluoride (PMSF), 1 mmol/L dithiothreitol, 1 mg/L aprotinin, 1 mg/L leupeptin, and 1 mg/L pepstatin A). After incubation for 10 min on ice, 0.1% NP 40 was added to the lysates and the tubes were shaken for 10 seconds on vortex. The cell homogenates were centrifuged at 12,000 g for 5 min at 4°C. The supernatants (cytoplasmic extracts) were harvested and stored at -70°C. Next, the nuclear pellets were resuspended in cold buffer B (20 mmol/L HEPES, pH 7.9, 420 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L phenylmethanesulfonyl fluoride, 1 mmol/L dithiothreitol, 1 mg/L aprotinin, 1 mg/L leupeptin, and 1 mg/L pepstatin A) and shaken at maximum speed at 4°C for 30 min on vortex. The lysates were centri-

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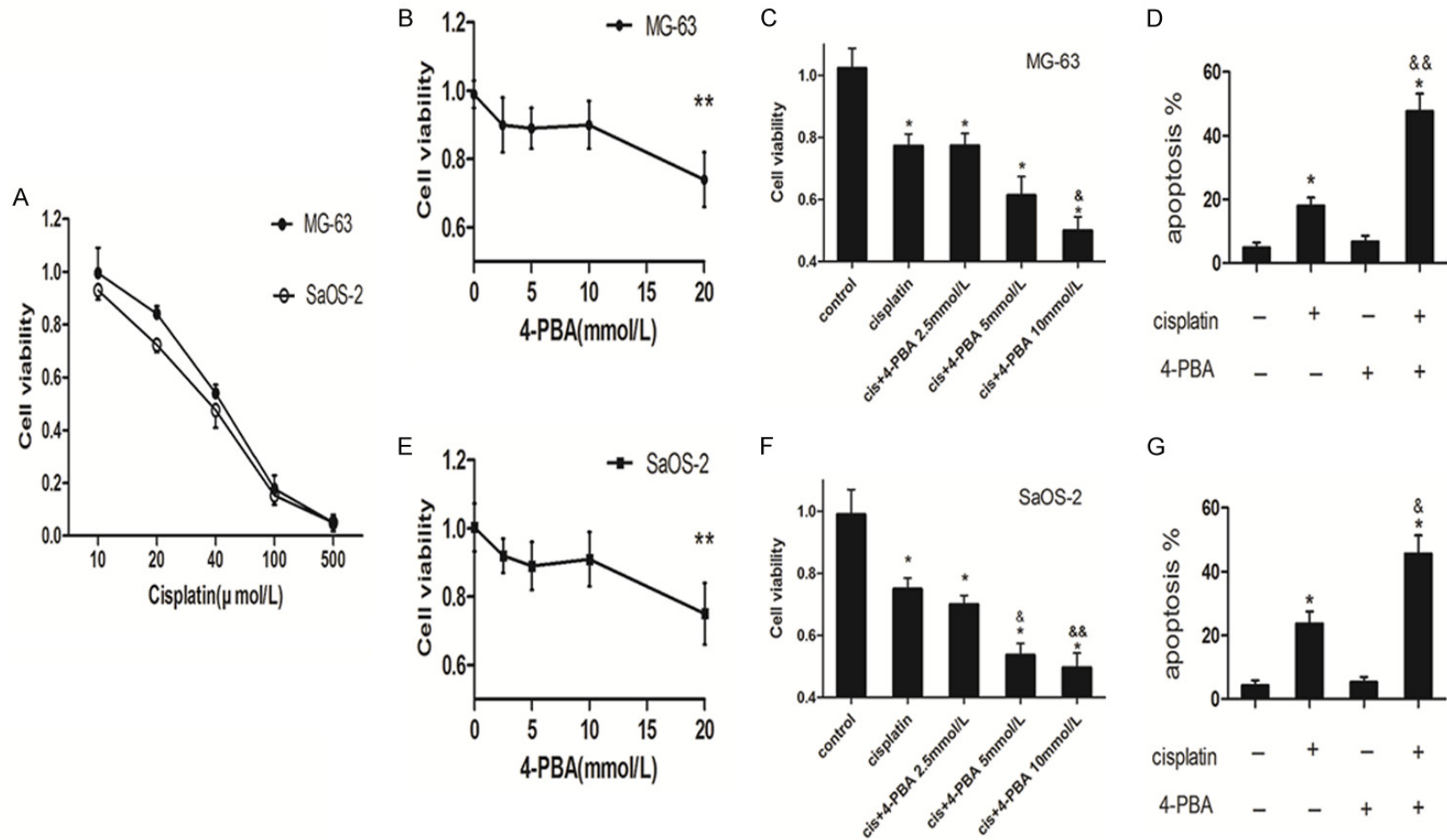


Figure 1. Attenuation of ER stress promoted cisplatin induced osteosarcoma cells apoptosis. A: Indicated osteosarcoma cells were treated with different concentration of cisplatin for 24 hours, then the cell viability was analyzed by MTT (n = 4). B and E: MG-63 cells and SaOS-2 cells were treated with indicated concentrations of 4-PBA for 24 hours and then cell viability was analyzed by MTT assay respectively (n = 3). C and F: MG-63 cells and SaOS-2 cells were treated with cisplatin (20 μmol/L) in the presence or absence of indicated concentrations of 4-PBA for 24 hours. Cell viability was analyzed by MTT assay (n = 3). D and G: MG-63 cells and SaOS-2 cells were treated with cisplatin (20 μmol/L) in the presence or absence of 4-PBA (10 mmol/L) for 24 respectively. Apoptosis was analyzed by the Annexin V assay by flow cytometry (n = 3). Data are represented as mean ± SD. *P<0.05, **P<0.01 compared to control; *&P<0.05, *&&P<0.01 compared to cisplatin alone.

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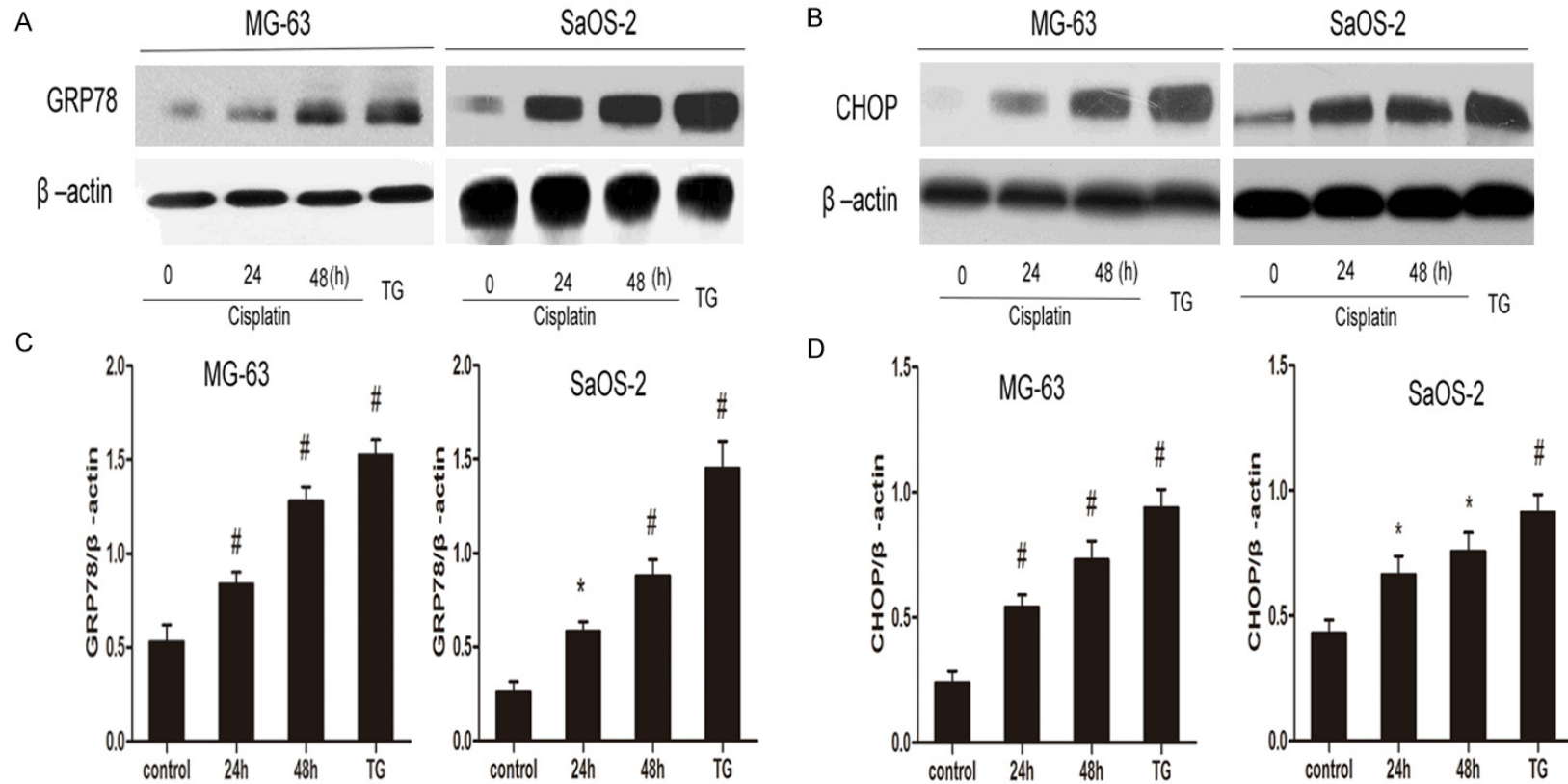


Figure 2. Effect of Cisplatin on levels of GRP78 and CHOP protein in osteosarcoma cells. MG-63 cells and SaOS-2 cells were treated with cisplatin (20 $\mu\text{mol/L}$) for indicated periods and TG (0.01 $\mu\text{mol/L}$) for positive control. Representative Western blot analysis of GRP78 protein level (A) and CHOP protein level (B) in OS cells treated with cisplatin. (C and D) Analyses of band intensity are shown as the relative ratio of GRP (C) and CHOP (D) to actin. Data are represented as mean \pm SD. B-actin was served as load control. * $P < 0.05$, # $P < 0.01$ versus control. All figures are representatives of the results of three independent experiments.

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fuged at 12,000 g for 5 min, and the supernatants (nuclear extract) were stored at -70°C. For western blotting analysis, total protein concentration was measured by the BCA assay (Pierce, Rockford, IL, USA).

Western blot analysis

Cell lysates were prepared with cell lysis buffer (RIPA buffer, 10 mmol/L Tris-HCl, pH 8.0, 10 mmol/L EDTA, 0.15 mol/L NaCl, 1% NP-40, 0.5% sodium dodecyl sulphate, 1 µg/ml aprotinin, 1 mmol/L PMSF) on ice for 30 min. The protein supernatants were collected by centrifugation at 10,000 g for 10 min. Total protein concentration was measured by the BCA assay. Each extract containing approximately 25-30 µg protein was subjected to the 10% polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) by wet transfer. The membranes were blocked by TBS-T (10 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 2 h at room temperature and incubated with primary antibodies (GRP78, 1:800 dilution; CHOP, 1:1000 dilution; β-actin, 1:1000 dilution; NF-κB p65, 1:1000 dilution; PCNA, 1:1000 dilution) overnight at 4°C. After three washes by TBS-T for 15 min, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. The blotting signals were detected by enhanced chemiluminescence (Pierce, Rockford, IL). β-actin and PCNA were acted as loading control.

Apoptosis detection by flow cytometry

The apoptotic cells were measured by Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Diego, USA). Briefly, 1×10^5 cells were seeded in 6-well plates and then incubated with indicated chemicals for indicated time. After treatment, approximately 1×10^6 cells were collected, washed twice with cold PBS, and then stained with Annexin V-FITC and PI according to the manufacturer's instructions. The resulting fluorescence was analyzed by flow cytometry (BD FACS Jazz, San Jose, CA).

Statistical analysis

Statistical analyses were performed using the SPSS 17.0 Statistical software. All data were presented as the mean ± SD. Statistical

comparisons were performed using one-way ANOVA. When significant values were found ($P < 0.05$), the Turkey's Honestly Significant Differences tests were used to compare the differences in two groups.

Results

Attenuation of ER stress promotes cisplatin-induced MG-63 cells and SaOS-2 cells apoptosis

As the UPR is of great importance to protect cells from apoptosis under stress, we explored the potential role of cisplatin-induced UPR in osteosarcoma cells. As showed in **Figure 1A**, the IC50 of cisplatin at MG-63 and SaOS-2 were 46.5 µmol/L and 39.1 µmol/L respectively. We prefer cisplatin at the concentration of 20 µmol/L in following study, because our previous report used cisplatin at 20 µmol/L [16]. **Figure 1B** and **1E** indicated that 4-PBA had an obviously cytotoxic effect on MG-63 cells and SaOS-2 cells at 20 mmol/L, whereas 4-PBA at the concentration of 2.5 mmol/L, 5 mmol/L and 10 mmol/L did not cause significant cell death. Then, 4-PBA treatment with the concentration of 2.5 mmol/L, 5 mmol/L and 10 mmol/L led to MG-63 cells and SaOS-2 cells more sensitive to cisplatin induced cell injury (**Figure 1C** and **1F**) and promoted cell apoptotic cell death (**Figure 1D** and **1G**). These data show that attenuation of ER stress promotes cisplatin-induced MG-63 cells and SaOS-2 cells apoptosis.

Cisplatin induces UPR in MG-63 and SaOS-2 cells

To explore whether cisplatin is able to trigger UPR induction in osteosarcoma cells, MG-63 cells and SaOS-2 cells were treated with cisplatin for indicated time. TG administration was used as positive control. Cisplatin treatment substantially elevated the expression of GRP78 (**Figure 2A** and **2C**) and CHOP (**Figure 2B** and **2D**) in MG-63 and SaOS-2 cells respectively. Then, MG-63 and SaOS-2 cells were treated with cisplatin in the presence or absence of 4-PBA (10 mmol/L). As expected, administration of 4-PBA reversed the increased GRP78 (**Figure 3A** and **3C**) and CHOP expression (**Figure 3B** and **3D**) in MG-63 and SaOS-2 cells respectively. Therefore, these findings show that cisplatin induces UPR activation in osteosarcoma cells.

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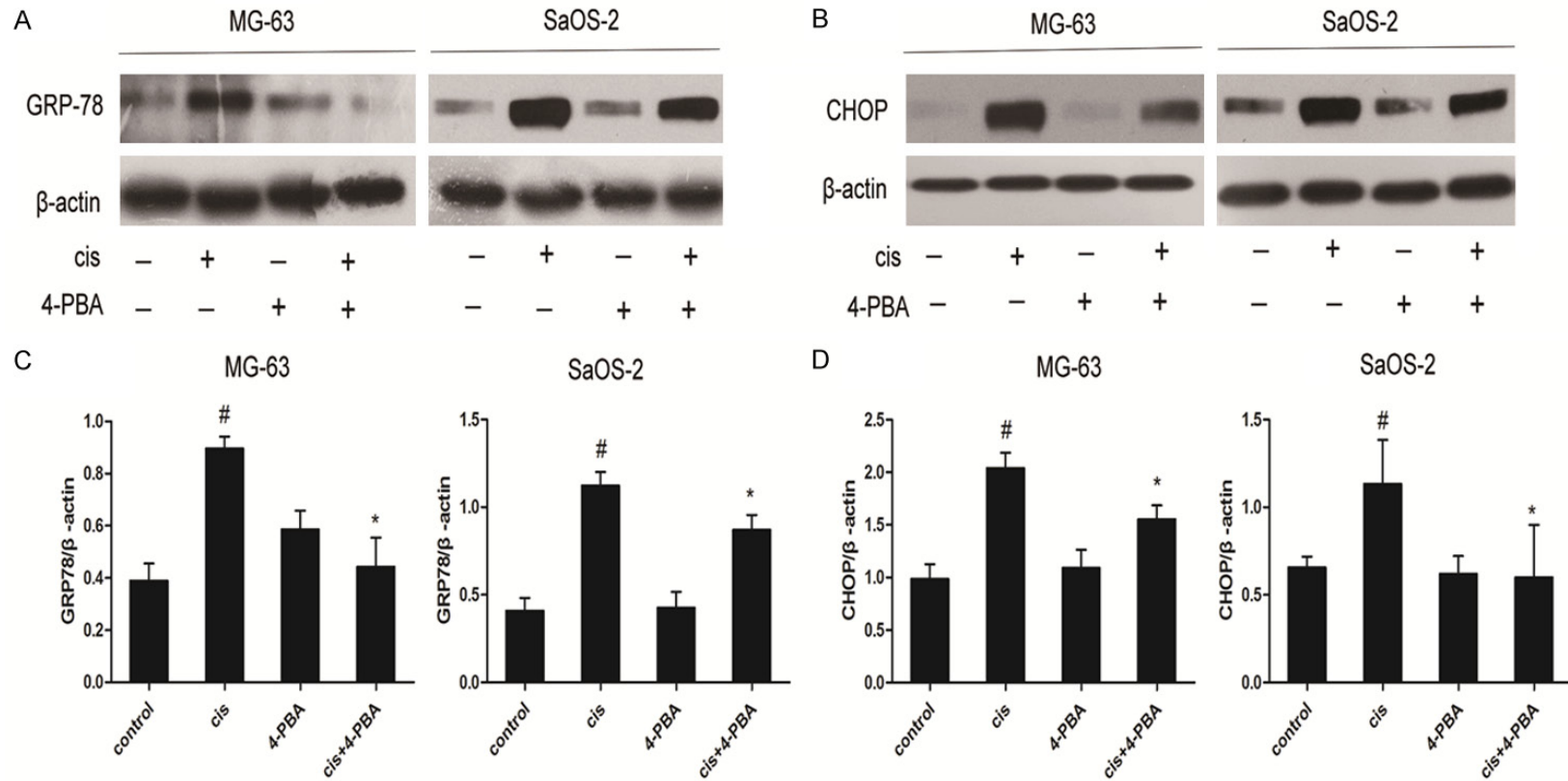


Figure 3. Effects of 4-PBA on the levels of GRP78 and CHOP protein in cisplatin-treated OS cells. MG-63 and SaOS-2 cells were treated with cisplatin (20 μmol/L) for 24 hours in the presence or absence 4-PBA (10 mmol/L). Representative Western blot analysis of GRP78 protein level (A) and CHOP protein level (B) in OS cells. Analyses of band intensity are shown as the relative ratio of GRP (C) and CHOP (D) to actin. Data are represented as mean ± SD. β-actin was served as load control. [#]P<0.05 versus control; ^{*}P<0.05 versus cisplatin. All figures are representatives of the results of three independent experiments.

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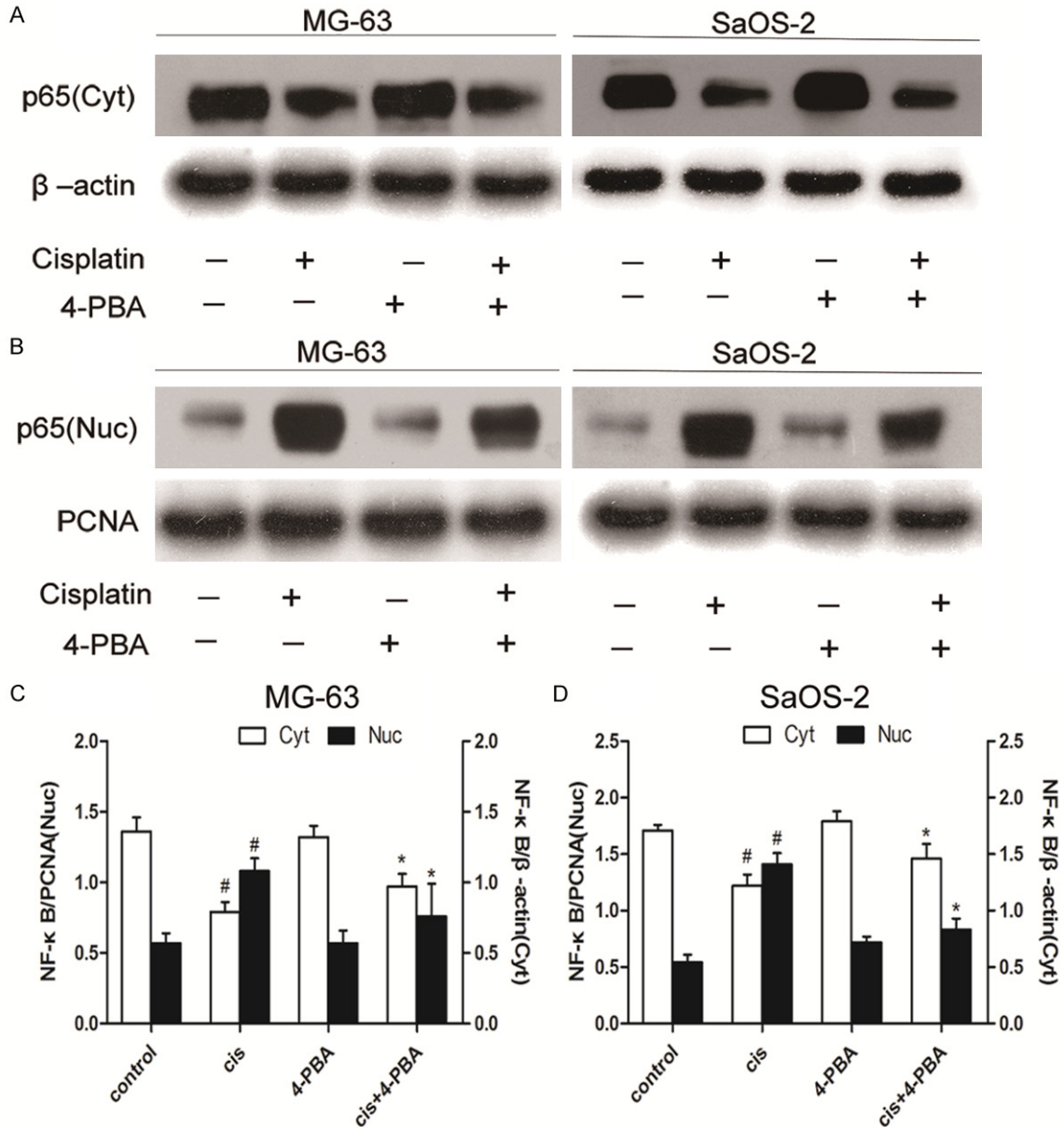


Figure 4. Effects of 4-PBA on the activations of NF-κB p65 in cisplatin-treated osteosarcoma cells. MG-63 and SaOS-2 cells were treated with cisplatin (20 μmol/L) for 24 hours in the presence or absence of 4-PBA (10 mmol/L). Representative Western blots of cytoplasmic NF-κB p65 subunit level (A) and nuclear NF-κB p65 subunit level (B) in OS cells. Analyses of band intensity are shown as the relative ratio of cytoplasmic NF-κB p65 to β-actin and nuclear NF-κB p65 to PCNA in MG-63 cells (C). Analyses of band intensity are shown as the relative ratio of cytoplasmic NF-κB p65 to β-actin and nuclear NF-κB p65 to PCNA in SaOS-2 cells (D). PCNA was served as nuclear load control. β-actin was served as cytoplasmic load control. Data are represented as mean ± SD. Figures of NF-κB p65 subunit are representatives of the results from three independent experiments. #*P*<0.05 versus control; **P*<0.05 versus cisplatin.

NF-κB is activated in OS cells treated with cisplatin

To validate whether NF-κB signaling is activated in MG-63 cells and SaOS-2 cells treated with cisplatin, the levels of NF-κB p65 in cytoplasm and nucleus were monitored. Administration of

cisplatin induced a moderate decrease of NF-κB p65 subunit level in cytoplasm (**Figure 4A**) and accumulation of NF-κB p65 subunit inside the nucleus (**Figure 4B**). In order to validate that NF-κB activation was related with UPR induction, we evaluated the activation of NF-κB in MG-63 and SaOS-2 cells following

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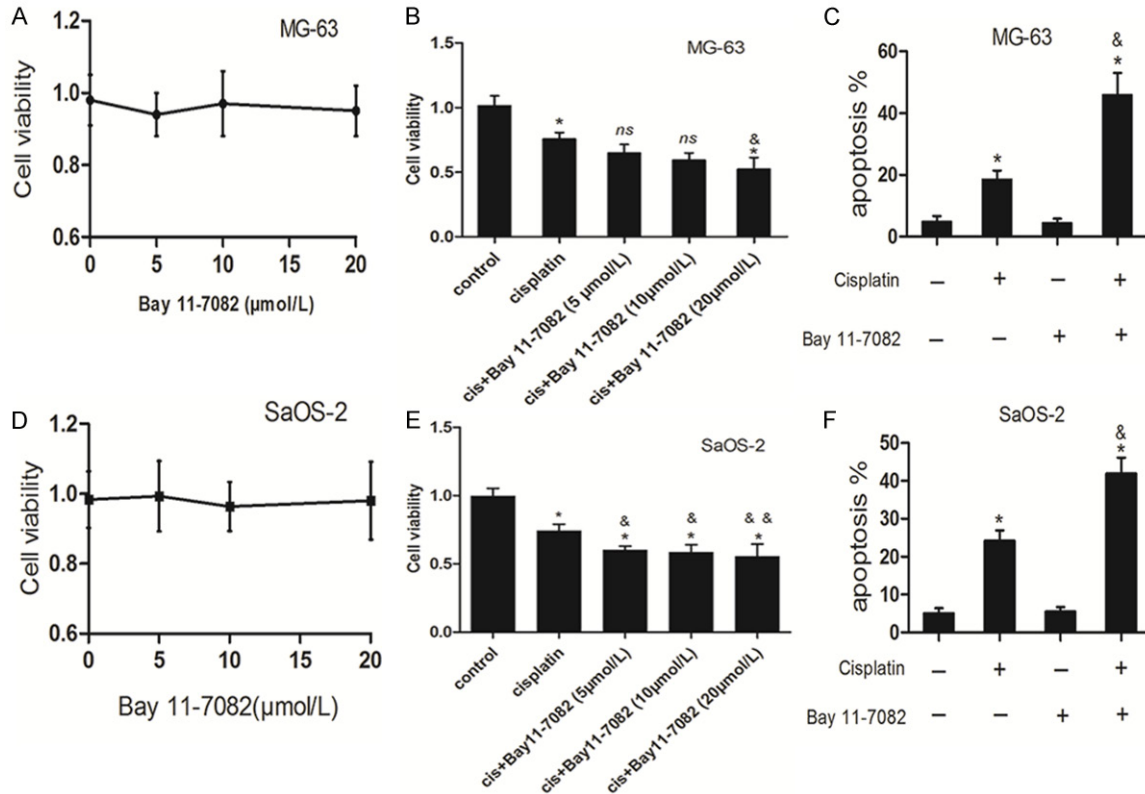


Figure 5. Activation of NF- κ B contributes to the cisplatin-treated osteosarcoma cells. MG-63 cells (A) and SaOS-2 cells (D) were treated with indicated concentrations of Bay 11-7082 alone for 24 hours and then cell viability was analyzed by MTT assay respectively ($n = 3$). MG-63 cells (B) and SaOS-2 cells (E) were treated with cisplatin (20 μ mol/L) in the presence or absence of indicated concentrations of Bay 11-7082 for 24 hours. Cell viability was analyzed by MTT assay ($n = 3$). MG-63 cells (C) and SaOS-2 cells (F) were treated with cisplatin (20 μ mol/L) in the presence or absence of Bay 11-7082 (20 μ mol/L) for 24 hours respectively. Apoptosis was analyzed by the Annexin V assay by flow cytometry ($n = 3$). Data are represented as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ compared to control; &* $P < 0.05$, &&* $P < 0.01$ compared to cisplatin alone; ns = no significance.

4-PBA treatment. As expected, 4-PBA treatment alone displayed little effect on the levels of cytoplasmic and nuclear p65 protein in OS cells. **Figure 4C** and **4D** showed that administration of 4-PBA decreased the levels of nuclear p65 protein and increased the levels of cytoplasmic p65 protein in cisplatin-treated OS cells. These findings support the fact that NF- κ B signaling is activated in cisplatin-treated MG-63 and SaOS-2 cells.

NF- κ B activation contributes to the cytoprotective role of UPR in cisplatin-treated OS cells

To validate whether NF- κ B signaling contributes to the cytoprotective role of UPR in cisplatin-treated osteosarcoma cells, MG-63 and SaOS-2 cells were pretreated with Bay 11-7082, a well-known inhibitor of NF- κ B. No significant cell death was observed after MG-63 and

SaOS-2 cells incubated with indicated concentration of Bay 11-7082 alone (**Figure 5A** and **5D**). **Figure 5B** indicated that co-treatment with cisplatin and Bay 11-7082 at 5, 10 μ mol/L showed no significant difference in cell viability when compared with that in cisplatin treatment alone in MG-63 cells. Indeed, Bay 11-7082 treatment at the concentration 20 μ mol/L led to MG-63 cells more sensitive to cisplatin-induced cell injury. Further, pretreatment with Bay 11-7082 (20 μ mol/L) obviously promoted the cisplatin-induced MG-63 cell apoptosis (**Figure 5C**). **Figure 5E** indicated SaOS-2 cells were more sensitive to cisplatin-induced cell injury after Bay 11-7082 addition. Cisplatin-induced SaOS-2 cells apoptosis was markedly increased following Bay 11-7082 administration (**Figure 5F**). These data suggest NF- κ B activation protects MG-63 and SaOS-2 cells from cisplatin-induced apoptosis.

Discussion

Although the therapeutic approaches such as neoadjuvant chemotherapy and surgical resection have developed rapidly, the cure rate of osteosarcoma still remains low. The developed drug resistance of OS is becoming a tough barrier to achieve the satisfied outcomes [17]. Drug resistance of OS attributes to various mechanisms including reduced intracellular drug concentration, dysfunction of membrane transport, inactivation of drug, anti-apoptosis, increased DNA repair and stem cell-like cancer cells induced chemoresistance [18]. In this study, we presented that UPR activation was another contributor to cisplatin resistance in OS. Mitigation of UPR sensitized OS cells to cisplatin. Meanwhile, activation of NF- κ B pathway contributed to the cytoprotective role of UPR in OS cells.

Cisplatin, a first class anti-tumor drug widely used in various cancers, targets the DNA to induce apoptosis through mitochondrial death pathway or Fas death pathway [19, 20]. Cisplatin-induced ER stress had been reported in human hepatocellular carcinoma cells and melanoma cells [21, 22]. Our study shows that cisplatin treatment induces the UPR activation. Moreover, the findings that 4-PBA mitigated cisplatin-induced increased expression of GRP78 and CHOP, provide additional support for this phenomenon. This observation consisted with previous report that indicated cisplatin treatment induced ER stress in rat kidney tissue *in vivo* [23]. It has been clear that activation of calpain in cisplatin manner is responsible for cisplatin induced ER stress [22].

In our study, attenuation of ER stress promoted cisplatin induced OS cells apoptosis. These results are in accord with recent reports [21-24]. It has been validated that UPR favors cancer cells adaption, escaping from cell demise and persistent growth in massive types of malignancies [25-27]. UPR aims to restore ER homeostasis during ER stress. However, if ER stress is severe and prolonger, cancer cells will succumb to UPR mediated apoptosis [28, 29]. The fact that UPR is cytoprotective as well as being cytotoxic has been supported by numerous evidences. For instance, the spliced X-boxing binding protein1 (XBP1s), downstream of IRE1, has been shown to contribute to the

angiogenesis, proliferation and drug resistance in tumors [30]. ATF6 activation enhances the transcription of genes encoding chaperones and enzymes that assist protein folding, which alleviates ER stress and improves the resistance of tumor to ER stress induced death [31, 32]. Meanwhile, ATF4, downstream of PERK/eukaryotic initiation factor 2 α (eIF2 α), promotes hypoxia resistance in tumors [33]. However, PERK dependent activation of CHOP dominates and leads to cell apoptosis in severe and prolonger ER stress [34]. Therefore, the double-edged sword role of UPR in cancer cells fate determination may depend on cell type and ER stress severity. The strategies that target UPR has mainly divided into two types: (1) enhancing misfolded proteins in ER lumen to initiate much more severe ER stress; (2) blocking UPR dependent pro-survival effect. Fortunately, these approaches have been confirmed to be effective to override the drug resistance in tumors [35]. In our study, UPR activation acts as a mechanism of survival in OS and targeting UPR shows a promising approach to overcome drug resistance in OS.

As NF- κ B plays a critical role in pro-survival and drug resistance in osteosarcoma cells [15], it is logical to present the hypothesis that NF- κ B signal is activated and protects osteosarcoma cells from cisplatin cytotoxicity. Our data suggest administration of cisplatin activates NF- κ B and this activation is suppressed following 4-PBA treatment. This phenomenon indicates that UPR contributes to NF- κ B activation, which consists with previous reports [36-38]. It has been clear that the PERK and IRE1, two arms of UPR, are responsible for NF- κ B activation. When UPR is initiated, phosphorylated eIF2 α by PERK suppresses general translation including the I κ B and NF- κ B. Because I κ B is characterized with shorter half-live than that in NF- κ B, general translational suppression augments the ratio of NF- κ B to I κ B. Therefore, NF- κ B dissociates from I κ B blockage and translocates into nucleus [36]. In response to ER stress, IRE1 undergoes autophosphorylation and binds to the adaptor tumor-necrosis factor- α -receptor-associated factor 2 (TRAF2). It has been found that IRE1/TRAF2 complex recruits and phosphorylates IKK. After that, IKK phosphorylates I κ B and elicits the ubiquitination and degradation of I κ B, leading to the nuclear delivery of NF- κ B [37]. Recently, the interplay of PERK and

IER1 activities was found to be essential for activation of NF- κ B in response to ER stress. Indeed, without IRE1 mediated basal phosphorylation of IKK, phosphorylated eIF2 α induced general translational suppression fails to decrease I κ B level enough to set a substantial number of NF- κ B free [39]. In our study, incubation with Bay 11-7082, an inhibitor of IKK, promotes cisplatin-induced OS cells apoptosis, which consisted with previous report [15]. It has been unveiled that NF- κ B contributes to anti-apoptosis and tumorigenesis through regulation of Bcl-xl, cFLIP and cIAP 1, cIAP2, cyclin D1, c-myc and Bfl-1 [40-42]. Some drugs that targeting NF- κ B signal has been demonstrated to sensitize tumors to anti-tumor agents [43-45].

In conclusion, treatment with cisplatin is able to induce UPR in OS cells and targeting UPR sensitizes OS cells to cisplatin. The cytoprotective role of UPR in OS cells may be through activation of NF- κ B pathway. Thus, these data suggest that targeting UPR and NF- κ B may be a potential strategy for osteosarcoma therapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jiandong Ni, Department of Orthopedic Surgery, The Second Xiangya Hospital, Central South University, 139 Renmin Road, Changsha, Hunan, China. Tel: 86-731-8529-2101; Fax: 86-731-8553-3525; E-mail: hpnjd@163.com

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