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ERK1/2/p53 and NF-κB dependent-PUMA activation involves in doxorubicin-induced cardiomyocyte apoptosis

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Abstract. – OBJECTIVE: Numerous studies have demonstrated that Doxorubicin (DOX) induces cardiomyocyte apoptosis, which is associated with DOX-induced acute and chronic cardiotoxicity. DOX activated ERP1/2 and NF-KB signals has been linked to DOX-induced apoptosis and cardiotoxicity. However, the underlying mechanisms responsible for DOX-induced apoptosis have not been completely elucidated. In this study, we determine whether both ERK1/2/p53-dependent and NF-κB dependent-PUMA activation was related to DOX-induced apoptosis in H9c2 cells.

MATERIALS AND METHODS: H9c2 cells were treated with DOX (1 μM) for 2-48 hours. To explore the effect of ERK1/2, NF-KB, P53 and PUMA on DOX-induced apoptosis in H9c2 cells, H9c2 cells were transfected with PUMA siRNA or p65 siRNA, or treated with PFT-α (a chemical inhibitor of p53), or PD98059 (ERK inhibitor) before DOX treatment. MTT, Flow cytometry, TUNEL, Western blot and EMSA assay was used to detect cell survival, apoptosis, protein expression and NF-KB activity.

RESULTS: DOX induced apoptosis and inhibited growth of H9c2 cells in a time-dependent manner. DOX activated ERK1/2, NF-KB, p53 and PUMA. Knockdown of PUMA completely blocked DOX-induced cell apoptosis and survival inhibition. Knockdown of NF-KB or ERK1/2 alone could partly block DOX-induced PUMA upregulation and cell apoptosis. However, knockdown of NF-KB and ERK1/2 together completely blocked DOX-induced cell apoptosis and PUMA upregulation. In addition, knockdown of ERK1/2 blocked p53-dependent PUMA upregulation.

CONCLUSIONS: DOX induced apoptosis and inhibited growth of H9c2 cells by activation of ERK1/2/p53 and NF-κB dependent-PUMA signaling pathway.

Key Words:
Doxorubicin, Apoptosis, ERK1/2, NF-KB, p53, PUMA.

Introduction

Doxorubicin (DOX) is the widely used anticancer agent. Although rapid progress has been made on the optimal usage of DOX for decades, its usefulness for this application can be limited by its ability to promote cardiotoxicity and heart failure[1,2]. But the mechanism by which DOX induces cardiac damage is still not completely elucidated.

Numerous mechanisms have been proposed, and apoptosis is believed to play a key role in this pathophysiology[3-5]. The mechanism of DOX-induced cardiomyocyte apoptosis has been extensively studied in both acute and chronic cardiotoxicity[7-9]. P53 is a tumor suppressor gene, *which* exerts its anticancer function by inducing cell apoptosis and cell cycle arrest in cancer cells. Wherease most chemotherapeutic agents functions by activation of p53, resulting in cytotoxicity. Scholars have shown[10-12] that ADR-induced cardiotoxicity and cardiomyocyte apoptosis is mediated by p53 protein. Although p53 tumor suppressor is needed for DNA damage-induced apoptosis, which of the many p53-regulated genes are required has remained unknown. Several p53-inducible genes, such as Apaf-1, caspase-9, Bax and IAP that play a role in the induction of apoptosis in response to p53 have been studied[13-15]. PUMA (p53 upregulated modulator of apoptosis) as a target for activation by p53 has recently found[16,17]. Expression of PUMA could result in a fast apoptosis that occurred much earlier than that resulting from expression of p53[18]. Li et al[19] have re-

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ported that DOX exposure could induce H9c2 cardiac myocytes apoptosis *in vitro* by activation of PUMA signal. We may suggested that DOX-induced apoptosis might through p53-PUMA signal pathway. In SH-SY5Y neuroblastoma cells, Zn2+ regulates the induction of PUMA through p53 and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways[20]. In cardiac myocytes, DNA lesions induced by ROS or directly by DOX activated ERK1/2, followed by increased phosphorylation of p53, the latter further up-regulated p53 downstream genes such as Bax[11,21]. We may suggest that ERK1/2 is involved in DOX-induced apoptosis, which might in part through activation of p53/PUMA signal pathway.

NF-kB is a transcription factor that regulates inflammatory and apoptotic pathways. It has dual role in regulation of apoptosis and organ protection[22]. Sun et al[23] have reported that PUMA was directly activated by p65 through the canonical NF-κB pathway following AKT inhibition. Wang et al[24] have demonstrated that PUMA is a direct target of NF-κB and mediates TNF-α-induced apoptosis *in vitro* and *in vivo*. As mentioned above, ERK1/2, NF-KB, p53 and PUMA may be related to DOX-induced apoptosis.

In the present study, we investigated how ERK1/2, NF-KB, P53 and PUMA regulated DOX-induced apoptosis in H9c2 cardiac myocytes.

Materials and Methods

Ethics Statement

Experiments were conducted according to the Declaration of Helsinki. This study was supported and approved by the Ethics Committee of Yishui Central Hospital of Linyi. All participants formally consented to participate in all stages of the study.

Cell Line and Culture

Rat embryonic ventricular myocardial H9c2 cells were purchased from Cell Bank (Chinese Academy of Sciences). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640, Gibco, Life Technologies Inc., Grand Island, NY, USA) with 10% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2. Cells were grown to 70% confluence and synchronized overnight in serum-free medium before experiments.

siRNA Transfection and Treatment

H9c2 cells were treated with Doxorubicin (DOX) (1 μM) for 2, 6, 12, 24 and 48 hours, respectively. To explore the effect of PUMA and NF-KB on DOX-induced apoptosis in H9c2 cells, H9c2 cells transfected with PUMA siRNA (2 μg) or p65 siRNA in OptiMEM (Gibco, Hangzhou, China) for 24 hours using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions, then treated with Doxorubicin (1 μM) for 2, 6, 12, 24 and 48 hours, respectively.

To explore the effect of ERK1/2 and p53 on DOX-induced apoptosis in H9c2 cells, H9c2 cells were preincubated with 20 uM Pifithrin-α (PFT-α), a chemical inhibitor of p53 for 6 hrs, or 20 nM ERK inhibitor, PD98059 for 6 h, then treated with DOX (1 μM) for 2-48h.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Viability of siRNA transfected or/and DOC or and PFT-α/ PD98059 treated H9c2 cells was assessed by MTT assay. After treatment, ~10,000 cells were seeded in a 96-well plates and incubated for another 24 hours. Then, 10 *μl* of MTT solution in PBS was added to the culture medium in each well at a concentration of 500 μg/ml and incubated at 37°C for 4 hours. Then, 100 μl of DMSO were added to the wells in the plate and incubated at 37°C overnight. The absorbance was measured at 570 nm in a Multiscan MS spectrophotometer (BioTek, Beijing, China).

Annexin V assay by Flow Cytometry

After siRNA transfection, inhibitor or/and DOX treatment, the cells were harvested and washed twice with cold PBS. Next, the cells were stained with Annexin V-FITC and 10 μl propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit (KEYGEN, Nanjing, China). The percentage of apoptotic cells was detected using a FACSCalibur Flow cytometer (BD, San Jose, CA, USA). All analyses were performed in triplicate.

TUNEL for Apoptosis Assay

To evaluate the apoptotic response, we performed the terminal deoxynucleotide transferase (TdT)-mediated biotin-dUTP nick end labeling technique using the commercially available *in situ* cell death detection kit (Boster, Wuhan, China). Briefly, after siRNA transfection, inhibitor or/and DOX treatment, 5,000 cells were washed, fixed with 4% buffered paraformaldehyde, and permeabilized with freshly prepared 0.1% Triton X-100 containing 0.1% sodium citrate. The cells were then incubated with TUNEL reaction mix-

ture for 1 h at 37°C in a humidified chamber. The slides were washed three times with PBS, and the incorporated biotin-dUTP was detected under a fluorescent microscope. Cell death was quantified as the relative percent of apoptosis compared to the controls.

Western Blot Assay

Western blotting was performed as previously described[25], with the antibodies for ERK1/2, pERK1/2,P53, PUMA and β-actin (Santa Cruz, Shanghai, China).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSA) was used to assess NF-κB activation. Briefly, nuclear extracts prepared from treated cells and tumor samples with Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) were incubated with biotin labeled double-stranded NF-κB oligonucleotide (5′-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGGGAGGCGTGG-3′; underline indicates NF-κB-binding sites) for 30 min at 37°C and EMSA was performed followed the instructions of LightShift chemiluminescent EMSA kit (Pierce, Beijing, China).

Statistical Analysis

Statistical significance of differences among groups was analyzed by the paired Student's *t*-test or one-way ANOVA followed by Newman-Keuls test. All data were represented as the mean ±SE of three different experiments. A probability of $p < 0.05$ was considered to represent a significant difference.

Results

Upregulation of PUMA by DOX Correlates with Apoptosis Induction in H9c2 cells

We treated H9c2 cells with doxorubicin (1 μM) for various intervals (2, 6, 12, 24 and 48 hours) as the previous report[19]. The peaks of PUMA protein induction were detected at 24 hours following 1 μM DOX treatment (Figure 1A), after 24 hours, PUMA expression was decreased. In contrast, DOX treatment did not upregulate other proapop-

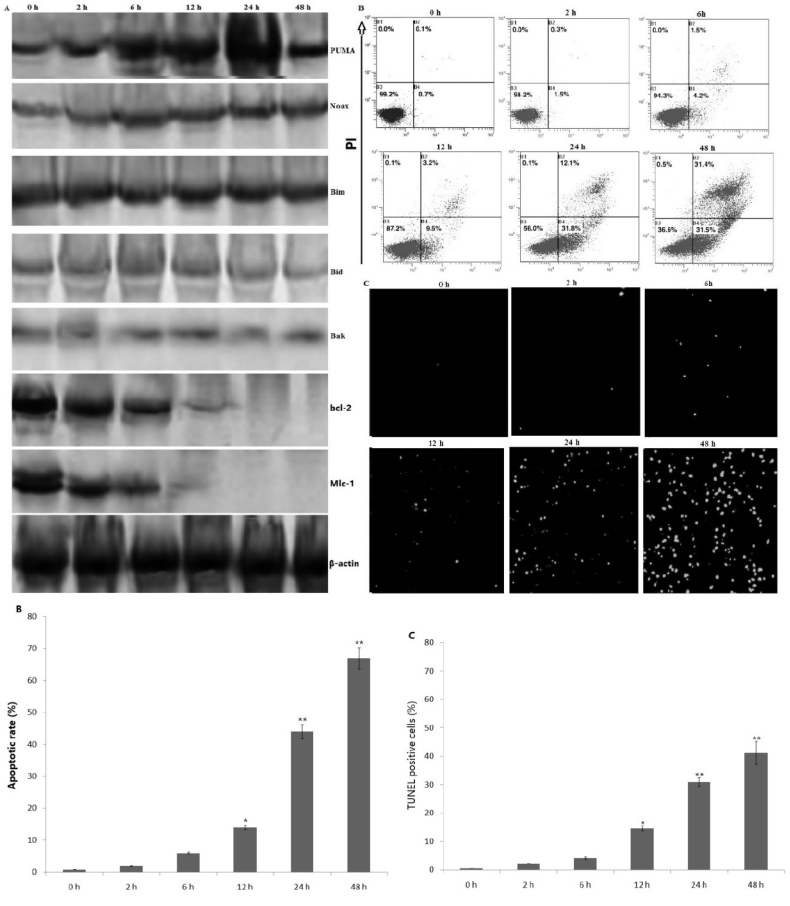

Figure 1. Effect of DOX on BH3-family protein expression and apoptosis of H9c2 cells. H9c2 cells with DOX (1 μM) for 2, 6, 12, 24 and 48 hours. ***A,*** BH3-family protein expression was detected by Western blot assay. ***B,*** Cell apoptotic rate was detected by flow cytometry assay. ***C,*** TUNEL analysis was used to test cell apoptosis. Histogram showing the number percentage of TUNEL-positive cells in total cell population, and bar graph represents the average value from there independent experiments; $^{*}p < 0.05$; $^{**}p < 0.01$.

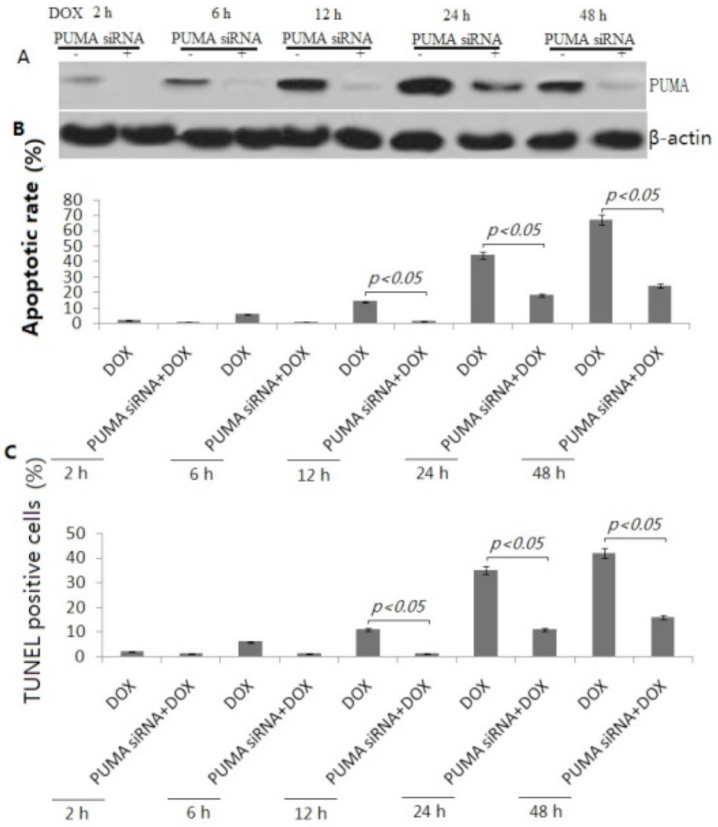

Figure 2. Effect of PUMA on DOX-induced apoptosis in H9c2 cells. H9c2 cells was transfected with PUMA siRNA or control siRNA for 24 hours, then treated with DOX (1 μM) for 2-48 hours. ***A,*** PUMA protein expression was detected by Western blot assay. ***B,*** Cell apoptotic rate was detected by flow cytometry assay. ***C,*** TUNEL analysis was used to test cell apoptosis, *vs.* control, $p < 0.05$.

totic Bcl-2 family members, including Bim, Bid, Bak and Noax, but reduced the expression of the antiapoptotic proteins Bcl-2 and Mcl-1 (Figure 1A). DOX caused time-dependent cell apoptosis in H9c2 cells. As shown in Figure 1B, following DOX treatment, many apoptotic H9c2 cells were shown by Annexin V/PI staining for FCM. The number of TUNEL-positive cells also increased with the duration of incubation (Figure 1C).

DOX Induces Apoptosis of H9c2 cells by PUMA-Dependent Pathway

We then investigated the role of PUMA in DOX-induced apoptosis in H9c2 cells. H9c2 cells was transfected with PUMA siRNA or control siRNA for 24 hours, then treated with DOX (1 μM) for 2-48 hours. As shown in Figure 2A, DOX-induced PUMA protein was completely blocked by PUMA transfection. Apoptosis induced by 1 μM DOX was significantly reduced in *PUMA* siRNA/H9c2 cells (Figure 2B). TUNEL staining confirmed the reduction of DOX-induced apoptosis in *PUMA* siRNA transfected H9c2 cells (Figure 2C).

DOX activated P53-dependent PUMA Expression

We first analyzed the mechanism of p53-dependent PUMA induction by DOX in H9c2 cells. H9c2 cells was exposure to 20 μM Pifithrin-α (PFT-α), a chemical inhibitor of p53 for 6 hours, then treated with DOX (1 μM) for 2-48 hours. The results showed that the peaks of p53 protein induction were detected at 12 hours following 1 μM DOX treatment (Figure 3). Treatment with 20 uM Pifithrin-α completely inhibited DOX-induced p53 upregulation, however, PUMA was partly inhibited with PFT-α treatment (Figure 3). It was indicated that DOX-induced PUMA upregulation was partly p53-dependent.

DOX activated NF-KB-dependent PUMA expression

The p65 subunit of NF-κB was recently identified as a transcriptional activator of *PUMA*. We next analyzed the mechanism of NF-KB-dependent PUMA induction by DOX in H9c2 cells. H9c2 cells was transfected with p65 siRNA or control siRNA for 24 hrs, then treated with 1

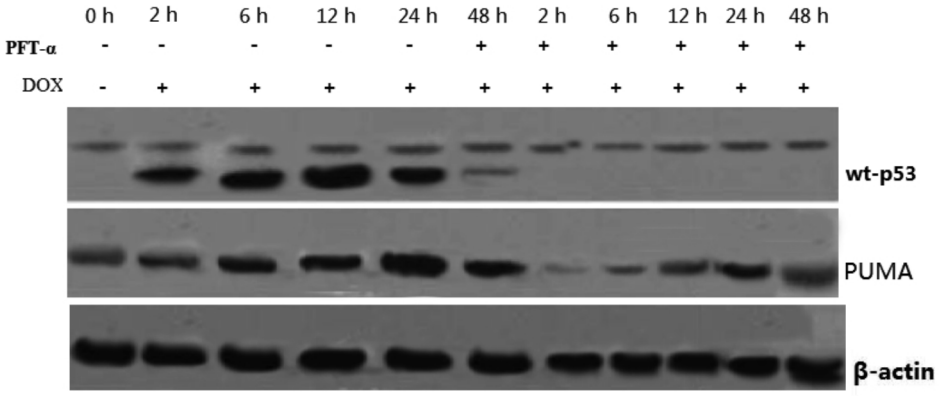

Figure 3. Effect of p53 on DOX activated PUMA expression. H9c2 cells was exposure to 20 uM Pifithrin-α (PFT-α) for 6 hours, then treated with Dox (1 μM) for 2-48 hours. P53 and PUMA protein expression was detected by Western blot assay.

μM DOX for 2-24 hrs. The results showed that the peaks of NF-KB activity and p65 expression were detected at 6 hours after DOX exposure (Figure 4A-4B). Knockdown of *p65* by siRNA completely abrogated NF-KB activity and p65 induction by DOX (Figure 4C), but partly abrogated PUMA induction by DOX (Figure 4D). It was indicated that DOX-induced PUMA upregulation was partly NF-KB-dependent. However, when the H9c2 cells was treated with p65 siRNA and Pifithrin-α together, DOX-induced PUMA was completely inhibited, suggested that PUMA activation by DOX is P53-dependent and NF-KB-dependent mechanism (Figure 4E).

DOX activated ERK1/2 dependent p53/PUMA signal pathway

H9c2 cells treated with DOX markedly induced ERK1/2 (T202/Y204) phosphorylation in a time-dependent manner. The peaks of phosphorylation of ERK1/2 induction were detected at 24 hours following 1 μM DOX treatment (Figure 4A). When the H9c2 cells was exposure to 20 nM ERK inhibitor PD98059 for 6 hours; then, treated with DOX (1 μM) for 2-24 hours, DOX induced ERK1/2 and PUMA expression was blocked (Figure 4B), but p65 phosphorylation and NF-KB activity did not affect (data not show), suggesting that ERK activationn mediates the activation of p53 and PUMA by DOX. PD98059 treatment did not affect DOX-induced P65 expression, suggesting that the NF-κB-dependent PUMA pathway is not involved in ERK1/2 activation by DOX.

Discussion

Doxorubicin (DOX), belonging to the anthracyclines, is one of the most widely used and successful antitumor drugs. Cardiotoxicity is a major limiting factor in anticancer therapy[26]. Acute or chronic cardiomyopathy represents for DOX-induced car-

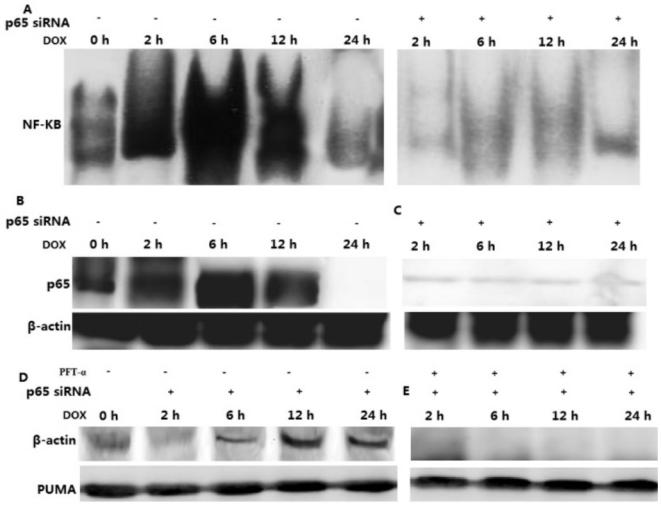

Figure 4. Effect of DOX on NF-KB activity and PUMA expression. ***A***, H9c2 cells was transfected with p65 siRNA for 24 hours, then treated with Dox (1 μM) for 2-24 hours. NF-KB activity was detected by EMSA assay. ***B, C***, p65 protein was detected by Western blot assay. ***D,*** E, H9c2 cells was treated with p65 siRNA and Pifithrin-α together, then treated with DOX (1 μM) for 2-24 hours. PUMA protein was detected by Western blot assay.

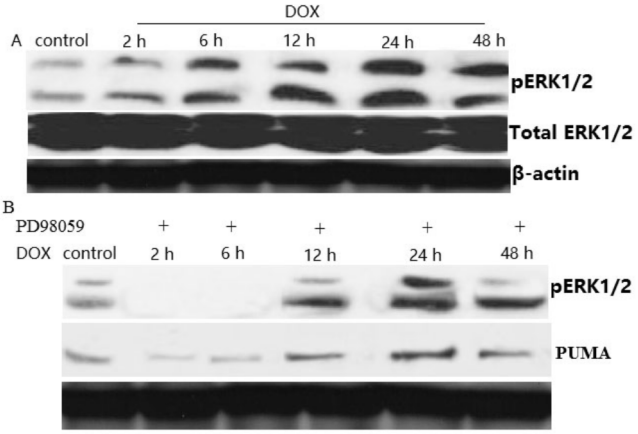

Figure 5. Effect of DOX on ERK1/2 dependent p53/PUMA expression. A, H9c2 cells was treated with 1 μM DOX for 2-48 hours. The phosphorylation of ERK1/2, total ERK1/2 and PUMA was detected by Western blot assay. B, H9c2 cells was treated with 20 nM ERK inhibitor PD98059 for 6 hours, then treated with 1 μM DOX for 2-48 hours. The phosphorylation of ERK1/2 and PUMA was detected by Western blot assay.

diotoxicity. The first is now rare, occurring after receiving high dose, and may present as acute tachyarrhythmias and acute heart failure while the latter is dose-dependent. Both acute and chronic DOX-induced cardiac toxicity may lead to cardiac dysfunction, cardiomyopathy, and eventually to severe heart failure and death[27]. Whereas DOX-induced cardiomyocyte apoptosis is the primary mechanism for DOX-induced heart failure and death[7-9].

Here, we found that Doxorubicin (DOX) significantly induced apoptosis of H9c2 cells *in vitro*, supporting that apoptosis is one of the reasons presenting DOX-induced cardiotoxicity. However, by what signaling that DOX induces apoptosis of H9c2 cells is not very clear.

PUMA is a member of the"BH3-only" branch of the Bcl-2 protein family members, which are shown to initiate cell apoptosis induced by a wide variety of stimuli by the mitochondria signaling pathway[27,28]. Previous studied[29-32] have found that elevated PUMA expression, either alone or in combination with chemotherapy or irradiation, induced profound toxicity to a variety of cancer cells, including lung, head and neck, esophagus and breast cancer cells. In our work, DOX induced apoptosis of H9c2 cells, followed by PUMA activity. However, knockdown of PUMA blocked DOX-induced cell apoptosis, suggested that PUMA is related to DOX-induced apoptosis of H9c2 cells.

Studies[11,12,33,34] have demonstrated that DOX induced not only p53 activation and cardiomyocyte apoptosis *in vitro* but also in p53 knockout mice and in adult mouse hearts expressing cardiomyocyte-restricted dominant-interfering p53. In our study, p53 was activated at 2 hours-24 hours, and reached the peak at 12 hours after DOX treatment. However, knockdown of p53 by Pifithrin-α (PFT-α), a chemical inhibitor of p53, blocked DOX-induced PUMA upregulation and DOX-induced cell apoptosis, suggested that p53 is related to DOX-induced apoptosis of H9c2 cells. Furthermore, DOX-induced cell apoptosis was achieved by p53-dependent PUMA upregulation.

It has found that DOX could activat ERK1/2, followed by increased phosphorylation of p53, and activated the intrinsic apoptosis pathway[11,21]. However, inhibitor of p53 effectively inhibited DOX-induced apoptosis in H9c2 cells, neonatal rat cardiomyocytes, and mouse hearts[11,21]. In our study, we found that knockdown of pERK1/2 by ERK inhibitor PD98059 blocked the activation of p53 and PUMA, and DOX-reduced apoptosis of H9c2 cells, suggesting that ERK1/2-p53/PUMA signal is related to DOX-induced apoptosis of H9c2 cells.

We also found in our investigations that knockdown of ERK1/2 or p53 partly involved DOX-induced PUMA activation, and DOX-induced cell apoptosis. Therefore, we suggested that other signals might be related to DOX-induced PUMA activation and cell apoptosis except for ERK1/2-p53/PUMA signal.

We further studied the effect of NF-KB on DOX-induced cell apoptosis. Our research found here that DOX treatment activated NF-KB and increase p65 nucleus translocation. However, knockdown of p65 by siRNA inhibited PUMA upregulation and DOX-induced cell apoptosis of H9c2 cells, suggesting that NF-KB/PUMA signal is related to DOX-induced apoptosis of H9c2 cells.

Some authors have reported that NF-kappaB plays an essential role in activation of wild-type p53 tumor suppressor to initiate proapoptotic signaling in response to overgeneration of superoxide[35], and cerebral ischemia[36]. In the present

study, we found treatment of H9c2 cells with Pifithrin-α (PFT-α) did not affect NF-KB activity and p65 translocation (data not shown). In addition, knockdown of p65 by siRNA did not affect p53 expression, suggesting NF-kB/PUMA signaling was independent of ERK1/2 and p53.

Althought knockdown of NF-KB or ERK1/2 could partly inhibit DOX-induced apoptosis and PUMA upregulation, knockdown of NF-KB in combination ERK1/2 inhibition could completely inhibit PUMA expression and DOX-induced apoptosis of H9c2 cells. It was demonstrated that NF-KB/PUMA and ERK1/2/P53/PUMA were involved in DOX-induced cell apoptosis.

Conclusions

Our investigation demonstrated that ERKs/p53/PUMA and NF-KB/PUMA signal transduction pathway is involved in doxorubicin-induced apoptosis in H9c2 cells. Knockdown of PUMA could completely inhibit doxorubicin-induced cardiomyocyte apoptosis. These findings contribute to our insights of doxorubicin-induced cardiac toxicity and identify new targets for strategies to treat and prevent doxorubicin-induced cardiomyopathy.

Acknowledgements

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Conflicts of interest

The authors declare no conflicts of interest.

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