European Review for Medical and Pharmacological Sciences 2013; 17: 2323-2329

Doxorubicin-induced apoptosis in H9c2 cardiomyocytes by NF-κB dependent PUMA upregulation

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Abstract. – OBJECTIVE: The cardiotoxicity of doxorubicin limits its clinical use in the treatment of a variety of solid tumors and malignant hematologic disease. Although the mechanism by which it causes cardiac injury is not yet known, apoptosis has been regarded as one of mechanisms underlying the cardiotoxic effects of doxorubicin. In the present study, we investigates the mechanisms of doxorubicin-induced apoptosis in H9c2 cardiomyocytes by NF-κB dependent PUMA upregulation.

MATERIALS AND METHODS: H9c2 cardiac myocytes was treated with 5-50 μM concentration of doxorubicin for 72 hours. p65 siRNA or PUMA siRNA was transfected into H9c2 cardiac myocytes, then treated with 50 μM doxorubicin for 72 hours. Apoptotic cells were detected using Flow cytometric analysis. MTT cytotoxicity test was used to detect the effect of doxorubicin on H9c2 cardiac myocytes. PUMA expression was detected by western blot assay. NF-kappaB binding activities was detected using electrophoretic mobility shift assays (EMSA).

RESULTS: Doxorubicin promotes apoptosis and inhibits proliferation in H9c2 cardiac myocytes in a dose and time-dependent way. Doxorubicin treatment increased NF-kappaB binding activities and PUMA expression. Inhibition of p65 by p65 siRNA decreased PUMA expression of doxorubicin-induced. Furthermore, p65 siRNA or PUMA siRNA-mediated suppression of p65 or PUMA results in inhibition of apoptosis of doxorubicin-induced.

CONCLUSIONS: It is demonstrated that PUMA is a direct target of NF-κB and mediates doxorubicin-induced apoptosis in vitro.

Key words:

Cardiac myocytes, Nuclear factor-κB (NF-κB), p53 upregulated modulator of apoptosis (PUMA), Doxorubicin, Apoptosis.

Introduction

Doxorubicin is an effective, widely used chemotherapeutic agent in the treatment of a variety of solid tumors and malignant hematologic disease[1]. However, cardiac toxicity, including the development of cardiomyopathy and clinical congestive failure, restricts the clinical use of the drug[2]. Therefore, an understanding of the mechanisms that underlie doxorubicin-induced cardiac injury is crucial for the development of strategies to inhibit its cardiotoxic action.

Studies have shown that treatment with doxorubicin results in transactivation into the nucleus to induce the expression of genes associated with cell arrest and apoptosis in the heart[3-7]. A molecular mechanism leading to doxorubicin-induced cardiomyopathy has been proposed to account for increasing oxidant production, altered calcium handling, and mitochondrial injury[3-7]. This is probably reflected by the profound cardiomyocyte damage in certain states, which interferes with mitochondrial function.

Nuclear factor-κB (NF-κB) is the best-known mediator of doxorubicin-associated cellular response[8-11]. NF-κB is a group of dimeric transcription factors comprising members of the NF-κB/Rel family, including p50, p52, p65 (Rel-A), Rel-B, and c-Rel[12]. Although p50 and p65 regulate the canonical NF-vB pathway, p52 and Rel-B are components of the noncanonical NF-κB pathway. The activity of NF-κB is normally kept in check by the IκB family of inhibitors, which bind to and sequester NF-κB in the cytoplasm[13]. Activation of NF-κB is triggered by IκB phosphorylation by IKK kinases and subsequent proteasomal degradation, which allows NF-κB to translocate to the nucleus, where it binds to the κB consensus sequences and modulates numerous target genes[14].

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A large body of evidence has demonstrated a protective role of NF-κB in doxorubicin-induced apoptosis in most tissues and cell types. NF-κB has been found to promote apoptosis under certain conditions by activating the expression of proapoptotic proteins, such as p53, Fas, Fas ligand, death receptor[13], and death receptor[14]. However, the mechanisms and physiological significance of NF-κB in apoptosis regulation remain controversial and poorly understood.

p53 upregulated modulator of apoptosis (PUMA) is a downstream target of p53 and a BH3-only Bcl-2 family member[15,16]. It is induced by p53 following exposure to DNA-damaging agents, such as γ-irradiation and commonly used chemotherapeutic drugs[17-19]. The proapoptotic function of PUMA is mediated by its interactions with antiapoptotic Bcl-2 family members, which lead to Bax/Bak-dependent mitochondrial dysfunction and caspase activation. In cardiomyocytes, mechanical stress enhanced apoptosis by PUMA expression upregulation[20-22]. Studies using PUMA-knockout cardiomyocyte cells and mice revealed that PUMA is essential for DNA damage, endoplasmic reticulum stress, ischemia/reperfusion-induced apoptosis[23-24]. However, the mechanisms and functions of PUMA induction remain unclear.

It has reported that PUMA is regulated by NF-κB after exposure to gemcitabine treatment[19], and proinflammatory cytokines and endoplasmic reticulum stress[25]. In the present study we investigated whether PUMA is activated by NF-κB in response to doxorubicin treatment,and whether PUMA is a critical mediator of doxorubicin -induced apoptosis *in vitro*.

Materials and methods

Cell lines and culture conditions

The embryonic rat heart-derived myogenic cell line H9c2 was obtained from the American Type Culture Collection. These cells were cultured in DMEM, supplemented with 10% FCS and 4 mmol/L glutamine. Cells were grown at 37°C in an atmosphere of 5% CO_2.

siRNA transfection

PUMA siRNA or p65 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection of PUMA siRNA or p65 siRNA was performed using Lipofectamine (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instructions. The final concentration of the siRNAs for transfection was 100 nmol/L. Transfected H9c2 cells were washed with PBS (phosphate buffered saline), and then incubated in new culture media for an additional 48 h for doxorubicin treatment and Western blot assays.

Doxorubicin treatment

H9c2 cells were treated with 5-50 μM doxorubicin for 72 hours, or 25 μM doxorubicin for 24-72 hours. PUMA siRNA/H9c2 cells or p65 siRNA/H9c2 cells were treated with 10-50 μM doxorubicin for 72 hours. Then the cells was used for further study below.

Western Blot Analysis

After specific treatments, H9c2 cells were incubated in lysis buffer containing Tris-HCl 20 mM (pH 7.5), 1% Triton X-100, ethylene diamine tetracetic acid (EDTA) 1 mM, NaCl 150 mM, 10% glycerol, Na3VO4 1 mM, NaF 50 mM, phenylmethyl sulfonylfluoride (PMSF) 1 mM and protease inhibitor mixtures for 20 min on ice. After insoluble debris was precipitated by centrifugation at 13000×g at 4°C for 15 min, the supernatants were collected. An equal amount of protein per sample (15 mg) was resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. The transferred membranes were blocked for 1 h in 5% nonfat milk in PBS containing 0.05% Tween 20 and incubated with appropriate primary antibodies (anti-p65 and anti-PUMA) and HRP-conjugated secondary antibodies. The immune complexes were detected with a SuperSignal West Pico Chemiluminescent substrate kit (Pierce, Rockford, IL, USA).

Electrophoretic mobility shift assay (EMSA)

After specific treatments, H9c2 cell nuclear extracts were prepared. Doublestranded oligonucleotides containing the sequences corresponding to NF-kB consensus site (5'-AGTTGAGGGGACTTTCCCAGGC-3' 3'-TCAACTCCCCTGAAAGGGTCCG-5') were 3'-end labeled with biotin. Binding reactions were carried out in a final volume of 20 ul. Nuclear extract (10 ug) was incubated with 3 μl buffer D (2 mM HEPES, 10 mM KCl, 50 uM EDTA, 0.02% NP40, 2% glycerol), 2 μl buffer F (20 mM HEPES, 60 mM KCl, 4% Ficoll 400, 2

mM DTT, 1 μg/μl BSA), polydI/dC (50 ng/μl) and 4 pmol biotin-labeled double-stranded NF-kB oligonucleotides or the 100-foldmolar excess of unlabeled oligonucleotide competitors. The mixtures were incubated for 20 min at room temperature, followed by another 10 min on ice. Samples were subjected to electrophoresis in 6% nondenaturating polyacrylamide gel in a 0.5× TBE (Tris/Borate/EDTA) buffer system. The gel was transferred to a nylon membrane (Amersham, Buckinghamshire, UK) by electroblotting. Detection of p65-DNA complexes were performed using the Chemiluminescent Nucleic Acid Detection Module (Pierce). In brief, after cross-linking, the blot was incubated in blocking buffer for 15 min at 37°C. Streptavidin-peroxidase was added for 15 min in blocking buffer at room temperature, and the blot was then washed six times in wash buffer. The blot was incubated for 5 min in equilibration buffer and developed with the chemiluminescent reagents provided with the kit.

Flow cytometric analysis for apoptotic quantification

After specific treatments cardiomyocytes were fixed with 70% ethanol and treated with RNase. Then nuclei were stained with propidium iodide (Molecular Probes, Eugene, OR, USA) and FITC Annexin V. DNA content was measured using by a FACS Calibur flow cytometer and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA). Ten thousand cells were counted in all assays. Apoptotic cells were quantified as the percentage of cells stained with Annexin V.

MTT assay.

After specific treatments, cell viability was measured using the MTT dye reduction assay as previously described[26].

Statistical analysis

The results are presented as mean±SD. Differences between various data sets were tested for significance using Student's *t*-test and $p < 0.05$ was considered significant.

Results

Doxorubicin enhances the expression of PUMA protein and NF-kB binding activities

H9c2 cells was treated with 10-50 uM doxorubicin for 72 hours or 50 uM doxorubicin for 24-72 hours. The PUMA protein expression (Figure 1A) and NF-kappaB binding activities (Figure 1B) in H9c2 cells was significantly increased in a dose-and time-dependent way.

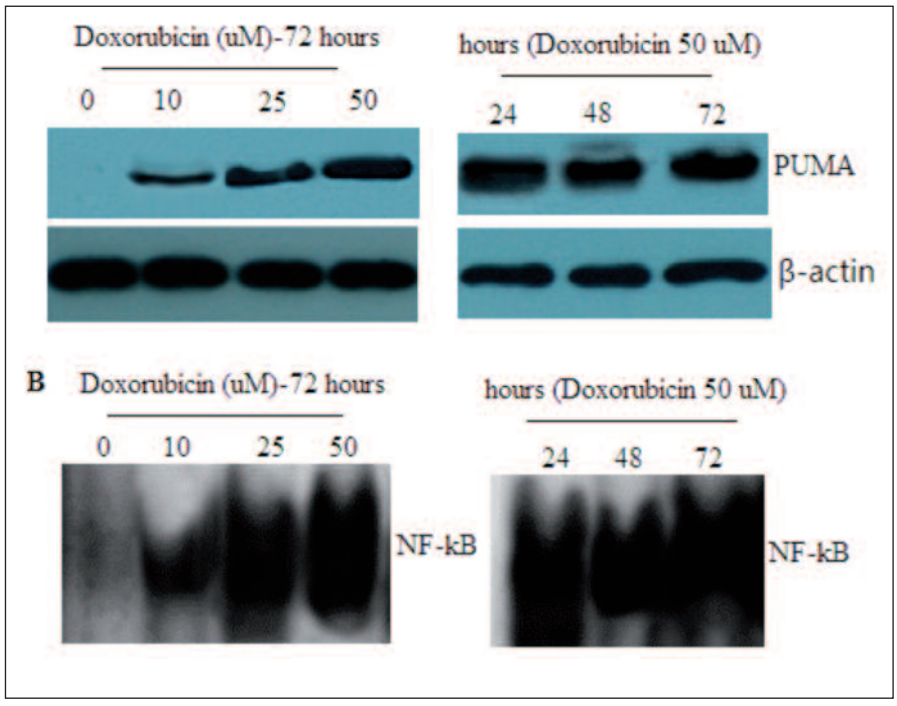

Figure 1. The effect of doxorubicin treatment on PUMA protein and NF-kB binding activities. H9c2 cells was treated with 10-50 μM doxorubicin for 72 hours or 50 μM doxorubicin for 24-72 hours.The PUMA protein was detected by western blot assay (**A**). NF-kB binding activities was detected by EMSA (**B**).

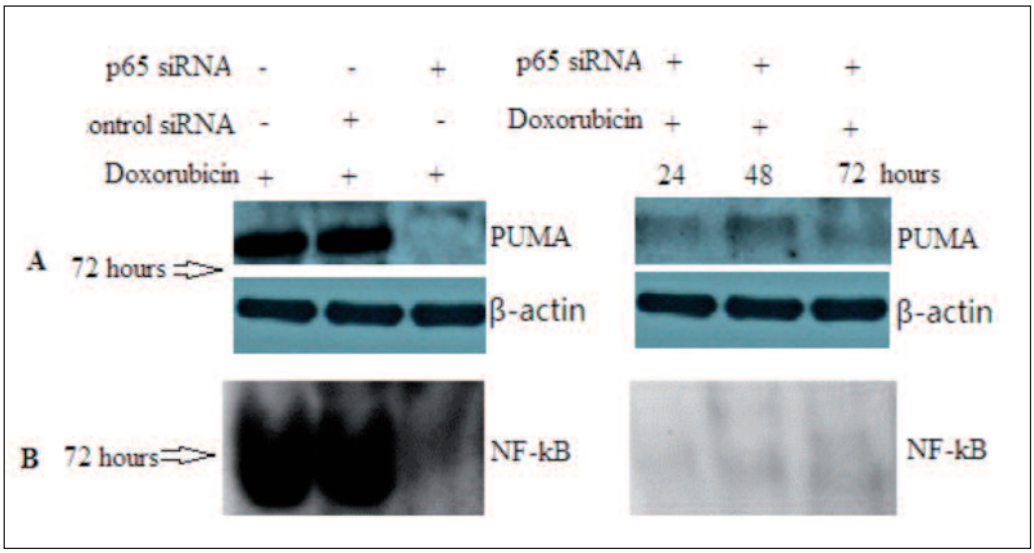

Figure 2. Doxorubicin enhances the expression of PUMA by NF-kB-dependent parthway. H9c2 cells was transfected with 100 uM siRNA p65 for 48 hours, then treated with 50 μM doxorubicin for 72 hours or 50 μM doxorubicin for 24-72 hours. The PUMA protein was detected by western blot assay (**A**). NF-κB binding activities was detected by EMSA (**B**).

Doxorubicin enhances the expression of PUMA by NF-kB-dependent parthway

H9c2 cells was transfected with 100 μM siRNA p65 for 48 hours, then treated with 50 μM doxorubicin for 72 hours or 50 uM doxorubicin for 24-72 hours. The PUMA protein expression (Figure 2A) and NF-kB binding activities (Figure 2B) in H9c2 cells was significantly decreased. However, when H9c2 cells was transfected with 100 nM PUMA siRNA, NF-kB binding activities was still increased after being treated with 50 μM doxorubicin for 72 hours or 50 μM doxorubicin for 24-72 hours (data not shown).

Doxorubicin induces apoptosis and decreases proliferation

H9c2 cells was treated with 10-50 μM doxorubicin for 72 hours. The apoptic cells was significantly increased in H9c2 cells in a dose-dependant way after doxorubicin treatment (Figure 3A). Furthermore, cell proliferation was significantly inhibited in H9c2 cells in a dose-dependent way (Figure 3B). Otherwise, H9c2 cells was treated with 50 μM Doxorubicin for 24-72 hours.The apoptic cells was significantly increased in a time-dependent way, and cell proliferation was significantly inhibited in H9c2 cells in a time-dependent way (data not shown).

To determine the involvement of PUMA in doxorubicin-induced apoptosis, H9c2 cells were transfected with siRNA targeting PUMA (or control; 100 nM; 48 hr) and treated with doxorubicin (10-50 μM; 72 hr). As shown in Figure 3A, in the presence of the control siRNA, doxorubicin significantly increased the percentage of cells undergoing apoptosis. By contrast, PUMA siRNA downregulated PUMA and attenuated the doxorubicin-induced upregulation of PUMA. Furthermore, doxorubicin-induced apoptosis was significantly inhibited by PUMA siRNA. Furthermore, PUMA siRNA inhibits the doxorubicin-induced proliferation inhibition (Figure 3B). Taken together, these results demonstrate that PUMA is required for sensitivity to doxorubicin-induced apoptosis and proliferation inhibition in H9c2 cells, and that PUMA is necessary for this effect.

Doxorubicin-induced apoptosis by NF-κB dependent PUMA upregulation

To determine the involvement of NF-κB in doxorubicin-induced apoptosis, H9c2 cells were transfected with siRNA targeting p65 siRNA (or control; 100 nM; 48 hr) and treated with doxorubicin (10-50 uM; 72 hr). As shown in Figure 3A, p65 siRNA downregulated p65 activity and attenuated the doxorubicin-induced upregulation of p65 activity. Furthermore, doxorubicin-induced apoptosis was significantly inhibited by p65 siRNA. p65 siRNA inhibits the doxorubicin-induced proliferation inhibition (Figure 3B). Furthermore, the PUMA protein expression in H9c2 cells was significantly decreased followed by decreased NF-kB binding activities (Figure 2).

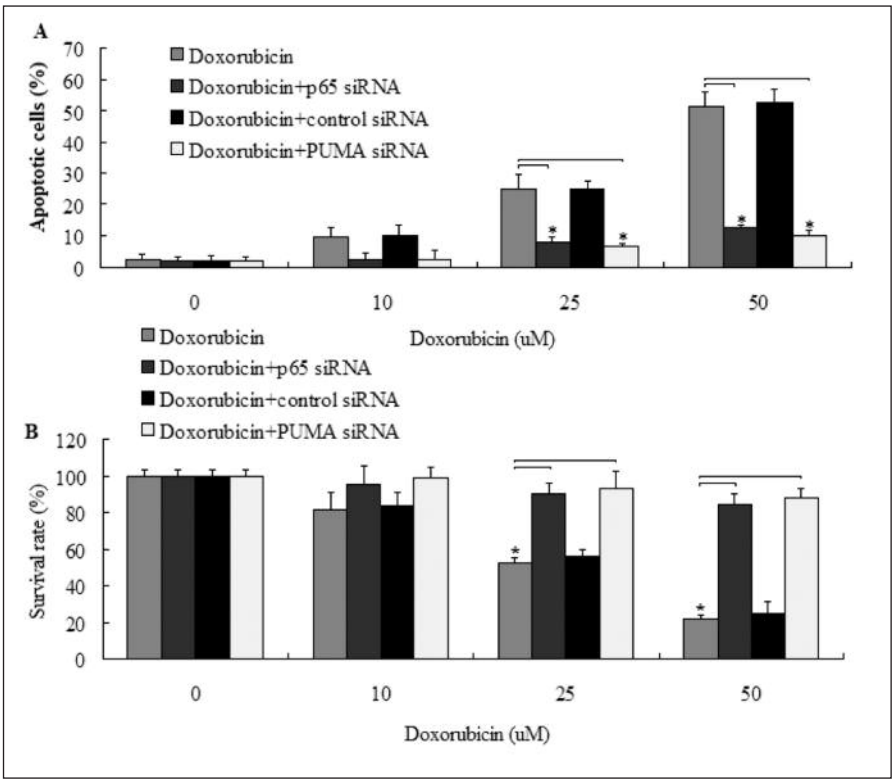

Figure 3. Doxorubicin-induced apoptosis in H9c2 cardiomyocytes by NF-κB dependent PUMA upregulation. A, H9c2 cells were transfected with siRNA targeting PUMA or siRNA p65 (or control; 100 nM; 48 hr) and treated with doxorubicin (10-50 μM; 72 hr) and apoptosis was determined by flow cytometric analysis. B. H9c2 cells were transfected with siRNA targeting PUMA or siRNA p65 (or control; 100 nM; 48 hr) and treated with doxorubicin (10-50 μM; 72 hr) and proliferation was determined by MTT analysis. $^{*}p < 0.05$.
PUMA is required for doxorubicin-induced apoptosis

Discussion

It was reported that apoptosis is the principal process in doxorubicin-induced cardiomyopathy[27]. In this study, we identified PUMA as a novel target of NF-κB and a critical mediator of doxorubicin-induced apoptosis in vitro in H9c2 cells. PUMA protein was consistently activated in cells treated with doxorubicin in a time- and does-dependent way. The induction of PUMA by doxorubicin required the p65 subunit of NF-κB. These results established the first case of direct regulation of PUMA by NF-κB in the doxorubicin response. Previous studies have shown that PUMA can be induced by hypoxia/reoxygenation in the cardiomyocytes[28], and targeted deletion of PUMA attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion[29]. A rapidly expanding body of evidence[30] supports the notion that cardiomyocyte death by apoptosis and necrosis is a primary mechanism of doxorubicin-induced cardiomyopathy and that other types of cell death, such as autophagy and senescence/aging, may participate in this process. Wold et al[31] has reported that doxorubicin impairs cardiac contractile property in single myocytes through an oxidative stress-mediated pathway. Our study revealed a new PUMA induction mechanism, which may be important in apoptosis triggered by doxorubicin.

Recent study[31] found that adriamycin induces myocardium apoptosis through activation of NF-kB in rat, and inhibition of NF-kB activation could decrease adriamycin-induced apoptosis in H9C2 cardiac muscle cells via[32-33]. We also demonstrated that the cardiacmyocyte apoptosis caused by doxorubicin was associated with the NF-kB activation.

Because PUMA is one of the most potent inducers of apoptosis[15], such cooperative effects may result from coordinated induction of PUMA by p65. Activation of NF-kB is known to render cancer cells resistant to anticancer drugs. Inhibition of NF-kB has been explored as an attractive

approach for anticancer therapies[34]. However, our data suggest that NF-kB inhibition can compromise PUMA induction by doxorubicin, which may be involved in inducting of H9C2 cell apoptosis and be beneficial for cardiacmyocyte protection therapies.

Conclusions

As PUMA are essential for triggering and completing apoptosis, our present results on the generation of PUMA by activation of NF-kB can be correlated with doxorubicin-induced H9c2 cell apoptosis. Our data suggest that PUMA functions as a novel link between the extrinsic and intrinsic apoptotic pathways.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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