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Adriamycin induces myocardium apoptosis through activation of nuclear factor κB in rat

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Abstract Adriamycin is one of the most effective and useful antineoplastic agents. Acute doxorubicin cardiotoxicity involved cardiomyocyte apoptosis. In this study, we investigated whether adriamycin induced myocardium apoptosis through activation of nuclear factor κB in rat. Forty male Wistar rats were randomly divided into five groups: control, ADR 5 mg/kg, ADR 10 mg/kg, ADR 15 mg/kg group and ADR + PDTC 200 mg/ml group. Myocardial apoptosis was detected by DNA fragmentation assay and TUNEL assay; Location and distribution of p-IκBα was observed by immunohistochemical assay; Myocardial expression of p-IκBα protein was assessed by Western blot analysis; Activity of NF-κB was evaluated by Electrophoretic Mobility Shift Assay. The myocardial apoptotic index, expression of p-IκBα, and binding activity of NF-κB increased significantly in ADR groups in dose-dependent manner. PDTC as a nonspecific inhibitor of NF-κB protected myocardium from apoptosis by inhibiting NF-κB activation. Adriamycin induces myocardium apoptosis through activation of nuclear factor κB in rat and NF-κB activation requires IκBα degradation.

Keywords Adriamycin · Nuclear factor-κ B · Myocardium · Apoptosis

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Introduction

The anthracycline antibiotic adriamycin (ADR) is one of the most effective and useful antineoplastic agents for the treatment of hematological as well as solid malignancies [1, 2]. However, its major adverse side effect is the frequent onset of acute and chronic myocardial injury [3, 4]. Recent studies showed that acute doxorubicin cardiotoxicity involved cardiomyocyte apoptosis [5]. Several researches made efforts to understand the signaling pathways responsible for ADR-induced apoptosis [6, 7]. One of the major signaling pathways is the nuclear transcription factor nuclear factor κB (NF-κB).

Nuclear factor-κB was first found in B-lymphocyte nucleus as a transcription factor that binds to an enhancer of the immunoglobulin κ-light chain gene. Since then, NF-κB has been identified in many different cells and is found to be activated by a wide range of inducers, including cytokines, ultraviolet irradiation, and chemotherapeutic agents. It has been shown that pyrrolidine dithiocarbamate (PDTC) can inhibit activity of NF-κB in vivo and in vitro studies [8, 9]. In quiescent cells, NF-κB is maintained as an inactive form in the cytoplasm where it is bound to IκB inhibitor proteins, which prevent NF-κB from entering the nuclei. Upon cellular activation by extracellular stimuli, IκB is phosphorylated and proteolytically degraded by proteases. This proteolytic process causes the release and translocation of NF-κB complex into the nucleus, where it can mediate gene transcription by binding to specific sequences in the promoter regions of its target genes. Products of these target genes initiate or regulate inflammatory response, apoptosis and carcinogenesis. NF-κB has been shown to promote or inhibit programmed cell death [10, 11]. The opposing effects of NF-κB are thought to be dependent on cell type and/or the nature of stimuli. It remains unclear whether NF-κB

regulates myocardial apoptosis in ADR-induced myocardial injury.

In this study we demonstrate that ADR causes apoptosis in adult rat myocardium. To understand the pathogenesis of ADR-induced apoptosis, we investigated activation of NF-κB in myocardium. This study demonstrates that adriamycin induces myocardium apoptosis through activation of nuclear factor κB in rat and NF-κB activation require IκBα degradation.

Materials and methods

Animals

Adult male Wistar rats weighing 150–170 g (total $n = 40$) were purchased from the Central Animal Laboratory of Harbin Medical University, China. Wistar rats were randomly divided into five groups: control ($n = 8$), ADR 5 mg/kg ($n = 8$), ADR 10 mg/kg ($n = 8$), ADR 15 mg/kg ($n = 8$) and ADR 15 mg/kg + PDTC 200 mg/ml group ($n = 8$). The rats had free access to standard rodent chow and water. The rats were given 5, 10, 15 mg/kg of ADR (Wanle Co Ltd Shenzhen, China) by i.p in ADR group. Instead of ADR, the same volume of physiological saline was injected into control rats. Rats in the ADR plus PDTC group were injected with 200 mg/kg PDTC (Sigma, USA) i.p. 1 h before administration of ADR. Rats were killed by exsanguination 24 h after the beginning of administration of ADR or saline. All experiments were performed according to Harbin medical university Animal Care and Use Committee guidelines.

DNA fragmentation assay

Apoptosis was evaluated by examining the characteristic pattern of DNA ladder generated in apoptotic myocardium using gel electrophoresis. Myocardial samples were homogenized in a lysis buffer containing 50 mmol/l Tris-HCl, pH 7.5, 10 mmol/l EDTA and 0.3% Triton X-100 with a polytron homogenizer on ice for 45 min. After centrifuging at 14,000 rpm for 5 min at 4°C, fragmented DNA in the supernatant was extracted with phenol/chloroform and precipitated at –20°C with ethanol/sodium acetate. The DNA fragments were separated by 1.8% agarose gel electrophoresis. The gel was examined and photographed with an Ultra Violet Products Gel Documentation System, and the electrophoresis results were analyzed by Kodak 2.0 software.

In situ terminal deoxynucleotidyl transferase assay (TUNEL method)

The fragmented DNA on the 3-mm sections was labeled with the ApopTag apoptosis detection kit (Roche, USA). Briefly, after pretreatment with proteinase K, paraffin-fixed slides were incubated with the reaction mixture containing working solution of TdT and digoxigenin-conjugated dUTP for 1 h at 37°C. Labeled DNA was detected by peroxidase-conjugated anti-digoxigenin conjugate antibody. The bound complex stained with a DAB based substrate. To quantitate the degree of apoptosis, apoptotic cells were counted by three independent observers blinded to the experimental protocol. The apoptotic index was expressed as the percent of the total number of myocardial cells.

Immunohistochemical assay

Paraffin sections of the heart were deparaffinized, endogenous peroxidase activity was inactivated with 3% H_2O_2 for 10 min. The primary antibody (rabbit anti-rat p-IκBα (ser 32), Santa Cruz, USA) or normal blocking serum was added and incubated overnight. Biotin-conjugated goat anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody and incubated for 30 min. An avidin-biotin enzyme reagent was sequentially added and incubated for 20 min. A peroxidase substrate was added and incubated until desired stain intensity developed. Finally, cover sections with a glass coverslip and observe by light microscope.

Western blotting analysis

Proteins were extracted from fresh-frozen left ventricle myocardium. Heart tissues were homogenized and then lysed in a lysis buffer (0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 5 mM aprotinin) for 1 h at 4°C. Total protein concentration was determined using a standard protein assay. Approximately 20 mg of protein was run on a 10% SDS-PAGE gel and resolved at 100 V for 2 h, and then transferred to a nitrocellulose membrane (Shanghai Huashun Corp, China) at 100 V for 2 h. The membrane was incubated with polyclonal rabbit anti-rat p-IκBα (ser 32) antibody (Santa Cruz Biotechnology, USA, diluted at 1:200), and visualized by the ProtoBlot®II AP system (Promega Corp, USA).

Electophoretic mobility shift assay

Nuclear proteins were extracted from myocardium, and electrophoretic mobility shift assay (EMSA) was performed to determine NF-κB/DNA binding activity. Nuclear extract was prepared according to Nuclear Extract Kit manufacturer's instructions (Active Motif Corp, USA). A double-stranded oligonucleotide with the sequence 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; 3′-TCA

ACT CCC CTG AAA GGG TCC G-5′ was end-labeled using [γ-^{32}p] dATP and T4 polynucleotide kinase according to the manufacturer's instructions (Promega Corp, USA). Gel Shift Assay was carried out according to Gel Shift Assay Systems protocol (Promega Corp, USA).

Statistical analysis

The data were analyzed using the program SPSS 11.5 for Window. Quantitative data were presented as mean ± SD. For comparison between multiple groups, data was analyzed by ANOVA, and with the Student-Newman-Keuls post hoc analysis. Values of $P < 0.05$ were considered significant.

Results

DNA ladder formation examined by agarose gel electrophoresis

No DNA ladder was detected in control group. Myocardial tissue showed distinct DNA ladder formation, a characteristic ladder pattern of apoptosis, with dose-dependent manner in ADR groups. DNA ladder appeared first in experiment when 5 mg/kg of adriamycin was injected. It became clearer when higher doses of adriamycin were treated, and the most prominent ladder exhibited at the point when 15 mg/kg was used (Fig. 1). In contrast to these findings, characteristic ladder pattern of apoptosis was seldom observed in the ADR + PDTC group (Fig. 1).

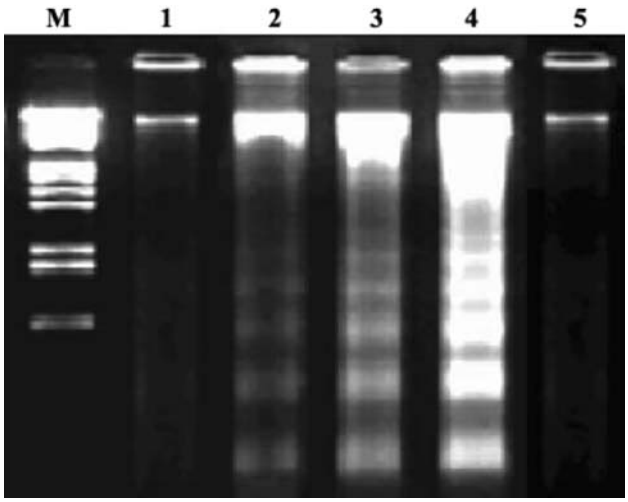

Fig. 1 The gel electrophoresis of DNA fragments extracted from myocardium. M: marker; 1: control group; 2: ADR 5 mg/kg; 3: ADR 10 mg/kg; 4: ADR 15 mg/kg; 5: ADR 15 mg/kg + PDTC 200 mg/ml

TUNEL assay for apoptosis

In general, cells undergoing apoptosis display a characteristic pattern of structural changes in nucleus and cytoplasm, including rapid blebbing of plasma membrane and nuclear disintegration. The nuclear collapse is associated with extensive damage to chromatin and DNA-cleavage into oligonucleosomal length DNA fragments. TUNEL methods were used to detect and quantify myocardial apoptosis in all experimental rat hearts, based on labeling of DNA strand breaks. TUNEL-positive apoptotic cells were a very rare event in control animals (Fig. 2A). In response to ADR, a dose-dependent induction of apoptotic cells was observed in rat hearts (Fig. 2B–D). Treatment with different dose of ADR (5, 10, and 15 mg/kg) increased the apoptotic index to 0.10, 0.14 and 0.17 respectively (Fig. 2F). Compared with the ADR group, apoptotic index was significantly reduced in ADR + PDTC group (Fig. 2E).

Location and distribution of phosphorylated I κ B α protein

Immunohistochemistry was performed to determine the histological localization of phosphorylated IκBα (p-IκBα) in the rat hearts. A control specimen showed the trivial immunoreactivity for p-IκBα (Fig. 3A). In contrast, there was a dramatic increase in staining for p-IκBα in ADR groups (Fig. 3B–D). p-IκBα protein with staining intensity was occasionally localized in the cytoplasm in ADR + PDTC group (Fig. 3E).

Expressions of phosphorylated IκBα protein

To investigate whether NF-κB activation was a result of increased p-IκBα protein in myocardium, the Western blot and semi-quantitative analyses using the NIH Image system for p-IκBα was performed. The Western blot with anti-rat p-IκBα antibody showed that levels of p-IκBα were higher in the rats with ADR injection than in the rats with physiological saline alone (Fig. 4). Moreover, the expressions of p-IκBα were correlated with dose of ADR. Pretreatment with PDTC suppressed the expression of this protein.

Electrophoretic mobility shift assay of NF-κB activation

The activation of NF-κB was measured in terms of its DNA-binding activity. Rats were injected with different dose of ADR, and the NF-κB activation in the nuclear extracts was examined by EMSA. As shown in Fig. 5A and B, ADR induced a dose-dependent activation of NF-κB.

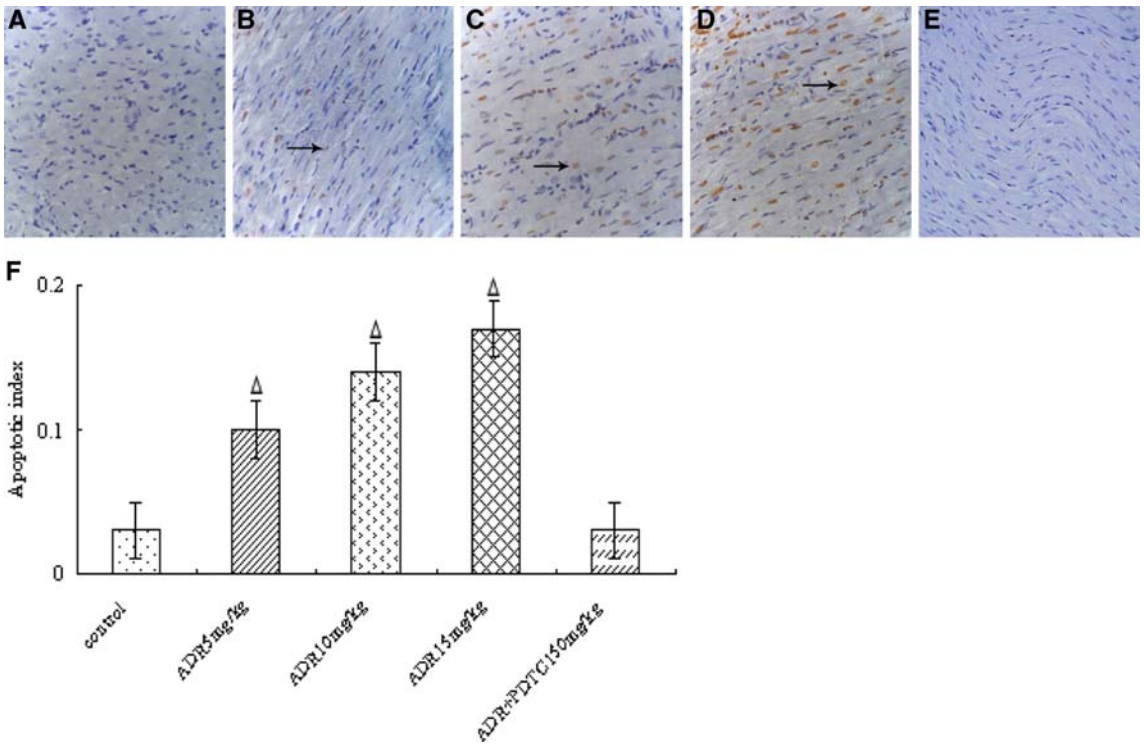

Fig. 2 Myocardial apoptosis of rats (40 times). (**A**) control group, (**B**) ADR 5 mg/kg group, (**C**) ADR10 mg/kg group, (**D**): ADR 15 mg/kg group (→ apoptotic cells), (**E**) ADR 15 mg/kg + PDTC 200 mg/ml. (**F**) the percentage of myocardium apoptosis in four groups. (Δ compared with control group, $P < 0.05$)

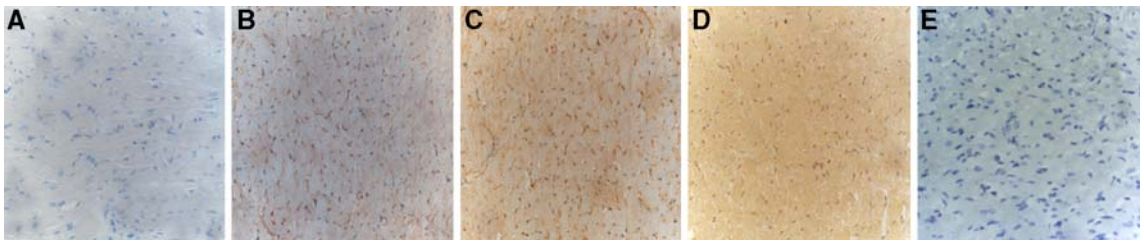

Fig. 3 p-IκBα immunohistochemistry of heart tissue in groups (40 times): (**A**) control group, (**B**) ADR 5 mg/kg group, (**C**) ADR10 mg/kg group, (**D**) ADR 15 mg/kg group, (**E**) ADR 15 mg/kg + PDTC 200 mg/ml

These findings suggest that ADR induces a dose-dependent increase in NF-κB activation in rat hearts. Pretreatment with PDTC suppressed NF-κB binding activity induced by ADR (Fig. 5).

Discussion

In the present study, we determined a significant induction of myocardial apoptosis, phosphorylation of IκBα and NF-κB activation in the early phase following ADR administration in rats. These findings strongly implicate that adriamycin induces myocardium apoptosis through activation of nuclear factor κB in rat and NF-κB activation require IκBα degradation.

The anthracycline antibiotic adriamycin is one of the most effective and useful antineoplastic agents for the treatment of hematological as well as solid malignancies [1, 2]. However, its practical therapeutic use is sometimes limited by the not infrequent induction of acute cardiotoxicity [3, 4]. Previous studies on the mechanisms of ADR cardiotoxicity have reported that the formation of free reactive oxygen radicals [12, 13], release of cardiotoxic cytokines [14, 15], cytoskeletal changes [16] and intracellular calcium overload [17, 18] might be involved in the mechanisms of ADR cardiotoxicity. Recently, evidence is accumulating that apoptotic mechanism is involved in acute and chronic myocytes loss in various heart disorders [19, 20]. In this study, the DNA ladder observed in gel electrophoresis showed that acute ADR administration

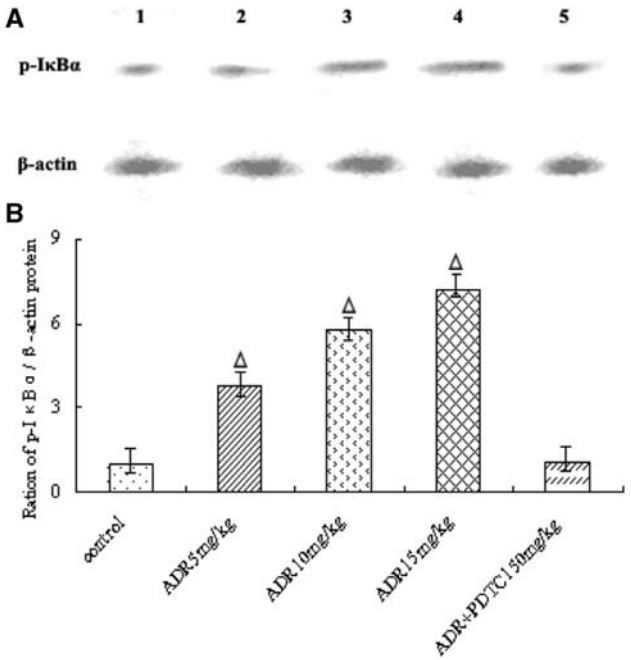

Fig. 4 The expressions of p-IκBα and β-actin proteins extracted from rat hearts: (**A**) Westernblot assay for the expressions of p-IκBα and β-actin proteins. Lane 1: control group, Lane 2: ADR 5 mg/kg group, Lane 3: ADR 10 mg/kg group, Lane 4: ADR 15 mg/kg group, Lane 5: ADR 15 mg/kg + PDTC 200 mg/ml. (**B**) Gel density quantification of different lanes shown in (A). (Δ compared with control group, $P < 0.05$)

induces myocardial apoptosis in vivo. To confirm the occurrence of apoptosis, alternative assay such as TUNEL method is required. These data showed that acute ADR administration induces myocardial apoptosis in dose-response manner. These results in present study are accordance with other researches [5]. Several researches made efforts to understand the signaling pathways responsible for ADR-induced apoptosis [6, 7]. One of the major signaling pathways is the nuclear transcription factor nuclear factor κB (NF-κB).

NF-κB has been reported to be involved in regulating ADR-induced apoptosis in various carcinomas, including T cell lymphoma [21], osteoclasts [22], melanoma [23], pancreatic cancer [24], bladder cancer [25], and breast cancer [26]. Activation of NF-κB induced by ADR in tumor cells results in resistance to apoptosis. Pyrrolidine dithiocarbamate (PDTC) has been shown as an inhibitor of NF-κB in vivo and in vitro studies [8, 9]. Our results showed that acute ADR administration induces NF-κB activation in dose-related manner in myocardium and PDTC as a nonspecific inhibitor of NF-κB protected myocardium from apoptosis by inhibiting NF-κB activation. Therefore, our data suggested that NF-κB activation is pro-apoptotic in adriamycin-induced myocardial injury.

Multiple pathways mediating NF-κB activation have been proposed. It is unknown, however, whether each stimulus activates NF-κB through the same or different signaling pathways. Reactive oxygen species have been implicated to stimulate IκBα degradation and NF-κB activation in myocardium [27]. Superoxide levels were significantly increased in ADR-induced myocardial injury [12]. Our findings indicate that ADR activates NF-κB through a mechanism that require IκBα degradation, as measured by immunohistochemistry and Western blot analysis with polyclonal antibody.

In a word, the present study indicates that adriamycin induces myocardium apoptosis through activation of nuclear factor κB in rat and NF-κB activation require IκBα

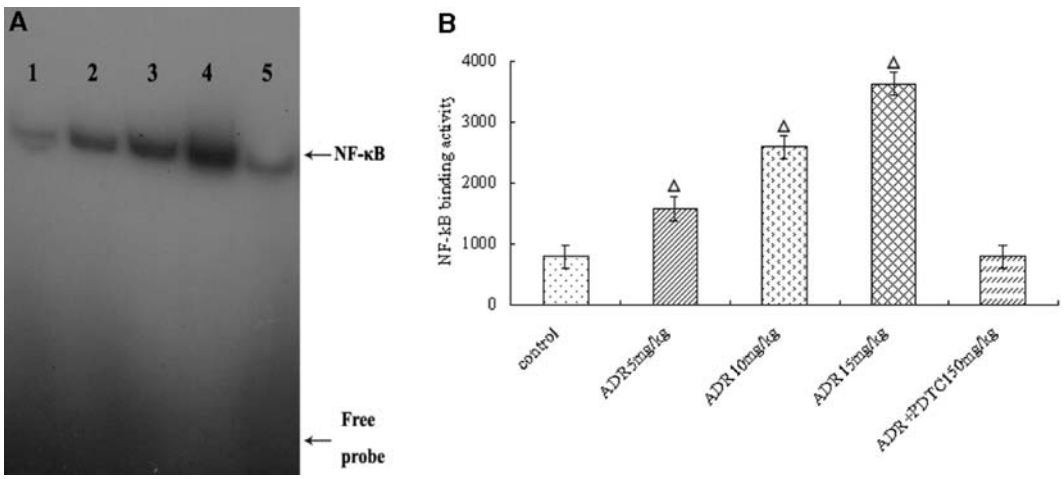

Fig. 5 NF-κB binding activity in rat hearts. (**A**) The EMSA assay for NF-κB activation. Lane 1: control group, Lane 2: ADR 5 mg/kg group, Lane 3: ADR 10 mg/kg group, Lane 4: ADR 15 mg/kg group, Lane 5: ADR 15 mg/kg + PDTC 200 mg/ml. (**B**) Gel density quantification of different lanes shown in (A). All gel density quantifications were made using the upper NF-κB bands. (Δ compared with control group, $P < 0.05$)

degradation. Regulation and control of NF-κB activation may be a powerful therapeutic strategy for reducing ADR-induced myocardial damage. In addition, controlled regulation of NF-κB activation maybe increases the sensitivity of tumor cells to antitumor therapy. However, intense research is needed to further elucidate the role and function of this important nuclear factor in health and pathogenesis.

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