

Activation of the p38 MAPK/NF- κ B pathway contributes to doxorubicin-induced inflammation and cytotoxicity in H9c2 cardiac cells

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Abstract. A number of studies have demonstrated that inflammation plays a role in doxorubicin (DOX)-induced cardiotoxicity. However, the molecular mechanism by which DOX induces cardiac inflammation has yet to be fully elucidated. The present study aimed to investigate the role of the p38 mitogen-activated protein kinase (MAPK)/nuclear factor- κ B (NF- κ B) pathway in DOX-induced inflammation and cytotoxicity. The results of our study demonstrated that the exposure of H9c2 cardiac cells to DOX reduced cell viability and stimulated an inflammatory response, as demonstrated by an increase in the levels of interleukin-1 β (IL-1 β) and IL-6, as well as tumor necrosis factor- α (TNF- α) production. Notably, DOX exposure induced the overexpression of phosphorylated p38 MAPK and phosphorylation of the NF- κ B p65 subunit, which was markedly inhibited by SB203580, a specific inhibitor of p38 MAPK. The inhibition of NF- κ B by pyrrolidine dithiocarbamate (PDTTC), a selective inhibitor of NF- κ B, significantly ameliorated DOX-induced inflammation, leading to a decrease in the levels of IL-1 β and IL-6, as well as TNF- α production in H9c2 cells. The pretreatment of H9c2 cells with either SB203580 or PDTTC before exposure to DOX significantly attenuated DOX-induced cytotoxicity. In conclu-

sion, our study provides novel data demonstrating that the p38 MAPK/NF- κ B pathway is important in the induction of DOX-induced inflammation and cytotoxicity in H9c2 cardiac myocytes.

Introduction

Doxorubicin (DOX) is widely used in the treatment of numerous types of human solid and hematological malignancies, including acute leukemia, lymphoma, Kaposi's sarcoma and bone tumors, as well as stomach, breast and ovarian cancer (1). However, the clinical use of DOX is limited by severe side effects, including cardiotoxicity, which leads to heart failure (2-4). The cause of DOX-induced cardiotoxicity is multifactorial; however, cardiac inflammation and the generation of oxidative stress are known to participate in this clinical event. DOX has been shown to induce a significant increase in the levels of inflammatory markers, including interleukin (IL)-6, tumor necrosis factor- α (TNF- α) (5-8) and cyclooxygenase-2 (COX-2) (9). In a murine model of DOX-induced heart failure, an inhibitor of COX-2 was able to improve left ventricular function and mortality (10), suggesting the involvement of COX-2 in DOX-induced cardiotoxicity.

Nuclear factor- κ B (NF- κ B) may also be a key contributor to DOX-induced cardiotoxicity. NF- κ B is a positive regulator of COX-2 expression in response to various cytokines and growth factors (11,12). The NF- κ B family is composed of five proteins, Rel A (p65), Rel B, c-Rel, NF- κ B1 (p50) and NF- κ B2 (p52), each of which may form homo- or heterodimers. NF- κ B is a dimeric transcription factor that regulates genes associated with stress responses, including inflammation, oxidative stress and apoptosis. DOX has been shown to induce NF- κ B (13-15). We have recently demonstrated that the inhibition of NF- κ B attenuates the cytotoxicity and levels of IL-6 and IL-8, as well as the overexpression of COX-2 in chemical hypoxia-treated HaCaT cells (16,17). These findings indicate the modulatory effect of NF- κ B on inflammatory factors. However, it is unclear whether there is an association between NF- κ B and inflammatory factors in DOX-induced cardiotoxicity.

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The role of p38 mitogen-activated protein kinase (MAPK) in DOX-induced cardiotoxicity has been examined in several studies (18-21). p38 MAPK is a subfamily of the MAPK superfamily. This subfamily is composed of four isoforms, p38 α , p38 β , p38 γ and p38 δ (22,23), and is important in the inflammatory stress response and cell differentiation (24,25). Kang *et al.* (18) have shown that the activation of p38 MAPK is implicated in DOX-induced apoptosis. DOX is able to activate p38 α and p38 β , which contribute to DOX-induced cardiomyocyte apoptosis by degradation of the transcriptional co-factor p300 (21). Our recent study has indicated that the activation of p38 MAPK is capable of enhancing the generation of reactive oxygen species (ROS) (26) and mediating chemical hypoxia-induced inflammation (data not shown), strongly indicating that p38 MAPK activation may contribute to the DOX-induced inflammatory response. The present study aimed to investigate the molecular mechanisms underlying DOX-induced inflammation in order to clarify the association between p38 MAPK and NF- κ B and the roles of these two pathways in the induction of inflammatory factors, including IL-1 β , IL-6 and TNF- α by DOX. The findings of the present study demonstrated that the p38 MAPK/NF- κ B pathway is critical in the induction of the inflammatory response in DOX-treated H9c2 cardiac cells.

Materials and methods

Materials. DOX, SB203580 and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Cell Counter kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). DMEM-F12 medium and fetal bovine serum (FBS) were purchased from Gibco-BRL (Carlsbad, CA, USA).

Cell culture and treatment. H9c2 embryonic rat cardiac cells (Sun Yat-sen University Experimental Animal Center, Guangzhou, China) were cultured in DMEM-F12 medium supplemented with 10% FBS at 37°C in an atmosphere of 5% CO₂. To examine the effects of PDTC and SB203580 on DOX-induced injury, H9c2 cells were pretreated with PDTC (a selective inhibitor of NF- κ B) for 30 min or SB203580 for 60 min prior to treatment with DOX.

Cell viability assay. After the H9c2 cells were cultured in 96-well plates and administered with the indicated treatments, 10 μ l CCK-8 solution was added to each well at a 1/10 dilution, followed by a 2-h incubation. The absorbance was measured at 450 nm with a microplate reader (Multiskan MK3 Microplate reader; Thermo Fisher Scientific Inc., Waltham, MA, USA). The mean optical density (OD) of five wells in the indicated groups was used to calculate the percentage of cell viability according to the following formula: Cell viability (%) = OD treatment group/OD control group \times 100. All the experiments were performed in triplicate.

Measurement of inflammatory cytokine levels using ELISA. The H9c2 cells were plated in 96-well plates. Following the administration of the indicated treatments, the relative content of each secreted inflammatory cytokine (IL-1 β , IL-6 and TNF- α) in the supernatant was measured using the Cytokine ELISA kit (Boster BioTech, Wuhan, China) according to the

manufacturer's instructions. The plates were read at a wavelength of 450 nm using a microplate reader (Multiskan MK3 Microplate reader; Thermo Fisher Scientific Inc.). The relative content of inflammatory cytokines in the culture medium was corrected by cell viability. All the experiments were performed in triplicate.

Western blot assay. Following the administration of the indicated treatments, the H9c2 cells were harvested and lysed, and the homogenate was centrifuged. After the total protein in the supernatant was quantified using the BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), the protein (30 μ g from each sample) was fractionated by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% free-fat milk in TBS-T for 1 h at room temperature, and then incubated with monoclonal rabbit primary antibodies specific to p38 MAPK (#2371; Cell Signaling Technology Inc., Beverly, MA, USA) and phosphorylated (p)-p38 MAPK (#4631; Cell Signaling Technology Inc.) (1:4,000), NF- κ B p65 (#4764; Cell Signaling Technology Inc.) and p-NF- κ B p65 (#3033; Cell Signaling Technology Inc.) (1:2,000) or GAPDH with gentle agitation at 4°C overnight and subsequent incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000 dilution) for 1.5 h at room temperature. Following three washes with TBS-T, the membranes were developed using enhanced chemiluminescence and exposed to X-ray films. To quantify protein expression, the X-ray films were scanned and analyzed with ImageJ 1.41o software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are presented as the mean \pm standard error (SE). Differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant result.

Results

DOX induces the activation of p38 MAPK in H9c2 cells. After the H9c2 cells were treated with 5 μ mol/l DOX for 15, 30 and 60 min, the expression levels of p-p38 MAPK increased in a time-dependent manner, indicating the activation of p38 MAPK by DOX treatment (Fig. 1). Alone, DOX at 5 μ mol/l did not alter the expression of total p38 MAPK.

DOX upregulates the phosphorylation of NF- κ B p65 in H9c2 cells. NF- κ B is important in regulating genes that contribute to the onset of oxidative stress and the inflammatory response. Therefore, we observed the effect of DOX on the phosphorylation of the NF- κ B p65 subunit (an essential step of NF- κ B activation). The results of the western blot analysis demonstrated that after the H9c2 cells were exposed to 5 μ mol/l DOX for 60 min, the expression levels of p-NF- κ B p65 significantly increased, reaching peak levels at 90 min, with the higher levels being sustained until 120 min (Fig. 2).

p38 MAPK participates in the activation of NF- κ B p65 by DOX in H9c2 cells. To examine the effect of the activation of p38 MAPK on the increased phosphorylation of NF- κ B p65 by

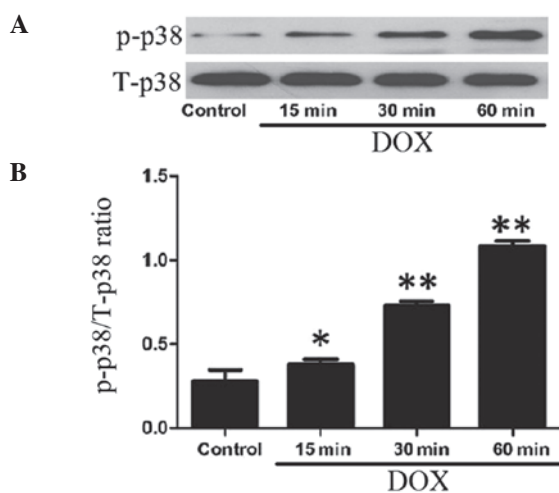


Figure 1. DOX increases the expression levels of p-p38 MAPK in a time-dependent manner in H9c2 cells. The H9c2 cells were treated with 5 μ M DOX for the indicated times. (A) Expression levels of p38 MAPK were examined by western blot analysis and (B) quantified by densitometric analysis for the results in (A) using ImageJ 1.41o software. The data are presented as the mean \pm standard error (n=3). *P<0.05 and **P<0.01 compared with the control group. DOX, doxorubicin; p-, phosphorylated; T-, total; MAPK, mitogen-activated protein kinase.

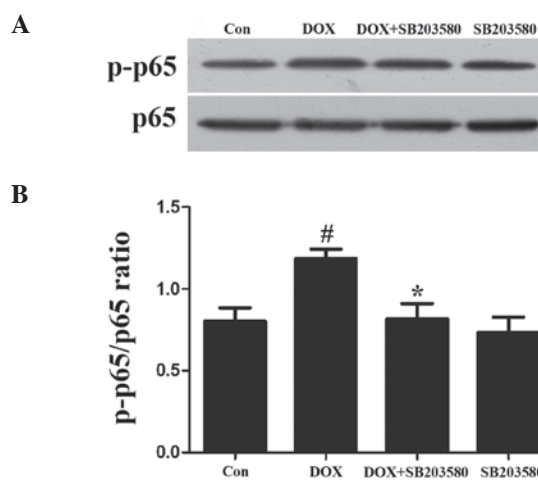


Figure 3. p38 MAPK inhibitor suppresses the DOX-induced phosphorylation of NF- κ B p65 in H9c2 cells. The H9c2 cells were treated with 5 μ M DOX for 90 min in the absence or presence of pretreatment with 3 μ M SB203580 for 60 min. (A) Western blot analysis was applied to detect changes in the expression levels of NF- κ B p65 and p-NF- κ B p65. (B) Densitometric analysis of the expression levels of p-NF- κ B in (A). The data are presented as the mean \pm standard error (n=3). #P<0.05 compared with the control group; *P<0.05 compared with the DOX treatment group. DOX, doxorubicin; p-, phosphorylated; Con, control; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B.

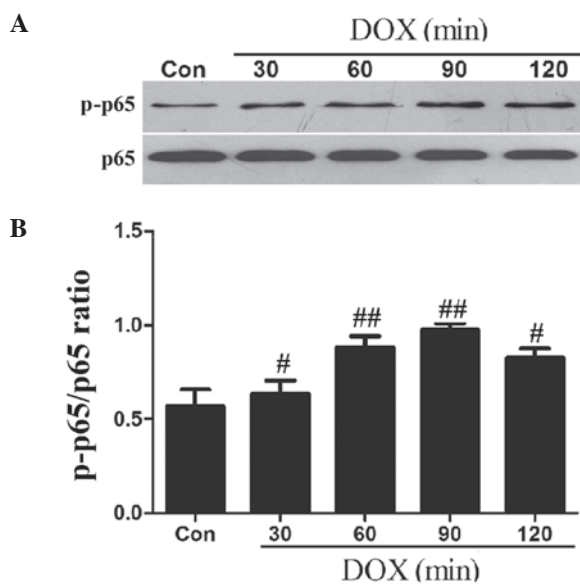


Figure 2. DOX upregulates the phosphorylation of NF- κ B p65 in H9c2 cells. The H9c2 cells were treated with 5 μ M DOX for the indicated times. (A) Expression levels of NF- κ B p65 were examined by western blot analysis and (B) quantified by densitometric analysis for the data in (A) using ImageJ 1.41o software. The data are presented as the mean \pm standard error (n=3). #P<0.05 and ##P<0.01 compared with the control group. DOX, doxorubicin; NF- κ B, nuclear factor- κ B; Con, control; p-, phosphorylated.

DOX, H9c2 cells were pretreated with 3 μ M SB203580, a specific inhibitor of p38 MAPK, for 60 min prior to exposure to 5 μ M DOX. As shown in Figs. 3A and 2B, the exposure of cells to 5 μ M DOX for 90 min markedly enhanced the expression levels of p-NF- κ B p65, which were attenuated by treatment with SB203580, suggesting the involvement of p38 MAPK in the DOX-induced activation of NF- κ B p65. SB203580 at 3 μ M alone did not change the basal expression level of p-NF- κ B p65 in H9c2 cells (Fig. 3A and B).

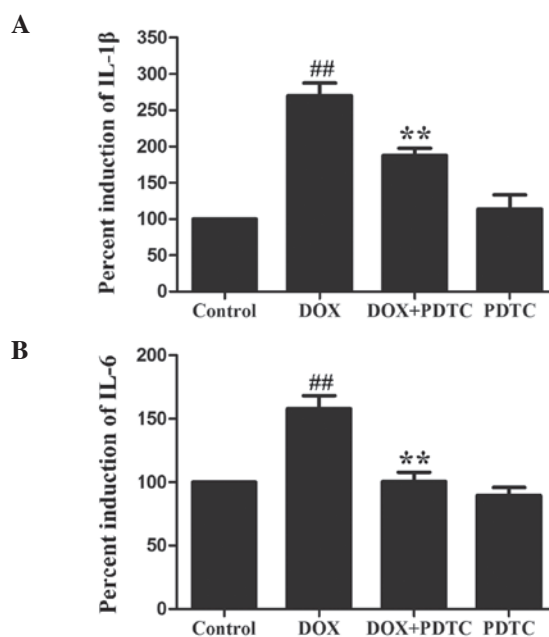


Figure 4. NF- κ B inhibitor suppresses the DOX-induced IL-1 β and IL-6 levels in H9c2 cells. The H9c2 cells were treated with 5 μ M DOX for 24 h in the absence or presence of pretreatment with 100 μ M PDTTC for 30 min prior to DOX treatment. ELISA was performed to detect the levels of IL-1 β and IL-6 in cell supernatants. The data are presented as the mean \pm standard error (n=3). ##P<0.01 compared with the control group; **P<0.01 compared with the DOX treatment group. DOX, doxorubicin; PDTTC, pyrrolidine dithiocarbamate; NF- κ B, nuclear factor- κ B; IL, interleukin.

Activation of NF- κ B p65 contributes to DOX-induced inflammation in H9c2 cells. The levels of IL-1 β and IL-6 were measured in response to DOX and PDTTC (an inhibitor of NF- κ B). Following the exposure of H9c2 cells to 5 μ M DOX for 24 h, IL-1 β (Fig. 4A) and IL-6 (Fig. 4B) levels were

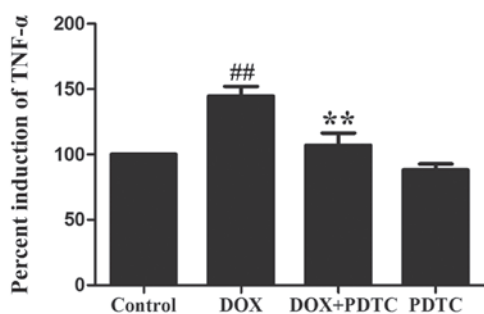


Figure 5. NF- κ B inhibitor reduces DOX-induced TNF- α secretion from H9c2 cells. The H9c2 cells were treated with 5 μ mol/l DOX for 24 h in the absence or presence of pretreatment with 100 μ M PDTC for 30 min prior to DOX treatment. ELISA was performed to detect the level of TNF- α in cell supernatants. Data are presented as the mean \pm standard error (n=3). ^{##}P<0.01 compared with the control group; ^{**}P<0.01 compared with the DOX treatment group. TNF- α , tumor necrosis factor- α ; DOX, doxorubicin; PDTC, pyrrolidine dithiocarbamate; NF- κ B, nuclear factor- κ B.

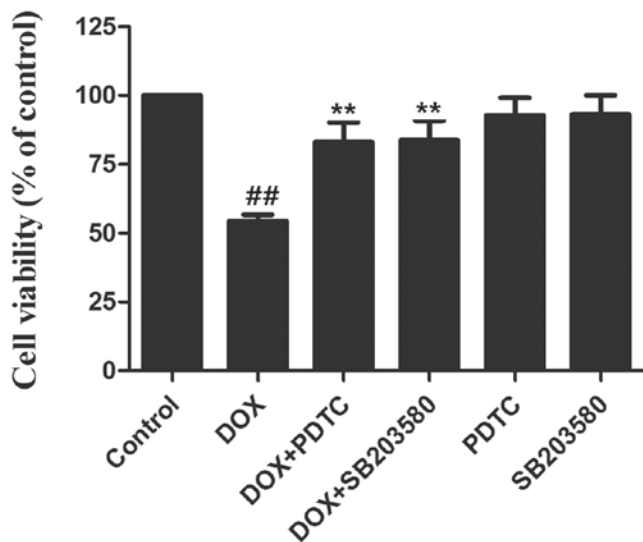


Figure 6. NF- κ B and p38 MAPK inhibitors protect H9c2 cells against DOX-induced cytotoxicity. The H9c2 cells were treated with 5 μ mol/l DOX for 24 h in the absence or presence of pretreatment with 100 μ M PDTC for 30 min or 3 μ M SB203580 for 60 min prior to DOX treatment. Cell viability was measured using the CCK-8 assay. Data are presented as the mean \pm standard error (n=3). ^{##}P<0.01 compared with the control group; ^{**}P<0.01 compared with the DOX treatment group. PDTC, pyrrolidine dithiocarbamate; CCK-8, Cell Counter kit-8; NF- κ B, nuclear factor- κ B; DOX, doxorubicin.

significantly increased. Pretreatment with 100 μ mol/l PDTC for 30 min prior to DOX exposure markedly ameliorated IL-1 β and IL-6 levels in H9c2 cells. Additionally, the exposure of H9c2 cells to 5 μ mol/l DOX significantly enhanced TNF- α (a proinflammatory cytokine) production (Fig. 5), which was reduced by pretreatment with PDTC. These results revealed that the DOX-induced inflammatory response is associated with the activation of NF- κ B p65.

The p38 MAPK/NF- κ B pathway is involved in DOX-induced cytotoxicity in H9c2 cells. To clarify the role of the p38 MAPK/NF- κ B pathway in DOX-induced cytotoxicity, H9c2 cells were pretreated with either SB203580 (3 μ mol/l) for 60 min or PDTC (100 μ mol/l) for 30 min before exposure

to 5 μ mol/l DOX for 24 h. As shown in Fig. 6, the exposure of H9c2 cells to DOX induced significant cytotoxicity, as indicated by the decrease in cell viability. However, the decreased cell viability was markedly inhibited by pretreatment with SB203580 or PDTC, indicating that DOX-induced cytotoxicity is mediated, at least partially, by the p38 MAPK/NF- κ B pathway.

Discussion

Since dose-related adverse effects, in particular cardiotoxicity, often limit the effectiveness of DOX in chemotherapy, alternative strategies using pharmaceutical agents have been investigated. Several of these agents, including dexrazoxan (27), angiotensin-converting enzyme (ACE) inhibitors (28), β -blockers (29) and vitamin E (30) have been tested in animal models and clinical studies to prevent or reduce these dose-related clinical events. However, to date, no single drug has clinically been capable of fully preventing DOX cardiotoxicity. Additional basic and clinical studies are required to validate the underlying mechanism of action of these agents. Results of the present study support the hypothesis that inflammatory responses to DOX treatment are mediated, at least partially, by the activation of the p38 MAPK/NF- κ B pathway, and that certain adverse inflammatory consequences induced by DOX may be ameliorated by inhibiting the p38 MAPK/NF- κ B pathway.

Recently, inflammation has been shown to play a role in DOX cardiotoxicity. DOX induces a significant increase in the levels of specific inflammatory cytokines and chemokines, including IL-1 β (31), IL-6, TNF- α (5-8,31), COX-2 (9) and CCL2/MCP-1 (31). COX-2 inhibitors are capable of improving left ventricular function and mortality in murine models of DOX-induced heart failure (10). Studies with IL-1 β -deficient mice have demonstrated that IL-1 β signaling is critical in DOX-induced increases in IL-6 and granulocyte colony stimulating factor (G-CSF) levels (31). Furthermore, DOX is able to induce the activation of NF- κ B (a positive regulator of COX-2 expression) (13-15), which contributes to cardiac inflammation and necrosis (32). Since the signaling pathway that induces the expression of the 35 kDa pro-IL-1 β is mediated by the activation of NF- κ B and p38 MAPK (33), we hypothesize that the activation of p38 MAPK and NF- κ B may modulate the inflammatory response in DOX-treated cardiomyocytes. The results of the present study confirmed our hypothesis. In agreement with previous studies (18-21), we demonstrated that the expression of p-p38 MAPK was markedly enhanced in DOX-treated H9c2 cardiac cells. In addition, the exposure of H9c2 cells led to DOX-induced activation of NF- κ B, which is consistent with previous studies (13-15). Notably, we observed that the pretreatment of cells with SB203580, a specific inhibitor of p38 MAPK, attenuated the increased activation of NF- κ B p65 by DOX, suggesting a modulatory effect of the p38 MAPK pathway on DOX-induced NF- κ B activation. Furthermore, our results showed that DOX significantly induced inflammatory responses, as indicated by an increase in the levels of IL-1 β , IL-6 and TNF- α . However, whether there is an association between the p38 MAPK/NF- κ B pathway and DOX-induced inflammatory markers is unclear.

To clarify the modulatory effects of NF- κ B activation on the levels of inflammatory markers, the H9c2 cells were

pretreated with PDTC, a selective inhibitor of NF- κ B, prior to exposure to DOX treatment. Firstly, we demonstrated that pretreatment with PDTC significantly reduced the levels of IL-1 β and IL-6 induced by DOX, highlighting the modulatory role of the NF- κ B pathway in the DOX-induced secretion of IL-1 β and IL-6 from H9c2 cells. IL-1 β is an initiator cytokine that is important in the regulation of the immune and inflammatory responses (34), and contributes to the DOX-induced increase in the levels of IL-6 and GCSF (31). Thus, elucidating the role of NF- κ B in the IL-1 β -mediated inflammatory response may present opportunities to inhibit the inflammatory consequences of DOX. This study also demonstrated that the activation of NF- κ B is necessary for the induction of IL-1 β and IL-6 by DOX in H9c2 cells. In addition, we observed that PDTC pretreatment had a notable inhibitory effect on the induction of TNF- α by DOX treatment, revealing the involvement of the NF- κ B pathway in the modulation of TNF- α induction. TNF- α , a proinflammatory cytokine, may cause apoptotic cell death, cellular proliferation, differentiation, inflammation, tumorigenesis and viral replication (35). Recent studies have demonstrated that DOX increases TNF- α expression (7,31,36,37). Notably, TNF- α is capable of activating NF- κ B (14). Daunorubicin, a DOX analogue, was demonstrated to strongly affect the potential ability of TNF- α to activate NF- κ B, suggesting a synergy between these two agents in this response (38). Based on our results and those of previous studies (7,14,31,36-38), we suggest that a cross-talk between the NF- κ B pathway and TNF- α exists, which may be important in DOX-induced inflammation. Further studies are required to confirm this hypothesis.

Additionally, we examined the role of the p38 MAPK/NF- κ B pathway in DOX-induced cytotoxicity. The findings of this study showed that the pretreatment of H9c2 cells with either SB203580 or PDTC prior to exposure to DOX markedly inhibited DOX-induced cytotoxicity, leading to an increase in cell viability. The results suggest that the induction of cardiac cytotoxicity and inflammation by DOX may share common mechanisms, including the p38 MAPK/NF- κ B pathway.

In conclusion, to the best of our knowledge, this is the first study to demonstrate the role of the p38 MAPK/NF- κ B pathway in the DOX-induced inflammatory response in H9c2 cells. A clearer understanding of the functional significance of this pathway may constitute a potential new therapeutic option to prevent DOX-induced cardiotoxicity. However, further clinical studies are required to verify whether this hypothesis is valid in patients.

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