Curcumin potentiates doxorubicin-induced apoptosis in H9c2 cardiac muscle cells through generation of reactive oxygen species

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A B S T R A C T

Doxorubicin (DOX) is a widely used chemotherapy agent. The major adverse effect of DOX treatment in cancer patients is the onset of cardiomyopathy and heart failure. Reactive oxygen species (ROS) are proposed to be responsible for DOX cardiotoxicity. Curcumin, a natural compound extracted from Curcuma Longa L., is known for its anti-oxidant properties. It has been identified as increased apoptosis in several cancer cell lines in combination with doxorubicin, but there are few studies about the effect of curcumin and doxorubicin on normal cardiac cells. Therefore, we evaluated the effects of curcumin on apoptosis induced by DOX in cardiac muscle cells. Pretreatment with curcumin significantly increased DOX-induced apoptosis of cardiac muscle cells through down regulation of Bcl-2, up-regulation of caspase-8 and caspase-9. The Bax/Bcl-2 ratio increased significantly after 1 h pretreatment with curcumin. As well, curcumin increases ROS generation by DOX. In response to DOX, NF-κB was activated. However, curcumin was able to inhibit NF-κB activation. In conclusion, our results indicated that pretreatment with nontoxic concentrations of curcumin sensitized H9c2 cells to DOX-mediated apoptosis by generation of ROS.

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1. Introduction

Doxorubicin (DOX) is an essential component of treatment in breast cancer, childhood solid tumors, soft tissue sarcoma and aggressive lymphoma. As with any other anticancer agent, DOX is associated with a number of unwanted side effects, especially serious cardiac toxicity (Gewirtz, 1999; Wold et al., 2005; Zordoky et al., 2010) which seems to be a multi-factorial process that leads to cardiomyocytes death as the terminal downstream events (Konorev et al., 2002; Tokarska-Schlattner et al., 2006). It has long been considered that DOX induce cardiotoxicity by redox activation of reactive oxygen species (ROS) which ultimately result in myocyte apoptosis (Minotti et al., 2004; Mizutani et al., 2005; Spallarossa et al., 2005). Some evidences indicate that DOX induces apoptosis in cardiomyocytes through activation of Nuclear Factor-κB (NF-κB), one of the most studied transcription factors in mammalian cells (Wang et al., 2002; Li et al., 2008). NF-κB dissociates from the inhibitory IκB and translocates from the cytoplasm to the nucleus, where it binds to the promoter elements and activate the expression of selected target genes involved in apoptosis such as Bcl-2 family proteins and caspase inhibitors (Karin and Ben-Neriah, 2000). Previous reports demonstrated that anti-oxidant natural substances including herbal medicines could inhibit DOX-induced apoptosis in cardiac cells by inhibition of ROS generation (Brookins Danz et al., 2009; Han et al., 2008; Kim et al., 2007). Curcumin (diferuloyl methane) is a principle coloring agent present in the rhizomes of Curcuma Longa L. It has been used for thousands of years in Southeast Asia and Indian folk medicine to treat various diseases and eradicate health problems (Duvoix et al., 2005). It has shown to have anti-oxidant, anti-inflammatory, anticancer and chemopreventive properties (Wang et al., 2009). This polyphenol compound is able to modulate several important signaling pathways such as NF-κB (Reuter et al., 2008). It was demonstrated that the use of curcumin may represent a promising approach to protecting cardiac cells against ischemia-reperfusion injury (Ciorillo et al., 2008). It was able to protect normal cells against some agents such as gamma radiation, haloperidol and 6-hydroxydopamine (Bishnoi et al., 2008; Srinivasan et al., 2006; Wang et al., 2009). Curcumin has been identified as increased apoptosis in several cancer cell lines in combination with doxorubicin (Choi et al., 2008; Chuang et al., 2002; Duvoix et al., 2005;
In our previous study, we evaluated the effect of curcumin on DOX cytotoxicity in normal cardiac cells. We showed pretreatment of cells with curcumin increased cytotoxicity of DOX in H9c2 cells through caspase 3 activation and reduction of mRNA expression of c-IAP1 (Hosseinzadeh et al., 2011). In the current study more details and methods used to explore the molecular mechanisms that mediated the enhancement of cell apoptosis by curcumin. Moreover, we investigated the role of NF-κB and ROS in potentiating effect of curcumin on DOX-induced apoptosis in H9c2 cardiac muscle cells.

2. Materials and methods

2.1. Materials

Curcumin, doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), 2,5 dichlorofluorescin diacetate (DCF-DA) and annexin V/FITC apoptosis detection kit were bought from Sigma Aldrich (St Louis, MO, USA). Cell culture medium, penicillin–streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Gibco, Grand Island, NY, USA). High pure RNA isolation kit was procured from Invitrogen (Carlsbad CA). Nuclear extraction and NF-κB was from Roche (Mannheim, Germany). Real-time RT-PCR kit was bought from Invitrogen (Carlsbad CA). BCA protein assay kit was from Pierce (Pierce, Bonn, Germany).

2.2. Cell culture conditions

The H9c2 embryonic rat heart derived cells, as an in vitro model for studies of doxorubicin cytotoxicity (L'Ecuyer et al., 2001) obtained from ATCC. The cells were grown in Dubblico modified Eagle's medium (DMEM ATCC) with 10% (V/V) heat inactivated FBS, penicillin G (100 U/ml) and streptomycin (100 mg/ml) at 37 °C in 95% CO2 humidified incubator. The medium was changed 2–3 days and subcultured when the cell population density reached to 70–80% confluence. Cells were seeded at an appropriate density according to each experimental design.

2.3. Cell viability assay

Stock solutions of curcumin and doxorubicin were prepared in dimethyl sulfoxide (DMSO). The final concentration of the vehicle in the medium was always 0.5%. Curcumin was added to H9c2 cell cultures at the desired concentration 1 h before treatment with doxorubicin then the medium was replaced with fresh medium containing doxorubicin for the time indicated for each experiment. The viability of cells was determined by MTT assay. Briefly, at appropriated time intervals, 20 μL of a 5 mg/ml MTT solution was added to each well. After 2 h incubation, the medium was carefully aspirated and the purple formazan crystals were solubilized with 100 μL DMSO. Optical density was measured at 570 nm (reference wavelength 630 nm) in a microplate reader (Bio-Tek, ELX 800, USA). The absorbance of the untreated culture was set at 100%.

2.4. Flow cytometry detection of apoptosis

Detection of apoptosis was performed by Annexin V-FITC apoptosis detection kit (Sigma, Aldrich) as follows. After treatment, cells were harvested, washed with cold PBS and resuspended in 1× binding buffer. Annexin V-FITC (5 μL) and propidium iodide (10 μL) were added to each cell suspension. The mixture was incubated for 10 min in the dark at room temperature. The stained cells were analyzed directly by flow cytometry using FloMax software (version 2.25) of PartecTM cytometry (Germany).

Table 1

<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2 (S74122)</td>
<td>5-TTGACGCTCTCCACACACATG-3</td>
<td>5-GGTGGAGGAACTCTTCAGGGA-3</td>
</tr>
<tr>
<td>Bax (NM017059)</td>
<td>5-TGCTCATGGCAACTTCAACT-3</td>
<td>5-ATGTATGCTTCGATGACGTC-3</td>
</tr>
<tr>
<td>Caspase-8 (AF288372)</td>
<td>5-TAAGACCTTITTAAGGGCCTTATTGGA-3</td>
<td>5-AGGATACRTGAACTTCAATGGTAC-3</td>
</tr>
<tr>
<td>Caspase-9 (AY027667)</td>
<td>5-GAGGGAAGCCCAAGCTGTC-3</td>
<td>5-GGCCACCTCAAGGCATTTGT-3</td>
</tr>
<tr>
<td>B-actin (AB028846)</td>
<td>5-TTCTGTCACATCGTCTGCT-3</td>
<td>5-GACAGATGCAGAAGGAT-3</td>
</tr>
</tbody>
</table>

2.5. Real-time RT-PCR analysis of apoptosis-related gene expression

Total RNA from H9c2 cells was extracted using high pure isolation kit (Roche, Mannheim, Germany) according to the manufacturer instructions. Quality and quantity of total RNA was assessed by spectrophotometer (NanoDropTM 2000, USA) and samples stored at –80 °C until use. Quantitative specific RNA expression was determined by real-time RT-PCR. The primers used are shown in Table 1.
was performed in one step with CYBR-green invitrogen kit. The primers used in this study were selected from already published studies (Table 1) (Druse et al., 2006; Kijima et al., 2004). The performances of all primer pairs were tested by primer concentration to determine the optimal reaction conditions. Thermal cycler conditions were 15 min at 50°C for cDNA synthesis, 10 min at 95°C followed by 40 cycles of 15 s at 95°C to denature the DNA and 45 s at 60°C to anneal and extend the template. Melting curve analysis was performed to ascertain specificity by continuous acquisition from 65°C to 95°C with a temperature transient rate of 0.1°C/s. All reactions were performed in triplicate in a Stratagene MX3000P system (USA). The value obtained for the target gene expression were normalized to β-actin and analyzed by the relative gene expression (ΔΔCT) method where 

\[ \Delta\Delta CT = (CT_{\text{target}} - CT_{\beta\text{-actin}})_{\text{unknown}} - (CT_{\text{target}} - CT_{\beta\text{-actin}})_{\text{Calibrator}} \]

2.6. Determination of intracellular ROS

Intracellular ROS levels were examined using DCF-DA. Briefly Cells treated as described above were incubated with 20 μM DCF-DA at 37°C for 30 min. After incubation with the fluorochrome, cell culture was washed and resuspended in cold PBS (Chen et al., 2008). The fluorescence intensity of 2,7-dichlorofluorescin formed by reaction of DCF-DA with intracellular ROS of more than 10,000 viable cells was analyzed by flow cytometry using a Partec TM cytometer (Germany) with standard Argon laser equipped with 488 nm excitation and 525 nm band pass(FL1) filter. The changing level of fluorescence integrity was assayed by FloMax version 2.25.

2.7. NF-kB activation assay

H9c2 cells were gently scraped with ice cold PBS then the nuclear proteins were extracted from 1 x 10^6 cells using nuclear extraction kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to manufacturer’s instruction. Protein concentration was determined by BCA protein assay kit (Pierce, Bonn, Germany). The DNA-binding capacity of NF-kB (p65 subunit) was measured in the nuclear extract of H9c2 cells by NF-kB transcription assay kit (Cayman Chemical Company) according to manufacturer procedure. Briefly, 20 μg of H9c2 nuclear extraction was added to designated wells which a specific double strand DNA (ds DNA) sequence (NF-kB response element) immobilized onto the bottom of the wells. The plate was incubated overnight at 4°C for binding the activated form of NF-kB to dsDNA. Next specific primary and secondary (conjugated with HRP) antibodies were added. After further washing, the developing solution was added (45 min, room temperature) with gentle agitation before adding stop solution. The absorbance of the reaction was read at 450 nm by a microplate reader (Bio-Tek, ELX800, USA).

2.8. Statistical analysis

Each experiment was performed at least three times, and the results were presented as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Turkey’s test was used to compare the differences between means. A probability value of p < 0.05 was considered to be statistically significant.

**Fig. 4.** The effect of curcumin on apoptosis induced by DOX in H9c2 cells. Flow cytometry detection of apoptosis with Annexin V/PI. (a) Representative of flow cytometry with annexinV/PI plots in different groups and (b) column bar graph of mean cell florescence for Annexin V-PI- (Viable cells), Annexin V+/PI- (apoptotic cells), Annexin V+/PI+ (necrotic cells). Data are expressed as the mean ± S.E.M of three separate experiments. #p < 0.05, ##p < 0.01 vs. control, *p < 0.05, **p < 0.01, ***p < 0.001 vs. DOX treated cells.
3. Results

3.1. Curcumin increases DOX-induced cytotoxicity in H9c2 cells

The H9c2 cells were treated with different concentrations of curcumin (5–50 µM) for 1 h. After 22 h the rate of cell growth inhibition was evaluated by MTT. As shown in Fig. 1 curcumin did not cause any cytotoxicity. However, doxorubicin inhibited cell proliferation in a dose dependent manner (Fig. 2). Interestingly, pretreatment with different concentrations of curcumin (5, 10 and 15 µM) for 1 h, increased significantly doxorubicin mediated cytotoxicity dose dependently (Fig. 3).

3.2. Curcumin enhances DOX-induced apoptosis in H9c2 cells

For improvement of MTT result and also characterizing the type of cell death involved in our experiments, FITC-Annexin V/PI double staining was performed. H9c2 cells treated with the highest concentration of curcumin (15 µM) alone did not show any detectable evidence of apoptosis. DOX (3 µM) significantly increased apoptosis to 18.367% ± 1.9 of control in H9c2 cells. Pretreatment with 5, 10 and 15 µM of curcumin significantly increased the number of apoptotic cells to 31.87% ± 4.2, 38.17% ± 1.89 and 49.29% ± 2.29, respectively. Curcumin pretreatment had no effects on the number of necrotic cells (Fig. 4A and B).

3.3. Curcumin modulates mRNA expression of some critical genes involved in apoptosis

Doxorubicin is known to affect the expression of a number of genes involved in apoptosis in cardiac cells (Wu et al., 2002). Curcumin has also the ability to regulate the expression of apoptotic genes (Woo et al., 2003). Therefore, to confirm above results and also to investigate how curcumin pretreatment increase DOX-induced apoptosis, we examined the mRNA expression of Bcl-2 protein family (Bcl2 and Bax) in H9c2 cells. Moreover, to determine which apoptotic pathway is activated by curcumin pretreatment, the mRNA expression of caspase-8 and caspase-9, the apical protease in extrinsic and intrinsic pathway respectively were analyzed (Chen et al., 2008). DOX significantly increased Bax mRNA

Fig. 5. The effect of curcumin on (a) Bcl2, (b) Bax mRNA expression and (c) Bax/Bcl-2 in H9c2 cells. Normalization relative to β-actin was performed. Levels of mRNA are expressed relative to control cardiomyoblast in the mean ± S.E.M values derived from three independent experiments. *p < 0.05, ###p < 0.001 vs. control, **p < 0.01, ***p < 0.001 vs. DOX treated cells.
expression to 1.31-fold of control and decreased Bcl2-mRNA expression to 0.427-fold of control (Fig. 5A and B). Also, DOX increased Caspase-9 expression compared to control, but did not affect Caspase-8 mRNA expression (Fig. 6A). When cells were pretreated with curcumin, the levels of Bcl2 mRNA expression were reduced significantly. Moreover, curcumin pretreatment up regulated Bax (15 μM), caspase-9 and caspase-8 (10 and 15 μM) compared to cells that had not been pretreated with curcumin (Fig. 6B). Furthermore the Bax/Bcl2 ratio increased 2.83-fold upon treatment with doxorubicin, while in cells that had been pretreated with 5, 10, and 15 μM curcumin, the Bax/Bcl2 ratio markedly increased to 5.23, 11.35 and 13.43-fold, respectively (Fig. 5C).

3.4. Curcumin-enhances DOX-induced ROS generation in H9c2 cells

In order to measure oxidative stress induced by DOX, fluorescent dye DCF-DA was used to measure ROS production. As anticipated, adding DOX (3 μM) to H9c2 cells caused a significant increase in ROS levels. It is interesting that the pretreatment with curcumin increased intracellular ROS levels in a dose dependent manner. This effect was dependent on the concentration of curcumin used (Fig. 7A and B). This result demonstrated that synergistic effect of curcumin on DOX-induced apoptosis mediated by ROS.

3.5. Curcumin inhibits DOX-induced NF-κB activation

Several reports have suggested that DOX-induced apoptosis in cardiac muscle cells may be mediated through activation of NF-κB (Wang et al., 2002). Therefore, we examined the NF-κB activation by an ELISA-based assay the DNA-binding capacity of NF-κB (p65 subunit) in nuclear extracts of H9c2 cells. We observed that NF-κB activity in H9c2 cells was sharply increased (6.86-fold) by incubation in the presence of 3 μM doxorubicin after 6 h. However, 1 h pretreatment with 5, 10 and 15 μM curcumin markedly attenuated NF-κB activation induced by DOX (Fig. 8).

4. Discussion

In this study, we aimed to assess the effect of curcumin pretreatment on doxorubicin-induced apoptosis in H9c2 cells. The results showed that curcumin itself was non-toxic. This was in line with earlier studies, that revealed low concentrations of curcumin had no cytotoxic effects on normal cells (Bush et al., 2001; Watson et al., 2008). Curcumin has been described as inhibitor of apoptosis in several reports (Bishnoi et al., 2008; Reuter et al., 2008; Wang et al., 2008), so it may serve as a potential protective agent against the DOX-mediated apoptosis in H9c2 cardiac myoblast cells. However, curcumin was not able to protect H9c2 cells against DOX-induced apoptosis, but rather potentiated the cell death (Hosseinzadeh et al., 2011).

To confirm that synergistic effect of curcumin, as noted in MTT and annexin V/PI assays, was due to apoptosis, we investigated apoptosis-related genes expression. The Bcl-2 family of proteins has emerged as a key regulatory component of the cell death process, acting to either inhibit (Bcl-2, Bcl-xl) or promote (Bax, Bcl-xS) cell death. They regulate release of cytochrome-c from mitochondrial intermembrane (Crompton, 2000; Spallarossa et al., 2004). In the current study, DOX induced apoptosis by down regulating anti-apoptotic Bcl-2 and up regulating pro-apoptotic Bax, thus leading to increase caspase-9 mRNA level. These results are consistent with previously described findings that DOX is able to induce apoptosis in rat cardiac H9c2 cells through an increase in expression of Bax and caspase-9 and decrease in mRNA expression of Bcl-2 (Han et al., 2008; Kim et al., 2007). In our study, it appears that apoptosis induced by doxorubicin occurred through caspase-9, the initiator caspase of mitochondrial pathway for apoptosis, rather than by caspase-8, the mediator of extrinsic pathway. Y.M. Jang et al. have previously described that extrinsic pathway plays a minor role in doxorubicin-induced apoptosis (Jang et al., 2004). Previous studies have shown that depending on the cell models, apoptosis from curcumin can follow different pathways dependent on caspase-9, the initiator caspase of mitochondrial pathway for apoptosis, rather than by caspase-8, or both (Bielak-Zmijewska et al., 2004; Bush et al., 2001; Karmakar et al., 2006; Woo et al., 2003). Also, curcumin is said to induce apoptosis in tumor cells by down regulating of Bcl-2 and up-regulating of Bax (Sen et al., 2005; Shishodia et al., 2005). Our findings demonstrated that curcumin pretreatment, at the level of gene expression, potentiated down regulation of Bcl-2 and up regulation of Bax and caspase-9. Also, curcumin pretreatment increased caspase-8 mRNA level, while DOX had no effect on it. Probably, the enhancement of cytotoxicity is due to involvement of the extrinsic and mitochondrial pathways.

Since the Bax/Bcl-2 ratio is an important regulator of apoptosis (Corsetti et al., 2008), it is relevant to note that curcumin pretreatment increased the Bax/Bcl-2 ratio compared to DOX alone in H9c2 cells.

Reactive oxygen species (ROS) are proposed to be responsible for DOX-induced apoptosis in cardiac cells (Mizutani et al., 2005).
Therefore, we decided to evaluate ROS production after pretreatment with curcumin. It is interesting that pretreatment with curcumin leads to an increase in the ROS generation. This result identified that synergistic effect of curcumin was mediated through the enhancement of ROS levels. The present findings corroborate similar findings by Ortiz–Ortiz et al. who recently reported that curcumin enhanced paraquat-induced apoptosis in N27 mesencephalic cells via generation of ROS (Ortiz-Ortiz et al., 2009). Also, other studies have shown that curcumin, although a potent anti-oxidant with ROS scavengering properties is able to increase ROS formation in different models (Javvadi et al., 2008; Woo et al., 2003). Nitric oxide removes O$_2$ to prevent the generation of free radicals by DOX and absence of iNOS caused an exacerbation of DOX cardiac injury (Cole et al., 2006). As curcumin is an extremely specific inhibitor of iNOS expression (Jung et al., 2006), it is suggested that the elevation of ROS generation could be due to a failure in the nitric oxide production in rat cardiomyocytes. Additionally, it has been convincingly shown that curcumin can induce

![Fig. 7](image1)

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**Fig. 7.** The effect of curcumin on DOX-induced ROS generation. Flow cytometry detection of ROS with DCF-DA. (a) Representative of flow cytometry with DCF-DA plots of H9c2 cells in different groups (b) Column bar graph of mean cell florescent for DCF-DA. Data are expressed as the mean ± S.E.M of three separate experiments (n = 4). ###p < 0.001 vs. Control, *p < 0.05, **p < 0.001 vs. DOX treated cells.

![Fig. 8](image2)

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**Fig. 8.** NF-κB (p65) DNA-binding capacity in nuclear extract of H9c2 cells. Cells were pretreated with three different concentrations of curcumin for 1 h followed by incubation with DOX for another 6 h. The data represented the mean ± S.E.M of three separated experiments. ###p < 0.001 vs. control, **p < 0.01, ***p < 0.001 vs. DOX treated cells.
glutathione depletion in the various cells (Santel, 2008; Thayyulla-thil et al., 2008). Therefore, depletion of glutathione following curcumin treatment may cooperate in exacerbation of ROS in H9c2 cells.

Previously, we demonstrated that the drug toxicity enhancement cannot be explained through alteration of cellular DOX levels by curcumin (Hosseinzadeh et al., 2011). To further explore the molecular mechanisms that mediated the enhancement of cell death, we evaluated the effect of curcumin on NF-kB (p65) activity in H9c2 cells, because it was indicated that NF-kB induces multiple factors to regulate apoptosis at many steps along the cell death cascade including cIAPI and 2, caspase-8 and the Bcl-2 like protein in various cellular model systems (Bednarski, 2009; Burstein and Ducket, 2003; Wang et al., 1998). In agreement with early reports, DOX sharply increased NF-kB activation, but curcumin pretreatment significantly decreased DOX-induced NF-kB activity. Based on these results, it is possible that the decrease in nuclear translocation of the transcription factor, NF-kB (p65 subunit), contributes in enhancing DOX-induced apoptosis by curcumin through down regulation of anti-apoptotic gene Bcl-2 and up regulation of pro-apoptotic genes in H9c2 cells. This is similar to what was observed in cancer cells, in which NF-kB inhibition increased apoptosis induced by DOX (Choi et al., 2008; Chuang et al., 2002; Minotti et al., 2004; Notarbartolo et al., 2005). Furthermore, it has been reported that curcumin exerts only slight reducing effect on constitutive NF-kB activation but inhibits its increase in response to doxorubicin in human hepatocellular carcinoma (Chuang et al., 2002). However, this study is in contrast with some reports that suggest NF-kB activation mediates apoptosis (Wang et al., 2002).

Taken together, our findings demonstrated that curcumin enhanced the apoptosis induced by DOX through generation of ROS, modulation of genes involved in apoptosis and inhibition of NF-kB activation. Further studies are in progress in our laboratory to determine other molecular mechanisms involved in synergistic effect of curcumin and doxorubicin in rat cardiac muscle H9c2 cells, because it is clear that curcumin influences other transcription factors like AP-1 and STATs (Reuter et al., 2008), that playing major role in the control of cell proliferation and survival.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References


