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Doxorubicin-Mediated Cardiotoxicity: Role of Mitochondrial Connexin 43

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Abstract Doxorubicin is the highly effective anthracycline, but its clinical use is limited by cardiotoxicity and consequent dysfunction. It has been proposed that the etiology of this is related to mitochondrial dysfunction. Connexin 43 (Cx43), the principal protein building block of cardiac gap junctions and hemichannels, plays an important role in cardioprotection. Recent reports confirmed the presence of Cx43 in the mitochondria as well. In this study, the role of mitochondrial Cx43 was evaluated 3 or 6 h after Doxorubicin administration to the rat heart cell line H9c2. Pharmacological inhibition of Hsp90 demonstrated that the mitochondrial Cx43 conferred cardioprotection by reducing cytosolic and mitochondrial reactive oxygen species production, mitochondrial calcium overload and mitochondrial membrane depolarization and cytochrome c release. In conclusion, our study demonstrates that Cx43 plays an important role in the protection of cardiac cells from Doxorubicin-induced toxicity.

Keywords Connexin 43 · Doxorubicin · Mitochondria · Hsp90

Introduction

Doxorubicin (Doxo) is a widely used antitumor drug, but its clinical use is compromised by the development of a severe form of cardiomyopathy and heart failure. Multiple mechanisms are involved in Doxo-induced heart failure, including an increase in cardiac oxidative stress [1] and intracellular calcium dysregulation [2]. Several reports indicate that alteration of mitochondrial function is the principal mechanism by which Doxo elicits its cardiotoxicity [3]. In fact, Doxo-induced cardiotoxicity seems to be caused by the generation of free radicals leading to impairment of cell calcium regulation [4], and bioenergetic failure [5, 6] in mitochondria of cardiac cells [7].

Connexin 43 (Cx43) is a predominant junctional protein in the intercellular communication channels that critically regulate intercellular translocation of ions and small molecules and is responsible for electrical communication between varieties of cell types, especially cardiomyocytes [8]. Additionally, Cx43 also participates in other cellular functions such as cell growth, differentiation and death/survival signaling, but the involvement of Cx43 in these functions may be partially or fully independent of its role in intercellular communication [9, 10]. In the last decade, the presence of Cx43 in the mitochondria (mCx43) of various cell types has been reported [11]. Rodriguez-Sinovas et al. [9] demonstrate that ischemic preconditioning induces Cx43 translocation from cytosol to mitochondria with a mechanism that involves heat shock protein 90 (Hsp90) and translocase of the outer membrane 20 (TOM 20). This system involves binding of the target protein to a chaperone (Hsp90/Hsp70), presentation to specific parts of TOM complex, and release into the inner mitochondrial membrane [12]. Translocation of Cx43 to mitochondria appears to be important for certain forms of cardioprotection. In fact, increased mCx43 has been linked to ischemic preconditioning and cardioprotection, possibly through the regulation of mitochondrial ion homeostasis and swelling [13]. In addition, studies have demonstrated the role of mCx43 in controlling apoptosis initiation by preventing the release of cytochrome c into the cytosol, and reducing its availability to activate caspase-3 cascade [14, 15].

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Since mCx43 plays an important role in triggering cardioprotection and Doxo affects mitochondrial homeostasis; the aim of our study was to evaluate, in an in vitro model, the possible role of mCx43 in Doxo-treated H9c2 cells.

Materials and Methods

Materials

Doxorubicin was purchased from Baxter manufacturing Spa (Officina di Sesto Fiorentino, Florence, Italy). Where not indicated otherwise, the products were purchased from Sigma (Italy).

Cell Culture

Cardiomyoblast cell line (H9c2) was purchased from the American Tissue Culture Collection (Manassas, VA, USA). Cells were subcultured weekly in 100-mm Corning dishes containing 10 ml Dulbecco's modified Eagle's Medium (DMEM; Gibco) with 10 % fetal bovine serum (FBS; Gibco) and antibiotics (25 U/ml penicillin and 25 U/ml streptomycin). Cells were seeded at a density required for each of the different experimental analysis and starved for 24 h in DMEM containing 1 % FBS.

Experimental Protocols

In order to evaluate cardiotoxic effects of Doxo, H9c2 cardiomyoblasts were treated with Doxo (0.25–0.5 and 1 μM) for 3 or 6 h in DMEM 1 % FBS.

In the experiments that included the use of the inhibitor of Hsp90, Radicicol (1 μM) was administered 30 min earlier Doxo.

Western Blot Analysis

Membrane proteins were extracted from the cells by freeze/thawing in lysis buffer (Tris–HCl 50 mM pH 7.4) containing 10 mM NaF, 150 mM NaCl, 1 % Nonidet P40, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate, leupeptin (10 μg/ml) and trypsin inhibitor (10 μg/ml). Protein content was estimated according to Bio-Rad Protein Assay (Bio-Rad, Milan, Italy), and 50 μg protein/lane were loaded onto an acrylamide gel and separated by SDS-PAGE in denaturating conditions. Blots were incubated with primary antibody anti-Cx43 (diluted 1:250; from Cell Signaling, DBA Italy) or anti-GAPDH, used as loading control (from Santa Cruz Biotechnology, DBA Italy). After incubation with the primary antibodies and washing in TBS/0.1 % Tween, the appropriate secondary antibody, either anti-rabbit or anti-mouse (both diluted 1:5,000), was added for 1 h at room temperature. Immunoreactive protein bands were detected by chemiluminescence using enhanced chemiluminescence reagents (ECL) in LAS 4000 (GE Healthcare).

Flow Cytometry Analysis

Cytosolic Cx43 was checked by fluorescence-activated cell sorting (FACSscan; Becton–Dickinson). H9c2 cells were cultured in a 24-well plate (5×10^4 cells/well) and allow to grow for 24 h; thereafter, the medium was replaced with fresh medium and cells were treated with Doxo (0.25–0.5 and 1 μM) for 3 or 6 h. Cells were then harvested with trypsin and treated with permeabilization buffer (containing 2 % FBS and PBS in the presence of sodium azide 0.1 %, 4 % formaldehyde and Triton X 0.1 %) after the treatment with fixing buffer (containing 2 % FBS and PBS in the presence of sodium azide 0.1 %, 4 % formaldehyde) for 20 min. The permeabilization of cells was performed for 30 min and then anti-Cx43 antibody was added [16]. Rabbit anti-mouse FITC antibody was used as a secondary antibody (eBioscience, CA, USA). Cells were then washed twice with fixing buffer and then analyzed by means of FACS. Data obtained were analyzed by means of Cell Quest software. Results were shown as percentage of positive cells.

Immunofluorescence Analysis with Confocal Microscopy

For immunofluorescence assay, H9c2 cells (1×10^6 per well) were seeded on coverslips in 12-well plate and allow to grow for 24 h; thereafter, the medium was replaced with fresh medium and cells were treated with Doxo (0.25–0.5 and 1 μM) for 3 or 6 h. Then, cells were fixed with 4 % paraformaldehyde in PBS for 15 min and permeabilized with 0.1 % saponin in PBS for 15 min. After blocking with BSA and PBS for 1 h, cells were incubated with mouse anti-Cx43 antibody (Santa Cruz Biotechnologies) or with rabbit anti-TOM20 for 2 h at room temperature. The slides were then washed with PBS for three times and fluorescein-conjugated secondary antibody (FITC) or Texas red-conjugated secondary antibody was added for 1 h, DAPI was used for counterstaining of nuclei. Coverslips were finally mounted in mounting medium and fluorescent images were taken under the laser confocal microscope (Leica TCS SP5).

Measurement of Intracellular Reactive Oxygen Species (ROS) and Mitochondrial Superoxide Evaluation with MitoSOX Red

ROS formation was evaluated by means of the probe 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA). H_2DCF-DA is a non-fluorescent permeant molecule that passively diffuses

into cells, where the acetates are cleaved by intracellular esterases to form H_2DCF and thereby traps it within the cell. In the presence of intracellular ROS, H_2DCF is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, H9c2 cells were plated at a density of 3.0×10^4 cells/well into 24-well plates and treated as described above. Cells were then collected, washed twice with phosphate buffer saline (PBS) buffer and then incubated in PBS containing H_2DCF-DA (10 μM) at 37 °C. After 45 min, cells fluorescence was evaluated using a fluorescence-activated cell sorting and analyzed with Cell Quest software.

In order to detect superoxide release from mitochondria MitoSOX Red was used. H9c2 cells were treated as described above and MitoSOX Red (2.5 μM) was added for 10 min before fluorescence evaluation by means of flow cytometry. This indicator is a fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells and, once targeted to the mitochondria, it is oxidized by superoxide and exhibits red fluorescence. MitoSOX is readily oxidized by superoxide but not by other ROS-generating systems.

Measurement Mitochondrial Membrane Depolarization with TMRE

Mitochondrial permeability transition pore (mPTP) opening was measured using FACS scan. Fluorescent dye, tetramethylrhodamine methyl ester (TMRE) was used for these experiments. Due to its positive charge TMRE penetrates and accumulates in the mitochondria in inverse proportion the membrane potential. For these experiments H9c2 cells were plated at a density of 3.0×10^4 cells/well into 24-well plates and treated with Doxo (0.25–0.5–1 μM) with or without Radicicol (1 μM) for 1–3 or 6 h. Cells were then collected, washed twice with phosphate buffer saline (PBS) buffer and then incubated in PBS containing

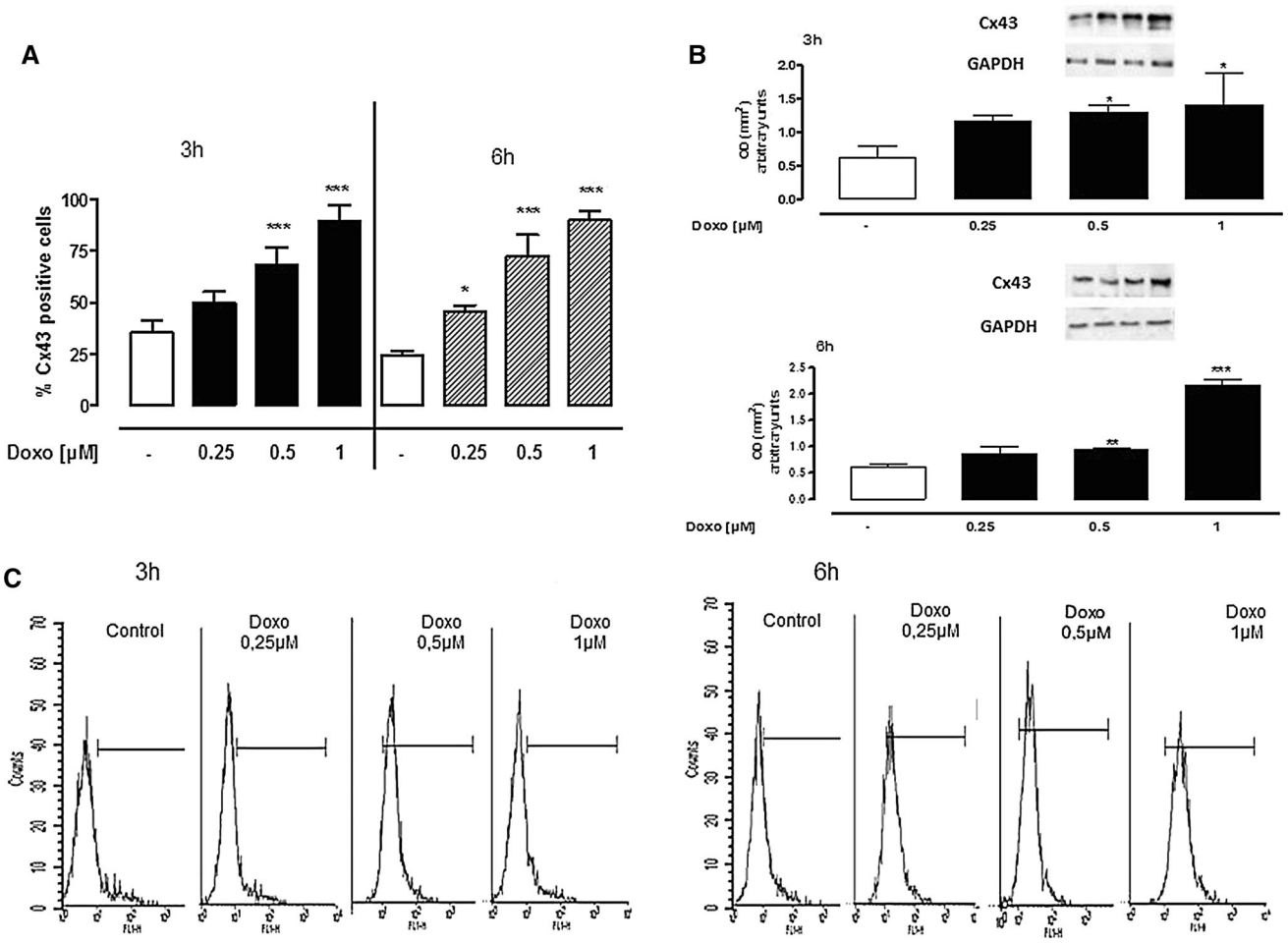

Fig. 1 Doxorubicin increases cytosolic Cx43 expression in H9c2 cells. H9c2 cells were treated with Doxo (0.25–0.5–1 μM) for 3 and 6 h and cytosolic Cx43 content (**a**) was checked by flow cytometry (BD FacsCalibur Milan, Italy) analysis. Cx43 expression was confirmed by Western blot analysis (**b**). Results are expressed as mean ± SEM from at least three independent experiments each performed in duplicate. Data were analyzed by Student's *t* test. $^*P < 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.001$ versus control. **c** Reports representative histograms for the flow cytometry analyses

TMRE (5 nM) at 37 °C. After 30 min, cells fluorescence was evaluated using a fluorescence-activated cell sorting and analyzed with Cell Quest software.

Measurement of Intracellular Ca^{2+} Signaling

Intracellular Ca^{2+} concentrations $[Ca^{2+}]i$ were measured using the fluorescent indicator dye Fura 2-AM, the membrane-permeant acetoxymethyl ester form of Fura 2, as previously described [17] with minor revisions. Briefly, H9c2 cells (5×10^5/multiwell 24 culture dishes) were incubated at 37 °C. Thereafter, the medium was replaced with fresh medium and Doxo (0.25–0.5–1 μM) was added, with or without Radicicol (1 μM). After incubation period (1–3 or 6 h), cells were washed in phosphate buffered saline (PBS), re-suspended in 1 ml of Hank's balanced salt solution (HBSS) containing 5 μM Fura 2-AM for 45 min. Thereafter, cells were washed with the same buffer to remove excess

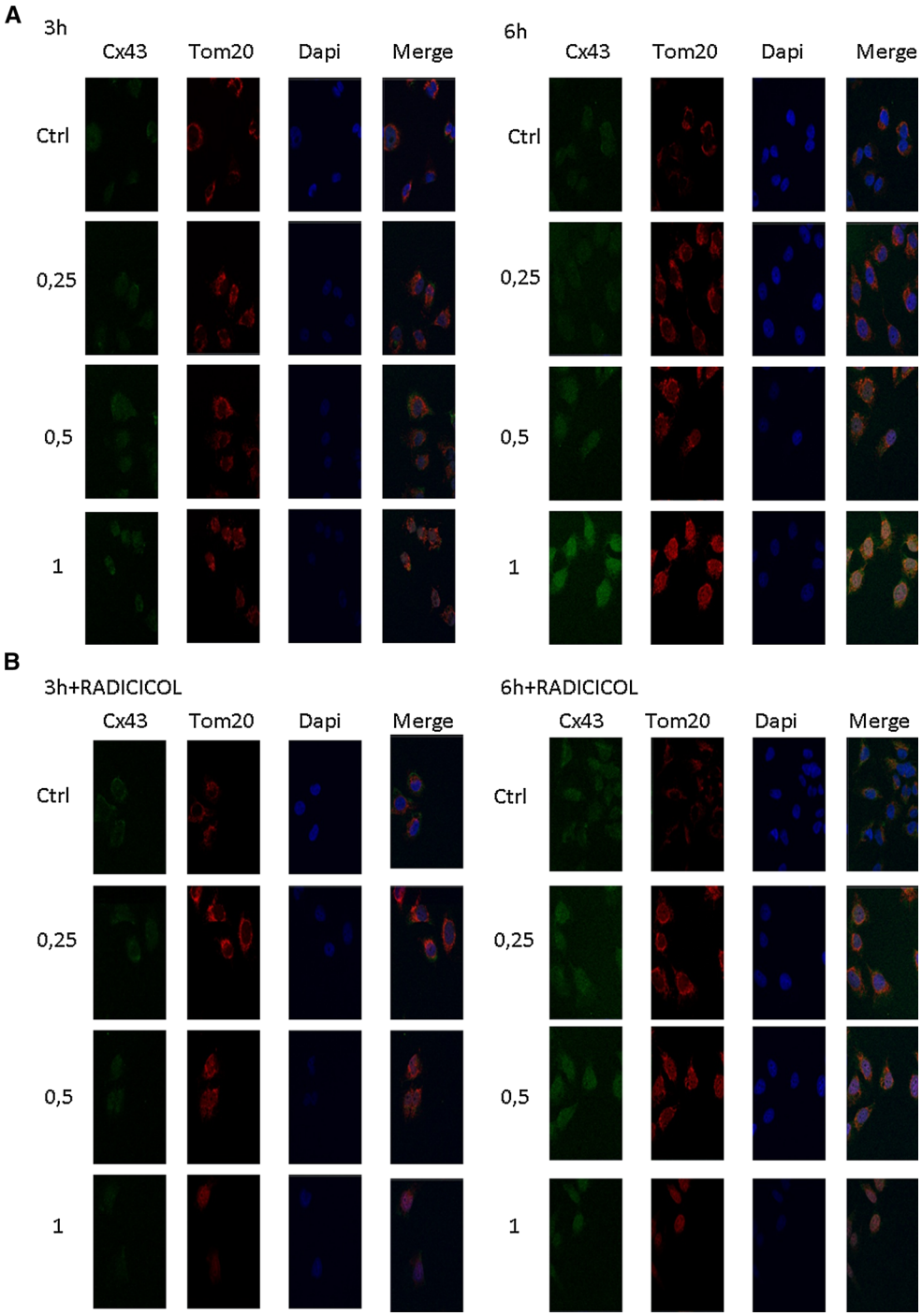

Fig. 2 Doxorubicin increases mitochondrial Cx43 localization. Doxo (0.25–0.5–1 μM) was administered for 3 and 6 h and mitochondrial localization of Cx43 was detected using immunofluorescence assay at confocal microscopy (**a**). Where indicated, Radicicol (1 μM) was administered 30 min before Doxo (**b**). *Scale bar* 10 μm. A representative of three experiments was shown

Fura 2-AM and incubated in Ca^{2+}-free HBSS/0.5 mM EGTA buffer for 15 min to allow hydrolysis of Fura 2-AM into its active-dye form, Fura 2. H9c2 cells then were transferred to the spectrofluorimeter (Perkin-Elmer LS-55). Treatment with ionomycin (1 μM final concentration), or with carbonyl cyanide *p*-trifluoromethoxy-pyhenylhydrazone (FCCP, 0.05 μM final concentration) was carried out by adding the appropriate concentrations of each substance into the cuvette in Ca^{2+}-free HBSS/0.5 mM EGTA buffer. The excitation wavelength was alternated between 340 and 380 nm, and emission fluorescence was recorded at 515 nm. The ratio of fluorescence intensity of 340/380 nm (*F*340/*F*380) was used to estimate intracellular free calcium [18]. Results are indicated as delta increase in fluorescence ratio (*F*340/*F*380 nm) induced by ionomycin-basal fluorescence ratio (*F*340/*F*380 nm) or FCCP-basal fluorescence ratio (*F*340/*F*380 nm).

Statistical Analysis

Statistical analysis was performed with the aid of commercially available software GraphPad Prism4 (GraphPad Software Inc., San Diego CA, USA). Results are expressed as mean ± SEM. Student's *t* test was used for statistical analysis. A value of $P < 0.05$ was considered as statistically significant.

Results

Doxorubicin Increased Mitochondrial Cx43 Localization

Intracellular staining, by using flow cytometry analysis, showed a significant ($P < 0.001$) increase in cytosolic Cx43

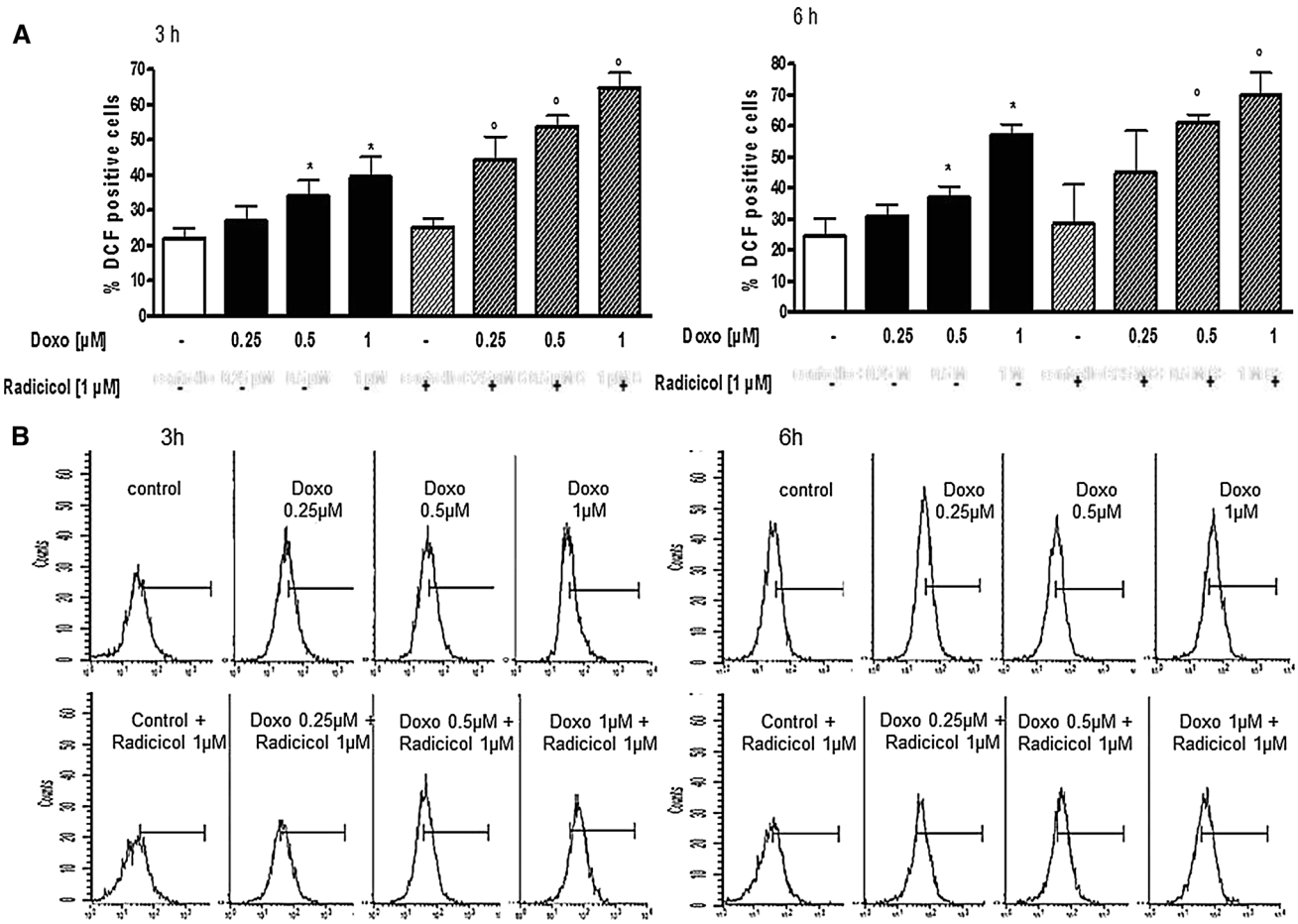

Fig. 3 Radicicol increases cytosolic ROS production in Doxorubicin treated H9c2 cells. ROS formation was evaluated by means of the probe 2′,7′ dichlorofluorescein diacetate (H_2DCF-DA) in H9c2 cells. Doxo (0.25–0.5–1 μM) was administered for 3 and 6 h. Where indicated, Radicicol (1 μM) was administered 30 min before Doxo. In **a** ROS production was expressed as mean ± SEM of percentage of DCF positive cells of at least three independent experiments each performed in duplicate. Data were analyzed by Student's *t* test. $^*P < 0.05$ versus control and $^\circ P < 0.05$ versus Doxo at the same experimental conditions. **b** Reports representative histograms for mitochondrial ROS detection

levels in Doxo-treated H9c2 cells (Fig. 1a, b). Data were confirmed by Western blot analysis (Fig. 1c). Immunofluorescence analysis revealed that Doxo administration induced an increase in mitochondrial Cx43 localization (Fig. 2a). As the import of proteins through the TOM/TIM system can be inhibited by a Hsp inhibitor [12], in our experimental model, H9c2 cells were pre-treated with Radicicol (1 μM), an Hsp90 inhibitor. As reported in Fig. 2b, Radicicol reduced mitochondrial Cx43 localization.

The Inhibition of Cx43 Translocation Increased ROS Production

To study the role of mCx43 in Doxo-induced cardiotoxicity, the main indicators of Doxo toxicity were evaluated both in absence and in presence of Radicicol (1 μM). Doxo (0.25–0.5–1 μM) significantly ($P < 0.05$) increased ROS production in a dose- and time-dependent manner. Pre-treatment with Radicicol significantly ($P < 0.05$) increased Doxo-induced ROS production (Fig. 3a, b) at all experimental time points.

Mitochondrial ROS production was evaluated by means of MitoSOX red. FACS analysis showed that Doxo (0.25–0.5–1 μM) significantly ($P < 0.05$) affect mitochondrial ROS production in a dose- and time-dependent manner. In H9c2 cells pre-treated with Radicicol, mitochondrial ROS production was significantly ($P < 0.05$) higher than those treated with Doxo alone (Fig. 4a, b).

The Inhibition of Cx43 Translocation Enhanced Mitochondrial Membrane Depolarization

Doxo (0.25–0.5–1 μM) administration induced a drastic, very fast and totally opening of the mitochondrial transition

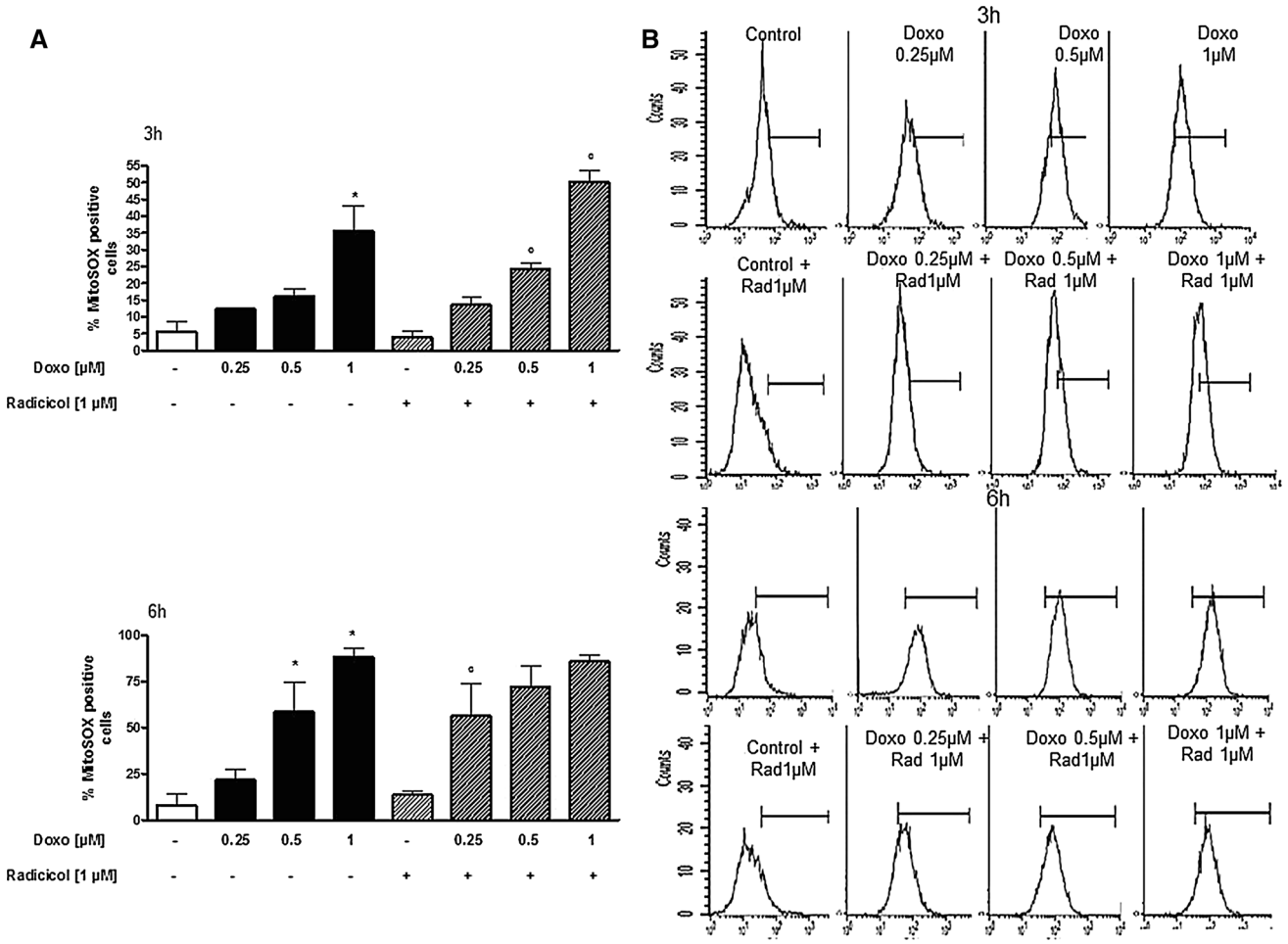

Fig. 4 Radicicol increases mitochondrial ROS production in Doxorubicin treated H9c2 cells. Superoxide production by mitochondria was evaluated by adding MitoSOX Red in H9c2 cells. Doxo (0.25–0.5–1 μM) was administered for 3 and 6 h. Where indicated, Radicicol (1 μM) was administered 30 min before Doxo. In **a** mitochondrial superoxide production was expressed as mean ± SEM of percentage of MitoSOX Red positive cells of at least three independent experiments each performed in duplicate. Data were analyzed by Student's *t* test. *$P < 0.05$ versus control, °$P < 0.05$ versus Doxo at the same experimental conditions. **b** Reports representative histograms for mitochondria superoxide detection. Mitochondrial ROS level was checked by means of flow cytometry analysis

pore, as demonstrated by means of TMRE. In fact, after 3 h of Doxo treatment, we observed total depolarization of the mitochondrial membrane. For this reason, we chose to analyze the effects of Doxo in a shorter experimental time point, 1 h. After 1 h of treatment, no significant differences between control cells and those treated with Doxo were observed, but, it is interesting to note that, in Radicicol pre-treated cells, mitochondrial membrane depolarization was similar to that observed in cells treated with Doxo alone for 3 h (Fig. 5a, b). It has been previously reported [19] that there is ample evidence in support of the oxidative alterations in mitochondrial calcium regulation as being responsible for cardiomyopathy induced by Doxo. So, to avoid the contribution of intracellular Ca^{2+} in Doxo-evoked signals, we carried out experiments in Ca^{2+}-free incubation medium (containing 0.5 mM EGTA). In Ca^{2+}-free medium, Doxo (0.25–0.5–1 μM) treatment significantly increased basal levels of $[Ca^{2+}]_i$ in a dose- and time-dependent manner. In fact, delta increase in $[Ca^{2+}]_i$ induced by ionomycin in Doxo-treated cells was significantly lower ($P < 0.001$) than control cells at all experimental time points, indicating higher levels of basal $[Ca^{2+}]_i$ (Fig. 6a).

To analyze the involvement of mitochondrial Ca^{2+} content in Doxo-evoked responses, cells were incubated with Doxo (0.25–0.5–1 μM) and then the mitochondrial calcium depletory, carbonyl cyanide *p*-trifluoromethoxy-pyhenylhydrazone (FCCP; 0.5 μmol/l), was added. As reported in Fig. 6b, in Doxo-treated H9c2 cells delta increase in $[Ca^{2+}]$i induced by FCCP was significantly ($P < 0.001$) lower than control cells. On the contrary, in Radicicol pre-treated cells, delta increase in $[Ca^{2+}]_i$ induced by FCCP was significantly ($P < 0.05$) higher than Doxo- treated cells at the same experimental time, indicating higher levels of mitochondrial $[Ca^{2+}]_i$ content.

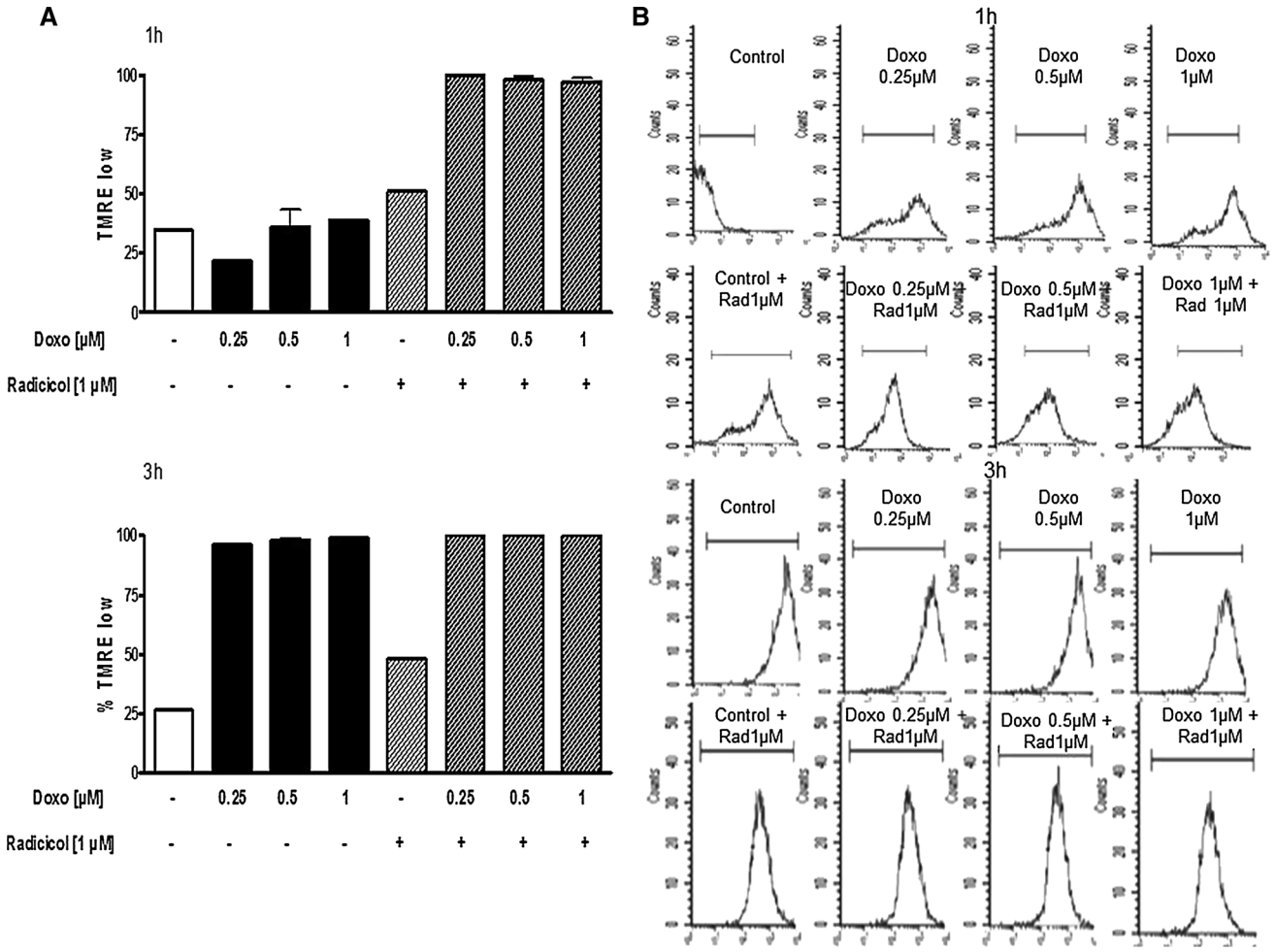

Fig. 5 Radicicol increases Doxorubicin-induced mitochondrial membrane potential collapse in H9c2 cells. Doxo (0.25–0.5–1 μM) was administered for 1 and 3 h. Where indicated, Radicicol (1 μM) was administered 30 min before Doxo. The mitochondrial membrane potential was evaluated by flow cytometry analysis with tetramethylrhodamina ethyl ester (TMRE), a cationic dye that gives a strong fluorescence signal. Results are expressed as mean ± SEM of fluorescence intensity of at least three independent experiments each performed in duplicate (**a**). **b** Reports representative histograms for mitochondrial membrane potential detection

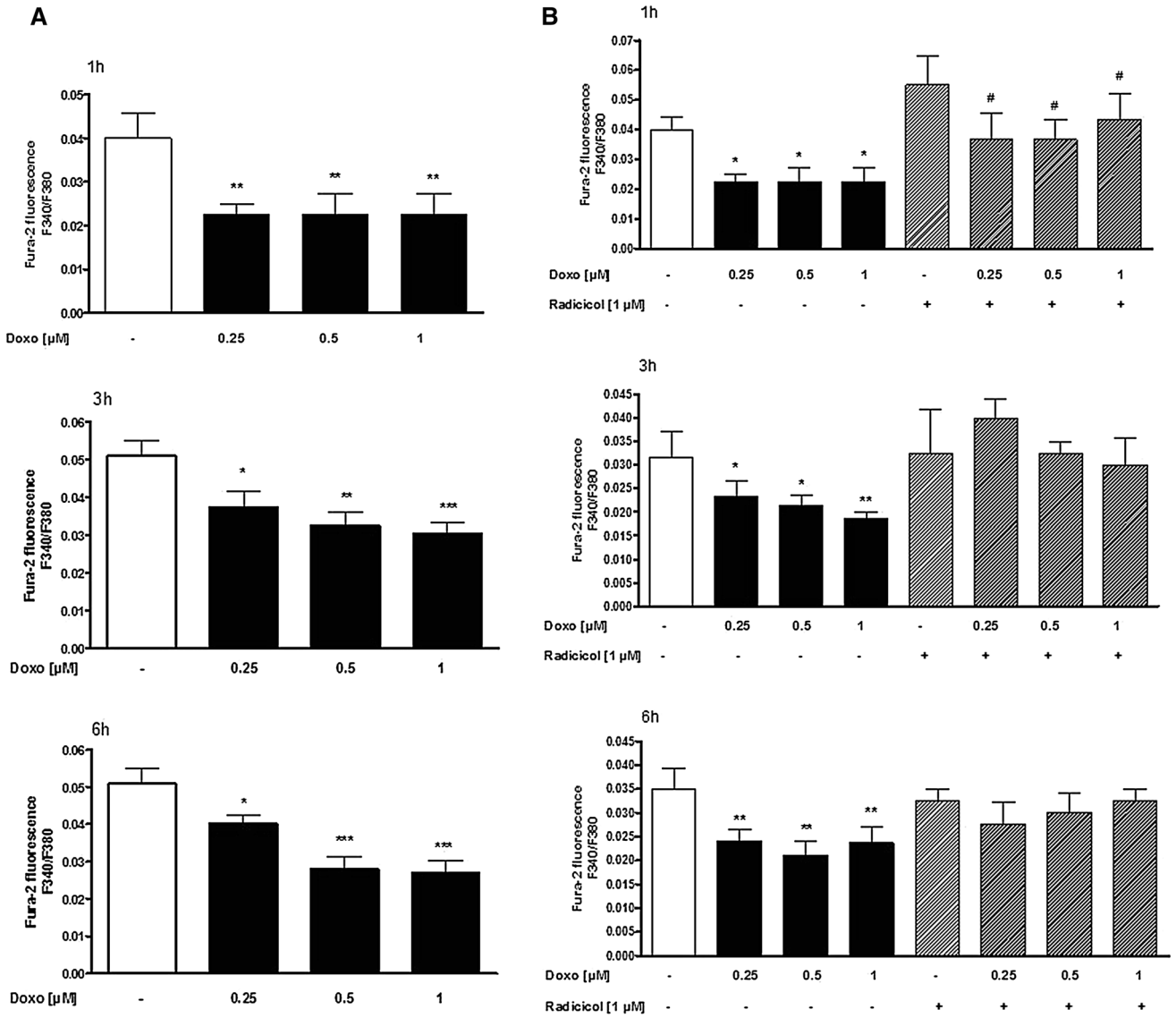

Fig. 6 Radicicol increases mitochondrial [Ca^{2+}] concentrations in Doxorubicin treated H9c2 cells. Doxo (0.25–0.5–1 μM) was administered for 1, 3 and 6 h. Where indicated, Radicicol (1 μM) was administered 30 min before Doxo. Intracellular calcium concentration was evaluated on H9c2 cells in Ca^{2+}-free medium (**a**). Effect of Radicicol pre-treatment on mitochondrial Ca^{2+} pool was evaluated on H9c2 cells in Ca^{2+}-free medium in presence of FCCP (0.05 μM) (**b**). Results were expressed as mean ± SEM of delta (δ) increase in Fura 2 ratio fluorescence (340/380 nm) from at least three independent experiments each performed in duplicate. Data were analyzed by Student's *t* test. $^{*}P < 0.05$ $^{**}P < 0.005$ and $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$ versus Doxo at the same experimental conditions

Doxo (0.25–0.5–1 μM) induced cytochrome c release in a dose- and time-dependent manner. Cytochrome c release induced by Doxo was significantly ($P < 0.05$) higher in Radicicol pre-treated cells (Fig. 7a, b).

Discussion

Doxo is one of the most widely used antineoplastic drugs, but its clinical use has serious drawback of cardiotoxicity [20]. There is growing support that disruption of mitochondrial function is the principal mechanism by which Doxo elicits its cardiotoxicity [4]. Studies in vivo and in vitro have shown that Doxo stimulates disturbances in cellular calcium homeostasis and mitochondrial calcium loading that are critical for its cardiotoxic effects [21]. Recently, mitochondrial Cx43 has been indicated as a novel regulator of mitochondrial function where inhibition with the gap junction blocker 18β-glycyrrhetinic acid results in the release of cytochrome C and myocyte apoptosis [22].

Cx43 is the major gap junction forming protein in the adult cardiac ventricles [23]. Even if most of the functions ascribed to Cx43 in cardiac pathophysiology are related to

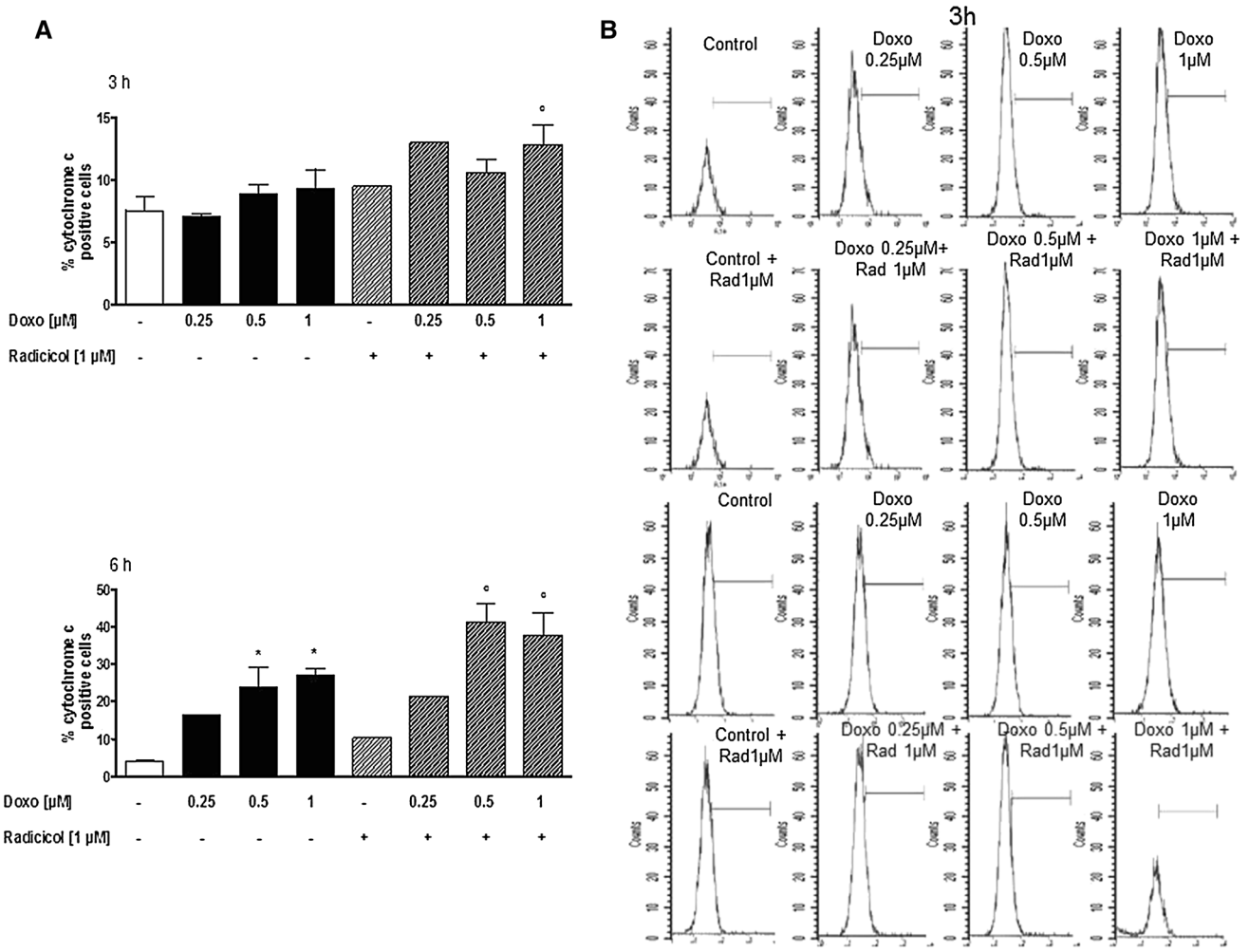

Fig. 7 Radicicol increased Doxorubicin-induced cytochrome c release in H9c2 cells. Doxo (0.25–0.5–1 μM) was administered for 3 and 6 h, where indicated, Radicicol (1 μM) was administered 30 min before Doxorubicin. Cytochrome c level into the cytosol was checked by flow cytometry analysis. Results are expressed as mean ± SEM from at least three independent experiments each performed in duplicate. Data were analyzed by Student's *t* test. $^{*}P < 0.05$ versus control $^{\circ}P < 0.05$ versus Doxo at the same experimental conditions

its function in a gap junction, recent literature reports roles and functions for Cx43 outside of intercellular communication [24, 25]. The presence of Cx43 onto mitochondria is well established and several studies report that mitochondrial content of Cx43 is enhanced by ischemia–reperfusion [12] as well as regulates apoptosis [23].

In our study, FACS analysis showed an increase in Cx43 content in the cytosol of Doxo-treated cells, and immunofluorescence analysis revealed an increase in mitochondrial Cx43 localization. Several reports indicate that increased mitochondrial content occurs via increased translocation of Cx43 from cytosol to mitochondria with a mechanism that involves Hsp90/TOM 20 machinery system and so can be inhibited by a Hsp90 inhibitor [12, 19]. In agreement with these authors, in our experimental model, mCx43 over-expression induced by Doxo was significantly reduced in the presence of Radicicol, an Hsp90 inhibitor, as demonstrated by confocal immunofluorescence microscopy.

It is well established that cardiotoxic effects of Doxo are due to increased mitochondrial calcium and reactive oxygen species, leading to oxidative stress, cell necrosis and induction of pro-apoptotic signaling pathways [22]. In order to evaluate the role of mCx43 in Doxo-induced cardiotoxicity, we have analyzed the main parameters of Doxo-induced cardiotoxicity both in absence and presence of Hsp90 inhibitor Radicicol. Our data show that Doxo induces a dose- and time-dependent increase in cytosolic and mitochondrial oxygen radical species, as demonstrated by means of DHCF and MitoSOX. Radicicol pre-treatment, significantly increases the effect of Doxo by accelerating both cytosolic and mitochondrial ROS production. Doxo

cardiotoxicity is also associated with disturbance in cellular calcium homeostasis and mitochondrial calcium loading [20]. Here we show that Doxo induces a significant increase in basal levels of [Ca^{2+}], in a dose- and time-dependent manner in H9c2 cells. Mitochondrial Cx43 appears to counteract the Doxo-induced mitochondrial calcium elevation. In fact, in agreement with data reported by Azarashvili et al. [26], in our experimental model, Radicicol pre-treatment significantly increases the net mitochondrial calcium accumulation induced by Doxo. Furthermore, in H9c2 cells pre-treated with Radicicol, mitochondrial calcium content was significantly higher than cells treated with Doxo alone.

Thus, our TMRE data support the notion that mCx43 hinders the accumulation of calcium into the mitochondria and delays the time to depolarization. In fact, it has been reported that mCx43 is involved in a signal transduction pathway that can prevent mitochondrial transition pore formation and thus prevents mitochondrial swelling, rupture of the outer mitochondrial membrane and release of apoptogenic mitochondrial contents [27]. Consistent with this, in our experimental model, the inhibition of Cx43 translocation by Hsp90 inhibitor also accelerates the release of cytochrome c.

In conclusion, our results suggest a novel role of Cx43 as a regulator of mitochondrial homeostasis. The main limitation of this work is that the observations were made in an in vitro system. Further in vivo studies need to better clarify if mCx43 could be a pharmacological target in Doxo-induced cardiotoxicity.

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