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Doxorubicin-induced reactive oxygen species generation and intracellular Ca^{2+} increase are reciprocally modulated in rat cardiomyocytes

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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DOX, doxorubicin; ROS, reactive oxygen species; RyR, ryanodine receptor; SR, sarcoplasmic reticulum

Abstract

Doxorubicin (DOX) is one of the most potent anticancer drugs and induces acute cardiac arrhythmias and chronic cumulative cardiomyopathy. Though DOX-induced cardiotoxicity is known to be caused mainly by ROS generation, a disturbance of Ca^{2+} homeostasis is also implicated one of the cardiotoxic mechanisms. In this study, a molecular basis of DOX-induced modulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was investigated. Treatment of adult rat cardiomyocytes with DOX increased $[Ca^{2+}]_i$ irrespectively of extracellular Ca^{2+}, indicating DOX-mediated Ca^{2+} release from intracellular Ca^{2+} stores. The DOX-induced Ca^{2+} increase was slowly processed and sustained. The Ca^{2+} increase was inhibited by pretreatment with a sarcoplasmic reticulum (SR) Ca^{2+} channel blocker, ryanodine or dantrolene, and an antioxidant, α-lipoic acid or α-tocopherol. DOX-induced ROS generation was observed immediately after DOX treatment and increased in a time-dependent manner. The ROS production was significantly reduced by the pretreatment of the SR Ca^{2+} channel blockers and the antioxidants. Moreover, DOX-mediated activation of caspase-3 was significantly inhibited by the Ca^{2+} channel blockers and α-lipoic acid but not α-tocopherol. In addition, cotreatment of ryanodine with α-lipoic acid resulted in further inhibition of the casapse-3 activity. These results demonstrate that DOX-mediated ROS opens ryanodine receptor, resulting in an increase in $[Ca^{2+}]_i$ and that the increased $[Ca^{2+}]_i$ induces ROS production. These observations also suggest that DOX/ROS-induced increase of $[Ca^{2+}]_i$ plays a critical role in damage of cardiomyocytes.

Keywords: apoptosis; calcium signaling; doxorubicin; myocytes, cardiac; reactive oxygen species

Introduction

An anthracyclin derivative, doxorubicin (DOX) is a highly effective anticancer drug that is widely used in treatment of a broad spectrum of cancers (Minotti *et al*., 2004). The clinical efficacy of this drug is compromised due to manifestation of atrial and ventricular arrhythmia and development of cumulative dose-dependent cardiomyopathy and heart failure (Singal and Iliskovic, 1998; Minotti *et al*., 2004). One of the proposed mechanisms of DOX-induced cardiac dysfunction is an oxidation of cellular components via formation of reactive oxygene species (ROS) (Minotti *et al*., 2004). Several mechanisms of DOX-mediated ROS generation have been proposed. The quinone moiety of DOX is prone to the generation of oxygen radicals through enzymatic mechanism utilizing mitochondrial respiratory chain and nonenzymatic pathway, which incorporates iron (Gutierrez, 2000; Shadle *et al*., 2000). In mitochondrial respiratory chain coupled to increased ROS production, several enzymes, including cytochome p450, NAD(P)H dehydrogenase, and endothelial nitric oxide synthase, have been implicated to catalyze the reductive metabolism of DOX (Doroshow and Davies, 1986; Childs *et al*., 2002).

Although less attention than ROS has been received, a number of studies has suggested that DOX- mediated alteration of Ca^{2+} homeostasis is one of the possible mechanisms of cardiotoxicity. Very recent studies have demonstrated that DOX-mediated ROS generation and apoptosis are inhibited by reducing intracellular Ca^{2+} level using a Ca^{2+}

chelator (Kalivendi *et al*., 2001; 2005). Direct effects of the drug on various Ca^{2+} channels have also been observed. DOX increases the open probability of sarcoplasmic reticulum (SR) calcium release channels (Zorzato *et al*., 1985; Kim *et al*., 1989), inhibits Na^{+}-Ca^{2+} exchanger (Caroni *et al*., 1981) or activates L-type cardiac calcium channel (Keung *et al*., 1991). All of these DOX effects on Ca^{2+} channels can lead to Ca^{2+} overload of cardiac cells that may render mitochondrial calcium overloading, resulting in alteration of energy metabolism and generation of reactive oxygen species (ROS). In addition, an increase in intracellular Ca^{2+} level promotes ROS generation (Przygodzki *et al*., 2005), and also modulates mitochondrial permeability transition by opening permeability transition pores, therewith releasing cytochrome c, a critical step for apoptosis (Petrosillo *et al*., 2004; Waring, 2005).

In this study, we have investigated the molecular basis of DOX-mediated regulation of $[Ca^{2+}]_i$ utilizing freshly isolated adult rat cardiomyocytes. The results have shown that DOX-mediated ROS formation increases $[Ca^{2+}]_i$ by releasing Ca^{2+} from SR via ryanodine receptor (RyR) and that ROS-induced rise of $[Ca^{2+}]_i$ generates further ROS.

Materials and Methods

Isolation of adult rat cardiomyocytes

Cardiomyocytes were isolated from Sprague-Dawley rats, weighing 210-240 g, by the method with a slight modification (Xie *et al*., 2003). Rat hearts were rapidly excised, cannulated, and subjected to retrograde perfusion on a Langendorff apparatus at 37°C with Ca^{2+}-free Krebs-Hanseleit (KH) buffer (10 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM pyruvate, 11 mM glucose, and 1 mM $CaCl_2$, pH 7.3) for 5 min and then with KH buffer containing 5 mM taurine, 0.075% collagenase Type II and 0.08 μg/l protease type XIV for 7-15 min and washed with KH buffer containing 0.2 mM Ca^{2+}. The left ventricle was removed, chopped into small pieces and further digested in a glass conical flask at 37°C for 10 min with shaking. The undigested tissues were removed through a 200 μm-mesh nylon sheet. The Ca^{2+} concentration in the cell preparation was gradually increased up to 1 mM. Isolated myocytes were pelleted by centrifugation at 60 × *g* for 2 min at room temperature and resuspended in a stabilizing buffer (pH 7.4) containing 20 mM HEPES, 137 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 15 mM glucose, and 10 mM 2,3-butanedione monoxime (BDM). The cell preparation was kept in the stabilizing buffer containing 1% bovine serum albumin (BSA) at room temperature for 1.5 h and then washed three times with MEM medium at 37°C. The cells were attatched on laminine (10 μg/ml)-coated confocal plates and incubated at 37°C in 5% CO_2/95% air humidified incubator before Ca^{2+} measurement.

Preparation of rat cardiac sarcoplasmic reticulum (SR)

Rat cardiac SR vesicles were prepared using a slight modification of a method reported previously (Chamberlain and Fleisher, 1988). Rats of either sex (250-300 g) were euthanized and the hearts were immediately removed, immersed in ice-cold saline, and trimmed off fat and connective tissues. The hearts were transferred in 5 volumes of imidazole buffer (0.29 M sucrose, 0.5 mM DTT, 10 mM imidazole-HCl, pH 6.9), homogenized (three times, a 15-s interval, high setting), and centrifuged at 3,800 × *g* for 15 min at 4°C. The supernatant was centrifuged at 27,900 × *g* for 15 min. The supernatant from second centrifugation was centrifuged at 119,200 × *g* for 2 h at 4°C. The pellets were collected and suspended in a buffer (0.29 M sucrose, 0.65 M KCl, 0.5 mM DTT, 3 mM NaN_3, 0.5 mM PMSF, 10 mM imidazole-HCl, pH 6.7). After 30 min on ice, the suspension was centrifuged to remove large aggregates at 4,400 × *g* for 10 min. The supernatants were then centrifuged at 120,000 × *g* for 100 min at 4°C. After removal of the supernatants by aspiration, the pellets were resuspended in a minimal volume of the same buffer and stored at -80°C until use. Protein concentrations were determined using the protein assay kit (Bio-Rad Laboratories, Hercules, CA), and known concentrations of BSA were used as the standard.

[^{3}H]ryanodine binding assay

Binding of [^{3}H]ryanodine (Du Pont-New England Nuclear, Boston, MA) to cardiac SR was performed as described previously (Takeda *et al*., 2003). Briefly, cardiac SR (70 μg) was incubated with 5 nM [^{3}H]ryanodine (specific activity, 50 Ci/mmol) at 37°C for 1 h in a binding buffer (25 mM imidazole, pH 7.4, 1 M KCl, 1 mM EDTA, and 1 mM $CaCl_2$). The reaction mixtures were quenched in an ice-water bath for 20 min and then fast filtered on GF-C (Whatman, International Ltd, Maidstone, UK) filters using a Millipore fast filter apparatus (Millipore Corp. Bedford, MA). The GF-C filters were pretreated with 1% polyenthylene glycol for 1 h. After five time washes with a buffer (25 mM imidazole, pH 7.4, and 1 M KCl), the radioactivity remaining on the filters was determined by a liquid scintillation counter. Nonspecific binding was determined in the presence of 5 μM unlabeled ryanodine.

Determination of Ca^{2+} uptake and release by SR

Measurement of Ca^{2+} release from SR vesicles was performed spectrophotometrically using a dye antipyrylazo III as a Ca^{2+} sensor according to the method described previously (Olson *et al*., 2000). Cardiac SR preparation (88 mg/tube) was preloaded with Ca^{2+} by adding 0.98 ml of a reaction solution (0.15 mM antipyrylazo III, 20 mM HEPES, 5 mM KCl, 5 mM $MgCl_2$, 5 mM sodium oxalate, 5 mM ATP, 5 mM NaN_3, pH 7.0) at 37°C. Subsequently, Ca^{2+} was loaded to SR by addition of 6 times of $CaCl_2$ (40 nM each). Calcium loading and release induced by DOX (3 μM) were monitored by the difference in absorbance between 710 nm and 790 nm using a Beckman 530 UV/VIS spectrophotometer. Various Ca^{2+} channel blockers were preincubated for 30 min prior to treatment with DOX.

Determination of $[Ca^{2+}]_i$

Levels of $[Ca^{2+}]_i$ in quiescent isolated adult rat cardiomyocytes were measured as described previously (Xie *et al*., 2003; Rah *et al*., 2005). Briefly, cardiomyocytes attached to laminine-coated plates were loaded with 5 μM Fluo 3-AM (Molecular Probe, Eugene, OR) in KH buffer containing 1% BSA at 37°C for 30 min, and the plates were washed three times with KH buffer and used for the treatment with various drugs. SR Ca^{2+} channel blockers, ryanodine (20 μM) (Xie *et al*., 2005) and dantrolene (2 μM) (Buyukokuroglu *et al*., 2004) and antioxidants, α-lipoic acid (100 μM) (Cao *et al*., 2003) and α-tocopherol (100 μM) (Ulker *et al*., 2003) were preincubated at 37°C for 20 min prior to treatment with DOX (3 μM). Signal of $[Ca^{2+}]_i$ in cardiomyocytes was measured every 6 s for 90 min on confocal microscope (Nikon) equipped with air-cooled argon laser system. The emitted fluorescence at 530 nm was collected using photomultiplier tube, and $[Ca^{2+}]_i$ was estimated according to the following equation after subtracting the background DOX fluorescence: $[Ca^{2+}]_i=(K_d \cdot R)/[(K_d/[Ca^{2+}]_0)-R+1]$, where R is the normalized fluorescence (F/F_0) and K_d is the dissociation constant of the Ca^{2+}-Fluo-3 complex (560 nM), and F is the observed fluorescence levels (Loughrey *et al*., 2003). Each tracing was calibrated for the maximal intensity (F_{max}) by addition of ionomycin (8 μM) and for the minimal intensity (F_{min}) by addition of EGTA (50 mM final) at the end of each measurement. During experiments, the temperature of dishes was maintained at 37°C using a microwarm plate.

Determination of ROS generation

Measurement of hydrogen peroxide formation was performed using a fluorescent probe in isolated adult rat cardiomyocyte as previously described (Cominacini *et al*., 2000). Briefly, cardiomyocytes attached to laminine-coated plates were loaded with 0.2 μM (final concentration) of the peroxide-sensitive fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probe, Eugene, OR) in HEPES buffer (pH, 7.4) containing 20 mM HEPES, 137 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 15 mM glucose, and 10 mM BDM at 37°C for 30 min, and the plates were washed two times with HEPES buffer and used for the treatment with various drugs. RyR blockers, ryanodine (20 μM) and dantrolene (2 μM) and antioxidants, α-lipoic acid (100 μM) and α-tocopherol (100 μM) were preincubated at 37°C for 20 min prior to treatment with DOX (3 μM). Fluorecsence of ROS in cardiomyocytes was measured every 5 min for 90 min using a fluorescence photometer, Spectra Max Gemini (Molecular Devices, Sunnyvale, CA).

Measurement of caspase-3 activity

The enzyme activity was measured using fluorogenic substrate, N-acetyl Asp-Glu-Val-Asp-7-amino-4- methylcoumarin (Ac-DEVD-AMC) using a method provided by the manufacturer (BD Biosciences). Cardiomyocytes were attached to laminine-coated plates at 37°C for 10 min. After removing unattached cells, the cells were further incubated at 37°C for 2 h in M199 medium (Gibco, Invitrogen, Grand Island, NY) supplemented with 2 mg/ml BSA, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 2 mM pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For the experiments, the cells were pretreated with various drugs (20 μM ryanodine, 50 μM dantrolene, 100 μM α-lipoic acid, or 100 μM α-tocopherol) at 37°C for 20 min prior to addition of DOX (3 μM). When α-lipoic acid was treated with ryanodine, α-lipoic acid was first treated for 20 min, and then the blocker was added and incubated for 20 min before addition of DOX. At the indicated time point, the cells were washed with ice-cold PBS (140 mM NaCl, 2.7 mM KCl, and 10 mM KH_2PO_4/K_2HPO_4, pH 7.5). Excess of PBS was removed by aspiration, and the cells were immediately frozen at -80°C after addition of 500 μl of a lysis buffer (10 mM Tris-HCl, 10 mM KH_2PO_4/K_2HPO_4, pH 7.5, 130 mM NaCl, 1% Triton X-100, and 10 mM sodium pytophosphate). The cells were harvested, homogenized, and centrifuged at 15,000 rpm at 4°C for 30 min. Supernatants were collected, and protein concentrations were determined as described above. Samples (100 μg protein) were incubated with the substrate (20 μM, final) at 37°C for 1 h. Fluorescence of 7-amino-4-methylcoumarin liberated from the fluorogenic substrate was determined at 380 nm excitation and 460 nm emission using a fluorescence photometer,

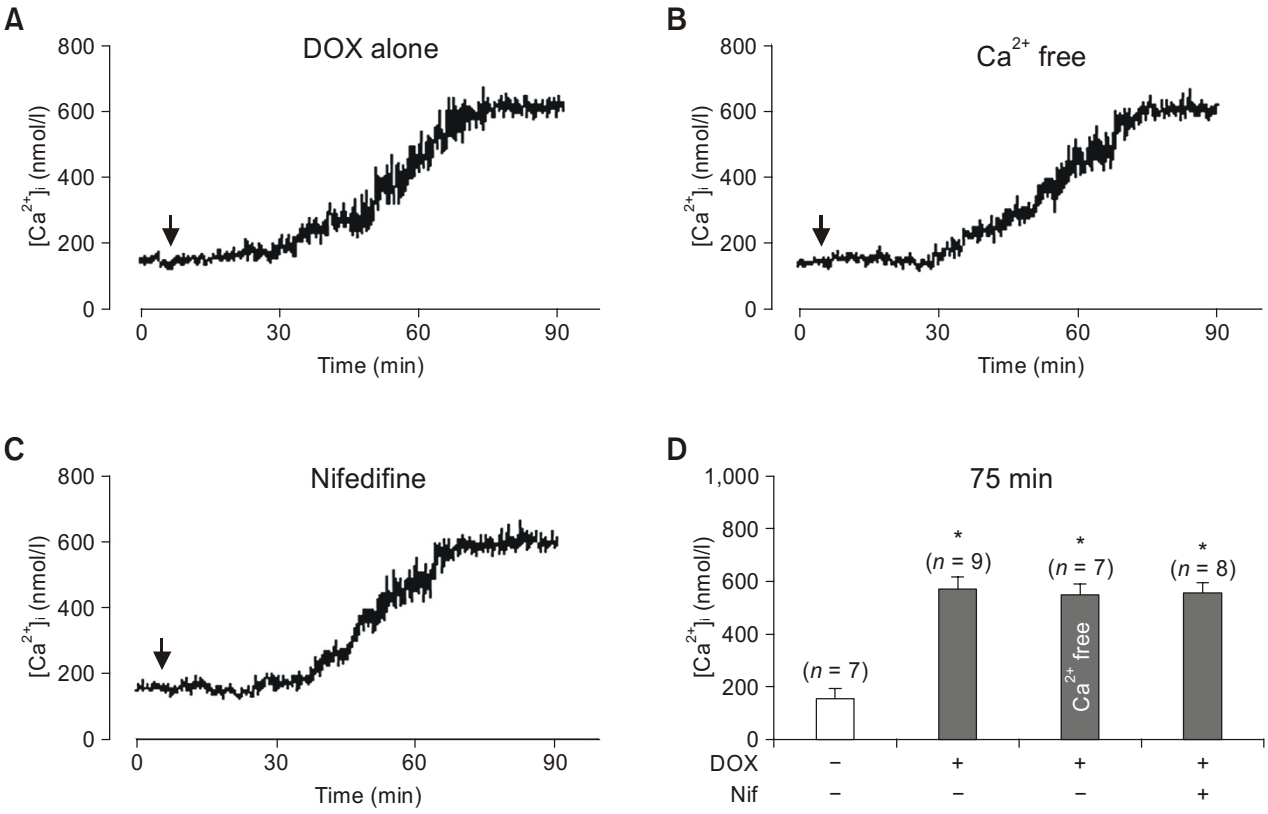

Figure 1. DOX induces Ca^{2+} release from intracellular stores. (A) Determination of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (1 mM). (B) The Ca^{2+} signal in the absence of extracellular Ca^{2+} (Ca^{2+} free) was determined using KH buffer, which was prepared without addition of $CaCl_2$. Concentration of DOX was 3 μM. (C) DOX does not activate L-type Ca^{2+} channel. L-type Ca^{2+} channel activation by DOX was determined in the presence of extracellular Ca^{2+} (1 mM) using a channel blocker, nifedipine (20 μM). An arrow indicates the time point at where DOX was added. (D) Changes of $[Ca^{2+}]_i$ are presented mean ± SEM. Nif indicates nifedipine. Cell numbers are presented in the parentheses. *, $P < 0.001$ versus basal $[Ca^{2+}]_i$.

Spectra Max Gemini (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA. Significant differences between groups were determined using the unpaired Student's *t* test. Statistical significance was set at $P < 0.05$.

Results

DOX induces Ca^{2+} release from intracellular Ca^{2+} stores in rat cardiomyocytes

Firstly, to elucidate the source of DOX-mediated increase of $[Ca^{2+}]_i$ in cardiomyocytes, Ca^{2+} signal was determined in the presence and absence of extracellular Ca^{2+}. Treatment of cardiomyocytes with DOX increased levels of $[Ca^{2+}]_i$ in the presence and absence of extracellular Ca^{2+} (Figure 1A and B). The DOX-induced Ca^{2+} increase was very slow, beginning to increase at approximately 30 min after DOX treatment, reached plateau at approximately 70 min, and sustained. The time to start Ca^{2+} rise was somewhat DOX concentration-dependent. The Ca^{2+} release begun to increase at approximately 60 min and 15 min after treatment with 1 μM and 5 μM DOX, respectively. In addition, DOX has been shown to activate L-type channel (Keung *et al*., 1991). Therefore, effect of a L-type Ca^{2+} channel blocker, nifedipine, on DOX-induced Ca^{2+} signal was examined. This blocker showed no effect on DOX-induced increase of $[Ca^{2+}]_i$ (Figure 1C). The Ca^{2+} levels at 75 min after DOX treatment are summarized in Figure 1D. These results suggest that DOX induces release of Ca^{2+} from intracellular stores in rat cardiomyocytes.

DOX induces Ca^{2+} release from isolated SR

A previous study has reported that DOX binds directly to RyR, thereby increases open probability (Saeki *et al*., 2002) and that DOX-induced Ca^{2+} increase shows a similar pattern of RyR-mediated

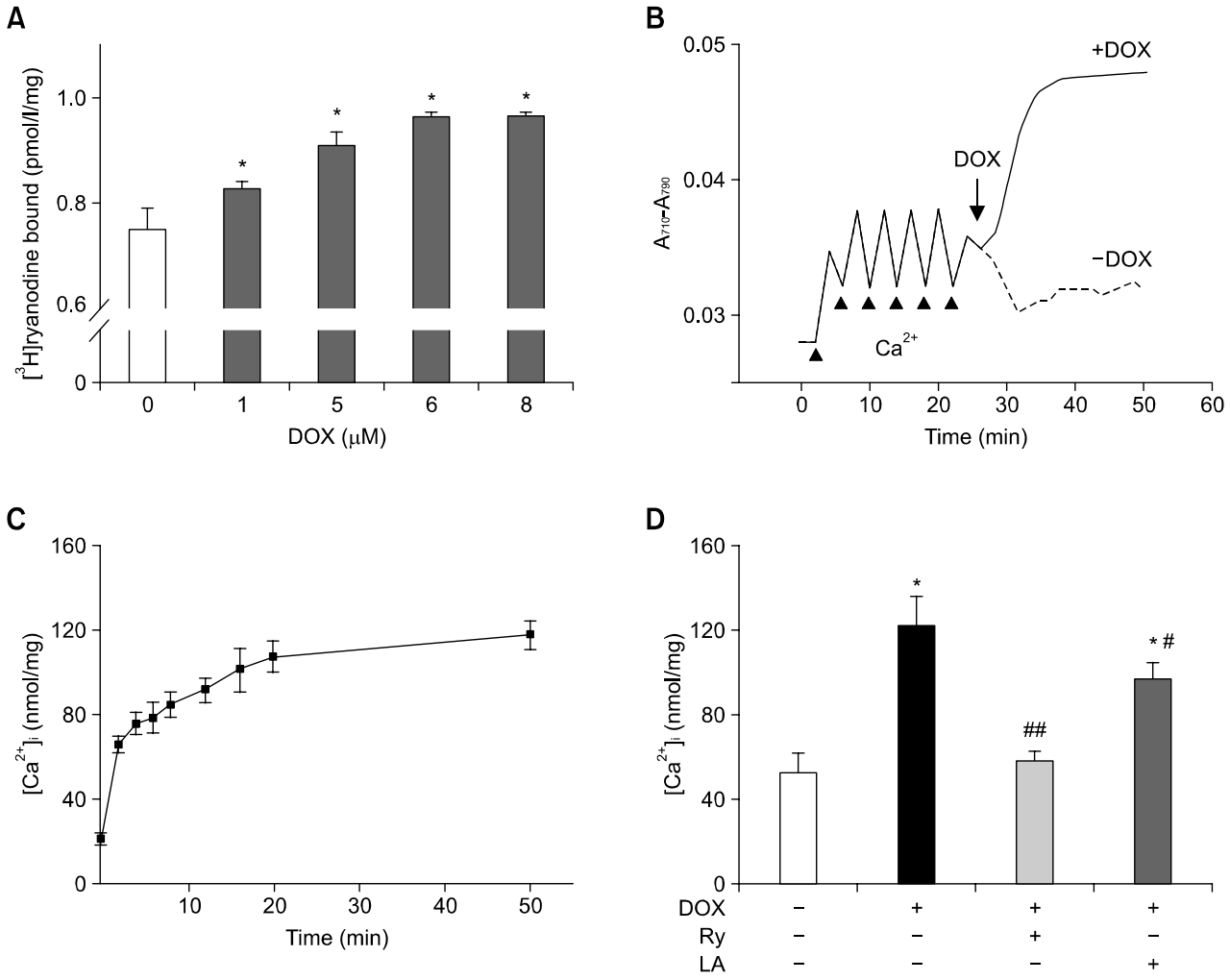

Figure 2. DOX induces Ca^{2+} release from SR via RyR. (A) DOX increases binding of ryanodine by SR. The binding of [^{3}H]ryanodine by SR was determined as detailed in Materials and methods. The error bar shows SEM from three independent experiments in duplicate. *, $P < 0.05$ and **, $P < 0.001$versus control. (B) Trace of Ca^{2+} uptake and DOX-induced increase of Ca^{2+} level in the reaction solution. Loading of Ca^{2+} (40 μM) was repeated 6 times. Ca^{2+} was added when the peak of Ca^{2+} fluorescence was reduced as indicated with an arrowhead. An arrow indicates the time point of DOX (3 μM) addition. The experiment was performed three times with similar results. (C) Time-dependent release of Ca^{2+} from SR. Ca^{2+} was loaded into SR as described in Materials and Methods. The Ca^{2+} release was monitored at indicated time after addition of DOX (3 μM). The error bar is SEM from three independent experiments. (D) DOX-induced Ca^{2+} release is blocked by pre-treatment with ryanodine (Ry) or α-lipoic acid (LA). The Ca^{2+}-loaded SR preparation (70 μg) was preincubated with 20 μM ryanodine (Ry) or 100 μM α-lipoic acid at 37°C for 15 min. The Ca^{2+} level in the reaction buffer was determined at 30 min after treatment of Ca^{2+}-loaded SR with DOX. The error bar shows SEM from three independent experiments in duplicate. *$P < 0.001$ versus control; $^{\#}P < 0.04$ versus DOX; $^{\#\#}P < 0.001$ versus DOX.

Ca^{2+} signal (Kim *et al*., 1989). The above data also suggest that DOX induces Ca^{2+} release from SR. An involvement of RyR in Ca^{2+} release was examined using SR preparations from rat hearts. The SR preparations showed high ryanodine binding activity (750 ± 5 nmole/mg), demonstrating that the preparation is SR-rich vesicles. Binding of [^{3}H]ryanodine by the SR vesicles was modestly increased in a DOX dose-dependent manner (Figure 2A), and maximum increase (28 ± 5%) of ryanodine binding was observed at 6 μM DOX. Uptake of Ca^{2+} by SR vesicles was observed, and treatment of these Ca^{2+}-loaded vesicles with DOX resulted in an increase of Ca^{2+} level in the reaction solution (Figure 2B). The Ca^{2+} release was gradually increased in a time-dependent manner, reached plateau at approximately 20 min after DOX treatment, and then sustained (Figure 2C). Interestingly, the DOX-induced Ca^{2+} release was substantially inhibited by pretreatment with high concentration of ryanodine (20 μM, this concentration of ryanodine blocks RyR) (Figure 2D). Treatment of α-lipoic acid, which is a multi-

functional antioxidant, reduced approximately 20% DOX-induced Ca^{2+} release (Figure 2D). Together, these results suggest that DOX induces Ca^{2+} release from SR mainly through RyR.

DOX-induced Ca^{2+} increase is inhibited by Ca^{2+} channel blockers and antioxidants in rat cardiomyocytes

The above observations that DOX stimulates Ca^{2+}

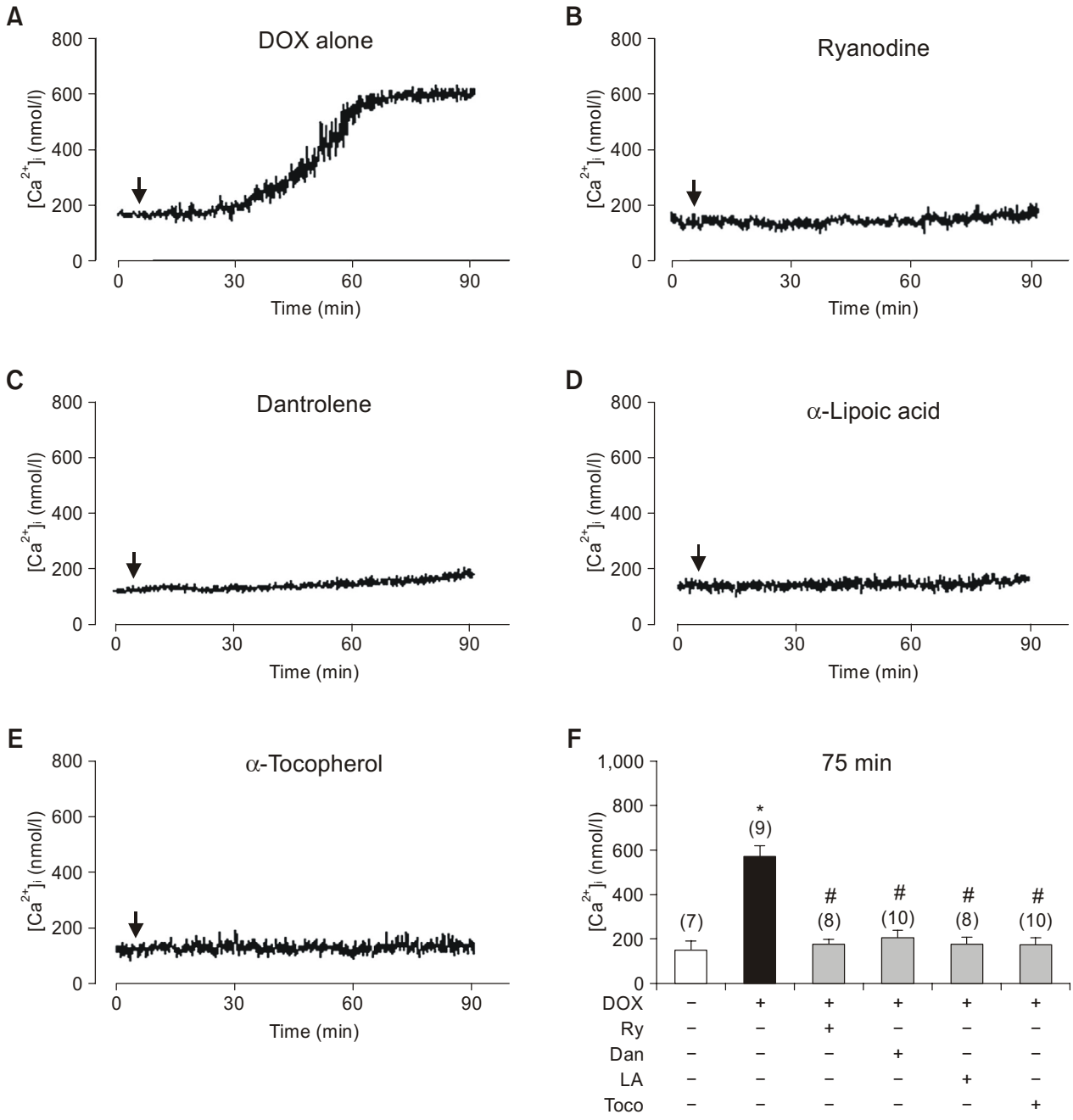

Figure 3. DOX-mediated increase of $[Ca^{2+}]_i$ is inhibited by the RyR blockers or antioxidants. Prior to addition of DOX (3 μM), cardiomyocytes were preincubated with and without ryanodine (Ry), dantrolene (Dan), α-lipoic acid (LA), or α-tocopherol (Toco) as described in Materials and Methods. (A) Inhibition of DOX-induced increase of $[Ca^{2+}]_i$. DOX-mediated increase of $[Ca^{2+}]_i$ is blocked by ryanodine (B), dantrolene (C), α-lipoic acid (D), or α-tocopherol (E). (F) A direct comparison of mean $[Ca^{2+}]_i$ at 75 min after treatment of DOX. The data are presented as mean ± SEM. Cell numbers are indicated in the parentheses. $^*P < 0.001$ versus basal $[Ca^{2+}]_i$; $^{\#}P < 0.001$ versus DOX-induced $[Ca^{2+}]_i$. An arrow indicates the time point of DOX addition.

release from SR via RyR were further evaluated using freshly isolated adult rat cardiomyocytes. Drugs with clearly defined effects on Ca^{2+} handling or ROS scavenging were used to explore DOX-mediated SR Ca^{2+} release. Pretreatment of a RyR blocker, ryanodine or dantrolene, inhibited DOX-induced Ca^{2+} rise (Figure 3B and C). Treatment of an antioxidant, α-lipoic acid or α-tocopherol, also blocked DOX-induced increase of $[Ca^{2+}]_i$ (Figure 3D and E). Blocking ability of SR Ca^{2+} channel blockers or antioxidants in DOX-induced Ca^{2+} rise observed at 75 min is summarized in Figure 3F. These data indicate that DOX-mediated increase in $[Ca^{2+}]_i$ is mainly due to opening of RyR and that there is a close link between Ca^{2+} release and ROS generation.

DOX-induced ROS generation is inhibited by the treatment of cardiomyocytes with antioxidants and Ca^{2+} channel blockers

To clarify a link between ROS generation and Ca^{2+} release induced by DOX, ROS production was determined as a function of time. Firstly, permeability of DOX was examined by determining intrinsic DOX fluorescence in cardiomyocytes. Presence of DOX in cardiomyocytes was found within 10 min and accumulated in the nucleus with time (Figure 4A). Similarly, the level of ROS was gradually increased in a time-dependent manner by the treatment of DOX (Figure 4B). The increase in ROS production was significantly reduced by the pretreatment with Ca^{2+} channel blockers, ryanodine and dantrolene (Figure 4C) or antioxidants, α-lipoic acid and α-tocopherol (Figure 4D). A direct comparison of ROS levels are summarized in Figure 4E. These results suggest that DOX-induced ROS generation is a gradual process involving Ca^{2+}.

Caspase-3 activation by DOX is inhibited by RyR blocker and antioxidant

Studies have shown that treatment of DOX results in activation of apoptotic pathways in which caspase-3 is activated (Yamaoka *et al*., 2000; Kalivendi *et al*., 2001; 2005; Jang *et al*., 2004). To examine the effect of Ca^{2+} rise and ROS generation induced by DOX on cardiomyocytes, caspase-3 activity was determined. Treatment of cardiomyocytes with DOX increased caspase-3 activity in a time-dependent manner, reaching maximum at approximately 7 h after DOX treatment (Figure 5A). The DOX-induced caspase-3 activation was significantly inhibited by the pretreatment with ryanodine or dantrolene (Figure 5B). Ryanodine or dantrolene alone had no effects on caspase-3 activity. α-Lipoic acid also reduced the DOX-induced caspase-3 activation whereas α-tocopherol did not. Failure of α-tocopherol to block DOX-mediated activation of caspase-3 is probably due to loss of antioxidant capacity during long-term incubation. Cotreatment of ryanodine with α-lipoic acid completely inhibited caspase-3 activity (Figure 5B).

Discussion

In this study, the molecular basis of DOX-induced intracellular Ca^{2+} regulation has been examined

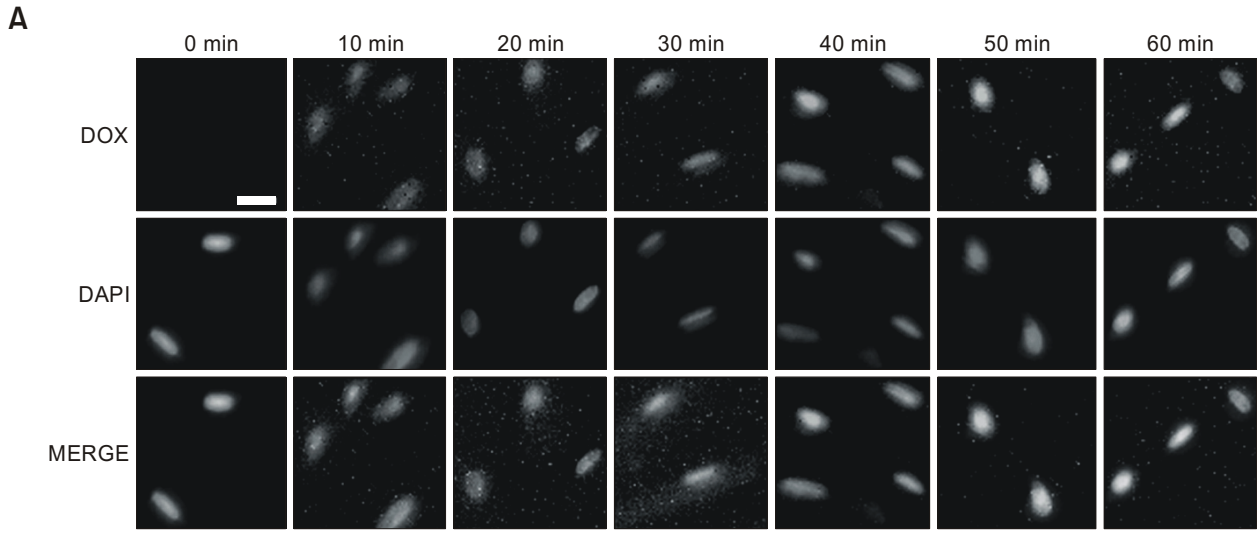

Figure 4. DOX-mediated ROS generation is inhibited by RyR blockers and antioxidants. (A) Permeability of DOX into rat cardiomyocytes. Cells were treated with 3 μM DOX, washed with PBS, and then intrinsic DOX fluorescence was observed under a fluorescent microscope. Bar indicates 10 μm.

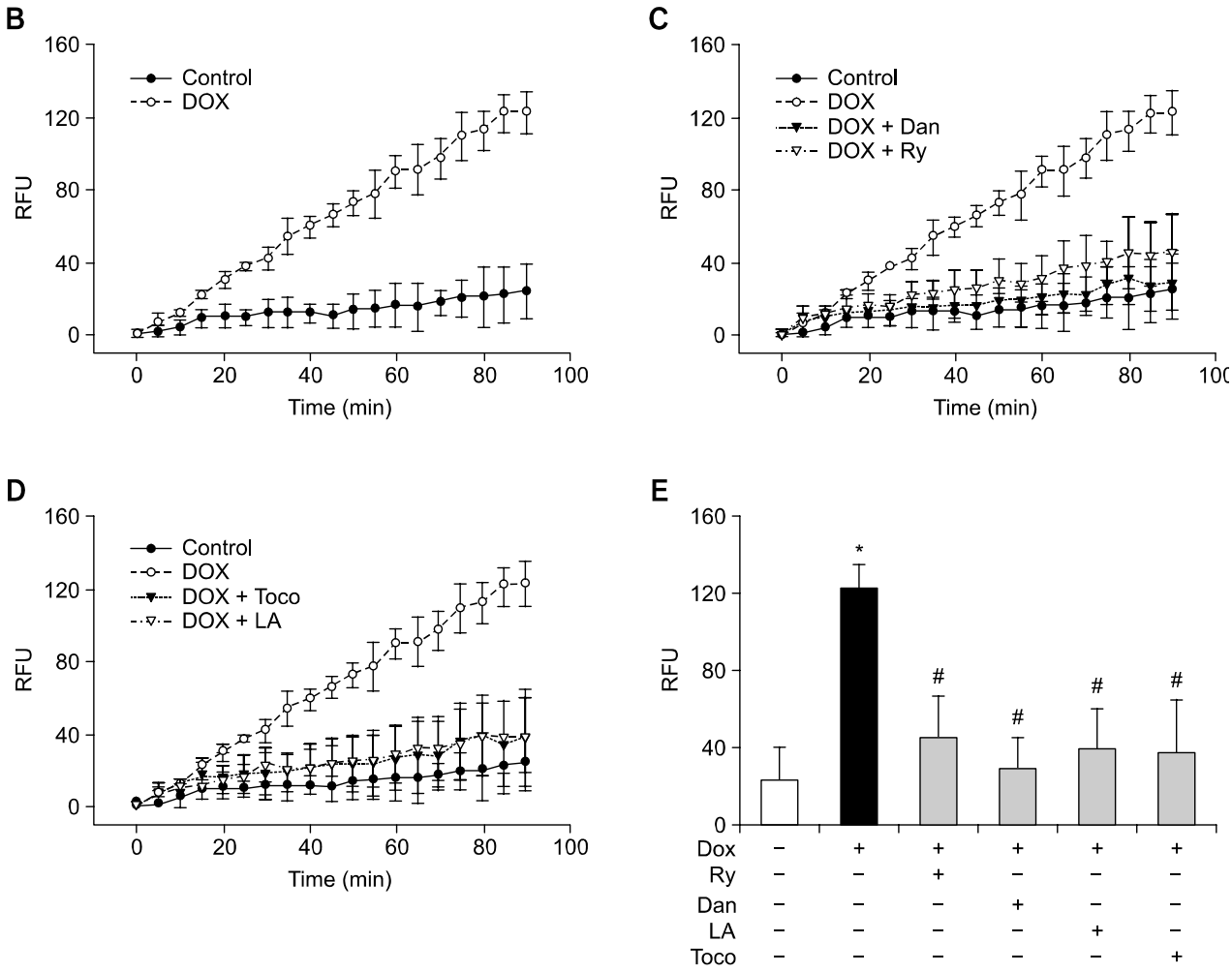

Figure 4. (B) Treatment of DOX increases ROS production. As an assessment of ROS generation by DOX, H_2O_2 levels were determined with the fluorescent probe H_2DCFDA as detailed in Materials and methods. (C) DOX-induced ROS generation is inhibited by ryanodine (Ry) or Dantrolene (Dan). Cells were pretreated with ryanodine or dantrolene at 37°C for 30 min. (D) DOX- induced ROS generation is inhibited by the treatment of α-lipoic acd (LA) and α-tocopherol (Toco). Prior to addition of DOX, cells were pretreated with α-lipoic acid or α-tocopherol as detailed in Materials and Methods. (E) A direct comparison of the ROS levels. Data are presented as mean ± SEM from three independent experiments. *$P < 0.001$ versus; #$P < 0.005$ versus DOX.

utilizing adult rat cardiomyocytes. The results have revealed that DOX increases $[Ca^{2+}]_i$ by releasing Ca^{2+} from SR through RyR and that there is a close link between DOX-induced ROS generation and an increase of $[Ca^{2+}]_i$. Evidence for a link between ROS generation and Ca^{2+} increase is that 1) DOX-induced increase of $[Ca^{2+}]_i$ is inhibited by pretreatment of antioxidants and 2) ROS generation is blocked by pretreatment with the RyR blockers. These findings suggest that the amount of ROS generated by DOX is an amalgamation of DOX plus Ca^{2+}-induced ROS production.

To date, the Ca^{2+} channel(s) opened by DOX remains unclear. Studies have reported that DOX activates L-type channel (Keung *et al*., 1991), inhibits Na^+-Ca^{2+} exchanger (Caroni *et al*., 1981), or releases Ca^{2+} from SR (Zorzato *et al*., 1985; Kim *et al*., 1989). Our results have shown that DOX-mediated increase of $[Ca^{2+}]_i$ is due to a release of Ca^{2+} from SR through ROS-mediated opening of RyR. The DOX-mediated Ca^{2+} signal was increased in the presence of a L-type channel blocker, nifedepine or absence of extracellular Ca^{2+}. The DOX-mediated Ca^{2+} release was blocked by pretreatment with an antioxidants, α-lipoic acid or α-tocopherol, indicating that ROS causes opening of the receptor. Although the causative mechanism of ROS-mediated RyR opening is not clearly understood, the receptor is known to be sensitive to oxidation due to presence of a large number of thiols (Favero *et al*., 1995). In addition, quinone-containing

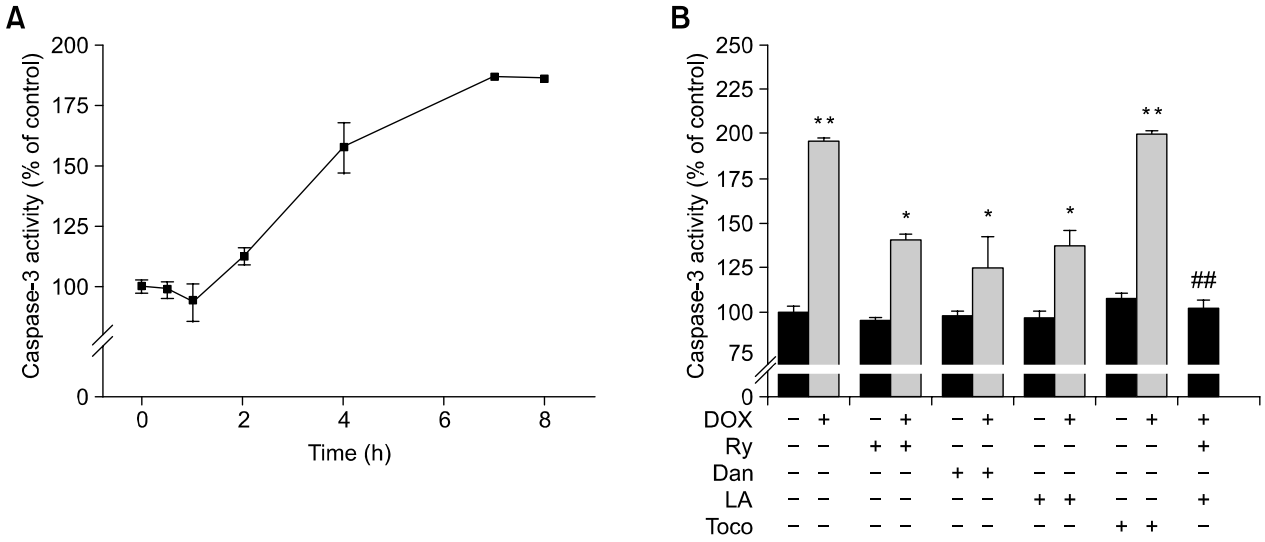

Figure 5. Treatment of cardiomyocytes with DOX stimulates caspase-3 activity. (A) Caspase-3 activity was increased in a time-dependent manner by treatment of DOX (3 μM). The caspase-3 activity was determined as detailed in Materials and methods. (B) DOX-stimulated caspase-3 activity is inhibited by treatment with RyR inhibitor or antioxidant. Cardiomyocytes were preincubated with 20 μM ryanodine (Ry), 2 μM dantrolene (Dan), 100 μM α-lipoic acid (LA), 100 μM α-tocopherol (Toco) or 20 μM ryanodine plus 100 μM α-lipoic acid at 37°C for 30 min. The data are presented mean ± SEM from three independent experiments in duplicate. *$P < 0.01$ and **$P < 0.001$ versus basal control (no drug treatment); $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ versus DOX.

compounds such as DOX, 1,4-benzoquinone, and 1,4-naphtoquinone are shown to open RyR by oxidizing essential thiols in RyR (Feng *et al*., 1999). Direct binding of DOX to RyR has also been reported (Saeki *et al*., 2002). Similarly, with the isolated SR preparation, DOX increased ryanodine binding to the receptor in a dose-dependent manner, indicating an increase of high affinity state of RyR for ryanodine. However, with cardiomyocytes, there was no indication that binding of DOX to the receptor opens RyR, since treatment of antioxidants blocked almost completely the Ca^{2+} rise mediated by DOX. One of interesting findings is that although RyR is most likely opened by ROS, the RyR blockers are able to inhibit the Ca^{2+} release, resulting in attenuation of ROS generation.

A large number of studies has suggested a close relationship between enhancement of $[Ca^{2+}]_i$ and ROS generation (Brookes *et al*., 2004). However, a link between DOX-mediated Ca^{2+} increase and ROS generation has not been studied. Our results indicate that DOX/ROS-mediated Ca^{2+} increase also generates ROS: DOX-induced ROS increase was inhibited by the treatment with high concentration of ryanodine or dantrolene. Consistent with the observations, studies have demonstrated that DOX-mediated ROS production is reduced by the treatment of endothelial cells and H9c2 cells with an intracellular Ca^{2+} chelator BAPTA (Kalivendi *et al*., 2001; 2005). The increase of ROS generation is also observed in cardiomyocytes or spleen cells by treatment with Ca^{2+} ionophore A23187 (Przygodzki *et al*., 2005) or thapsigargin (Yip *et al*., 2005), respectively. In addition, antioxidant properties of Ca^{2+} channel blockers have been observed with *in vivo* and *in vitro* studies (Buyukokuroglu *et al*., 2001; 2004; Umemoto *et al*., 2004; Ysunari *et al*., 2005). The molecular mechanism by which the increased Ca^{2+} level promotes ROS production is not clearly understood. It has been shown that accumulation of Ca^{2+} in mitochondria promotes the generation of ROS (Brookes *et al*., 2004). DOX-mediated enhancement of Ca^{2+}-sensitive nitric oxide synthase expression leads to generation of ROS in endothelial cells (Kalivendi *et al*., 2001). Recently, it has been demonstrated that dual oxidase-2, which belongs to NAD(P)H oxidase family, has an intrinsic Ca^{2+}-dependent H_2O_2 generating activity (Ameziane-El-Hassani *et al*., 2005). A similar Ca^{2+}-sensitive ROS generating enzyme(s) may exist in rat cardiomyocytes.

In this study, as an assessment of cellular effect of DOX-induced Ca^{2+} rise and ROS generation on cardiomyocytes, caspase-3 activity has been examined. The data indicate that DOX/ROS-mediated increase of $[Ca^{2+}]_i$ plays a critical role in cardiomyocyte apoptosis. DOX-stimulated caspae-3 activity was significantly blocked by ryanodine and dantrolene. Interestingly, antioxidants used in this study showed different effects on the modulation of Ca^{2+} signal and

apoptosis. α-Lipoic acid and α-tocopherol inhibited DOX-induced increase in $[Ca^{2+}]_i$. However, DOX-mediated activation of caspase-3 was reduced by α-lipoic acid, but not by α-tocopherol. Although reason for these different effects of the antioxidants is not clearly understood, it may due to different reducing capacity of the antioxidants. α-Lipoic acid and its reduced form, dihydrolipoic acid (DHLA), have been referred to as "a universal antioxidant" that functions in both membrane and aqueous phases (Packer *et al*., 1995). Both α-lipoic acid and DHLA have substantial antioxidant properties. These include their ability to directly quench a variety of reactive oxygen species, inhibit reactive oxygen-generators, and spare other antioxidants. α-Tocopherol, including other vitamins (vitamin A and C) has been shown to exhibit limited reducing power in inhibiting DOX-induced cardiomyophathy and apoptosis (Ulker *et al*., 2003; Minotti *et al*., 2004). In addition, we have also found that cotreatment of ryanodine and α-lipoic acid further attenuates DOX-stimulated caspase-3 activation blocked by treatment of individual agent, indicating that the activation of casapase-3 by DOX is largely due to ROS and Ca^{2+}.

Cardiodysfunction induced by DOX therapy is a continuing clinical problem. Our results demonstrate that DOX/ROS stimulates opening of RyR, resulting in a rise of $[Ca^{2+}]_i$. The data have also suggested that ROS produced by DOX is an amalgamation of ROS generated by both DOX and the increased Ca^{2+} and that the DOX/ROS-mediated Ca^{2+} increase plays a key role in DOX-mediated caspase-3 activation. These findings also provide a molecular basis of alteration of Ca^{2+} homeostasis by DOX.

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