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**Mitochondrial Translocator Protein (TSPO) ligands prevent
doxorubicin-induced mechanical dysfunction and cell death in isolated
cardiomyocytes**

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ABSTRACT

Contractile dysfunction and subsequent development of cardiomyopathies are well known limiting factors in the treatment of cancer with doxorubicin and have been linked to mitochondrial dysfunction. Here, using adult isolated paced cardiomyocytes, we have demonstrated that ligands of translocator protein (TSPO) 4'-chlorodiazepam and TRO40303 prevented the doxorubicin-induced alterations in contractility and improved cardiomyocyte viability. This cardioprotective effect was closely associated with both a potent reduction in reactive oxygen species production and inhibition of mitochondrial permeability transition pore opening. Thus, preventive administration of TSPO ligands may represent a novel pharmacological strategy to protect the heart during doxorubicin treatment.

Keywords: cardiomyocytes, doxorubicin, mitochondria, permeability transition pore, ROS, TSPO ligands.

Abbreviations: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid ; CoCl₂, cobalt chloride; CsA, cyclosporin A; DCF, dichlorofluorescein ; DCFH-DA, 2,7-dichlorodihydrofluorescein-diacetate ; CDZ, 4'-chlorodiazepam ; IMAC, inner membrane anion channel; mPTP, mitochondrial permeability transition pore ; \cdot OH, hydroxyl radical; PI, propidium iodide ; ROS, reactive oxygen species ; TSPO, translocator protein

1. Introduction

Doxorubicin, a quinone-containing anthracycline, is widely used as a chemotherapeutic agent for the treatment of a large spectrum of human neoplastic diseases. However, its administration in humans is limited by the risk of severe cardiotoxicity (Olson & Mushlin, 1990; Singal & Iliskovic, 1998; Minotti et al., 2004) including transient arrhythmias, non-specific electrocardiographic abnormalities, pericarditis and transient depression of left ventricular function (Ferreira et al., 2008). A large part of these abnormalities results from an impaired contractility and subsequent development of cardiomyopathies secondary to alterations in cellular structure (Lim et al., 2004), cell death (Minotti et al., 2004; Childs et al., 2002; Keefe et al., 2001) with ceramide accumulation, mitochondrial dysfunction, cytochrome c release (Childs et al., 2002; Delpy et al., 1999; Kalyanaraman et al., 2002) and generation of reactive oxygen species (ROS) (Tsang et al., 2003).

Mitochondrial dysfunction has been identified as an important event in doxorubicin-induced damage (Lebrecht et al., 2007; Montaigne et al., 2010) including loss of ATP synthesis, increased ATP hydrolysis, impairment of ionic homeostasis and ROS generation (Doroshov & Davies, 1986; Sawyer et al., 1999; Timolati et al., 2006). Indeed, mitochondrial respiratory chain is a major source of ROS production during doxorubicin treatment through inhibition of respiratory complexes (Berthiaume & Wallace, 2007; Tokarska-Schlattner et al., 2005). ROS production leads to the reduction in oxidative phosphorylation capacity and ultimately to an increase in the permeability of mitochondrial membranes through opening of the mitochondrial transition pore (mPTP), a multiprotein complex, leading to mitochondrial swelling and subsequent release of pro-apoptotic factors (Halestrap, 2009; Zoratti & Szabo, 1995).

In this context, any pharmacological strategy preventing mitochondrial dysfunction during doxorubicin treatment has therapeutic potential among which are the ligands of the translocator protein (TSPO) formerly named the peripheral benzodiazepine receptor. TSPO is a 18-kDa protein located in the outer mitochondrial membrane and a putative component of the mPTP multiprotein complex (Gavish et al., 1999; Papadopoulos et al., 2006). TSPO is abundant in steroidogenic tissues where it mediates the transport of cholesterol from the outer to the inner mitochondrial membranes (Papadopoulos et al., 1990). TSPO is also present in non-steroidogenic tissues such as the heart where its function remains unknown. Recently, several studies have shown that TSPO ligands prevented apoptosis, reduced mitochondrial permeabilization and ultimately the incidence of arrhythmias as well as left ventricular dysfunction and infarct size following myocardial ischemia-reperfusion in rats (Obame et al., 2007; Schaller et al., 2010; Xiao et al., 2010).

In this study, we investigated the possible preventive effect of TSPO ligands against doxorubicin-induced cardiotoxicity in isolated adult mouse and rat cardiomyocytes. Two structurally unrelated TSPO ligands were used, the benzodiazepine, 4'-chlorodiazepam, (Johnson et al., 1986) and the new ligand, TRO40303, that binds specifically to the cholesterol site of TSPO, which is different than the 4'-chlorodiazepam binding site (Schaller et al., 2010), on contractile performance and cell death of isolated adult cardiomyocytes treated with doxorubicin.

2. Materials and methods

2.1. Animals

All animal procedures used in this study were in accordance with the European Community Council Directive (86-609/87-848 EEC) and recommendations of the French Ministère de l'Agriculture. Male wistar rats (260-280g) were purchased from Janvier (Le Genest Saint Isle, France). Cyclophilin D knockout (CypD KO) mice were a generous gift from the Laboratory of Michel Ovize (INSERM U1060, Lyon, France; Li et al., 2012).

2.2. Isolation of rat and mouse ventricular myocytes

Single ventricular cells were obtained from animals as described in the AfCS Procedure Protocols PP00000125. Animals (200 - 250 g) were anesthetized with a solution of pentobarbital (60 mg/kg) and received heparin (200 UI/kg). The hearts were excised and retrogradually perfused on a Langendorff apparatus with calcium-free oxygenated (95% O₂, 5% CO₂) perfusion buffer (in mM : NaCl 113, KCl 4.7, KH₂PO₄ 0.6, Na₂HPO₄ 0.6, MgSO₄ 1.2, NaHCO₃ 12, KHCO₃ 10, HEPES 10, taurine 30, 2,3-butanedione monoxime 500, glucose 5.5, with pH adjusted to 7.4 with NaOH 1 N at 37 °C). Then, hearts were perfused for a few minutes with the same calcium-free perfusion buffer supplemented with 0.25 mg/mL of liberase blendzyme TM (Roche diagnostics, IN, USA), 0.14 mg/mL of trypsin (Invitrogen, Illkirch, France) and 12.5 µM of calcium chloride. The left ventricle was removed and further dissected into small pieces, the cellular dissociation being achieved by gentle mechanical agitation in a stopping buffer (in mM: NaCl 113, KCl 4.7, KH₂PO₄ 0.6, Na₂HPO₄ 0.6, MgSO₄ 1.2, NaHCO₃ 12, KHCO₃ 10, HEPES 10, taurine 30, 2,3-butanedione monoxime 500, glucose 5.5, CaCl₂ 12.5 µM and bovine calf serum 10% with pH adjusted to 7.4 with NaOH 1 N at 37°C). Extracellular calcium was added incrementally up to 1.0 mM. All cells studied were rod-shaped, had clear cross-striations and lacked any visible vesicles on their surfaces under observations with an optical microscope.

2.3. Measurement of myocyte contractility

Myocytes were transferred to a warmed (37°C) and continuously perfused cell chamber located on the stage of an inverted microscope (Nikon, Paris, France). The chamber was perfused with a physiological buffer containing (in mM) NaCl 140, KCl 5.4, CaCl₂ 1, MgCl₂ 0.8, HEPES 10 and glucose 5.6 (pH = 7.4 at 37°C).

Myocytes contraction was induced once per second (1 Hz) by platinum field electrodes that were placed in the cell chamber attached to a stimulator (Grass S88K, Grass Instruments, Trappes, France). Cell images were continuously acquired through a x 40 objective lens and transmitted to a 240 samples/s charge coupled device (CCD) video camera (Myocam, Ionoptix, Milton, MA, USA). The output from the CCD camera was displayed on a video monitor.

Myocytes were selected according to the following criteria (Kim et al., 1999): a rod shaped appearance with clear striations and no membrane blebs, no spontaneous contractions when stimulated in 1 mM calcium buffer, steady diastolic length and contractile amplitude at basal stimulation rates. Sarcomere length was measured using a video motion edge detector (DSI 200, Ionoptix, Milton, MA, USA) and data were acquired at 240 samples/s. The camera images were converted to sarcomere length measurements by the video sarcomere detector and were analyzed by the data-acquisition system. Contractility was analyzed using an IonWizard data acquisition system (IonOptix). For sarcomere shortening, absolute twitch amplitude was measured as the difference between the systolic and the diastolic sarcomere length; sarcomere length shortening percentage was expressed as the ratio of absolute twitch amplitude to diastolic sarcomere length. Shortening velocity (-dl/dt) was used to measure the maximum velocity of sarcomere shortening during contraction and relengthening velocity

(+dl/dt) was used to assess the maximum velocity of sarcomere relengthening during relaxation.

2.4. Measurement of intracytosolic calcium concentration

Intracytosolic calcium concentration was measured with a calcium ion-sensing system (Ionoptix, Milton, MA) by means of the fluorescent calcium indicator fura-2 (Invitrogen, Illkirch, France), using a dual-excitation fluorescence. The cells were loaded with 5 μ M of the membrane-permeant ester form of fura-2-AM, for 20 min at room temperature.

The fura-loaded myocytes were excited at 360 nm and 380 nm, and the resulting fluorescence was measured at 510 nm. The ratio of the fluorescence excited at 360 nm to that excited at 380 nm was independent of the intracellular fura-2 concentration, cell geometry, and excitation light intensity and reflected intracytosolic calcium concentration.

The fluorescence excited at 360 nm was independent of intracytosolic calcium concentration changes. During a typical data collection run, the 360 nm light was presented to the myocytes for 0.5 s at the beginning and at the end of the run, which lasted 250 ms. Between these two points, only the 380 nm light was presented, and the fluorescence was acquired by the system. At each of these sampling points, a calculated 360 nm excited fluorescence was determined, using an interpolation of the 360 nm excited fluorescence collected at the beginning and at the end of the data collection run. The fluorescence ratio was formed by dividing the calculated 360 nm excited fluorescence by the measured 380 nm excited fluorescence. Changes in intracellular Ca^{2+} were analyzed using an IonWizard data acquisition system (IonOptix). For the ratio of fluorescence intensities of fura-2 AM (360nm/380nm), the amplitude was measured as the difference between baseline and peak

length; Ca^{2+} transient amplitude was expressed as the ratio of absolute amplitude to transient baseline.

2.5. Measurement of mPTP opening in adult cardiomyocytes and cell viability

Direct assessment of mPTP opening in cardiomyocytes was made by means of the established loading procedure of the cells with calcein acetoxymethyl ester (calcein-AM) and CoCl_2 resulting in mitochondrial localization of calcein fluorescence (Katoh et al., 2002; Petronilli et al., 1999). Cells were loaded with 1 μM calcein-AM for 30 min at 37°C in 2 ml of M199 medium, pH 7.4, supplemented with 1 mM CoCl_2 . To determine cell death, cells were co-loaded with propidium iodide (5 μM), which permeates only the damaged cells. For detection of calcein fluorescence, a 460 to 490 nm excitation and a 510 nm emission filters were used. Propidium iodide fluorescence was excited at 520 to 550 nm and recorded at 580 nm. Photographs were taken using an inverted fluorescence microscope (Olympus IX-81, Rungis, France). Measurements of calcein and propidium iodide fluorescence intensities were performed for each image using a digital epifluorescence imaging software (Cell P; Olympus, Rungis, France). Fluorescence was integrated over a region of interest ($\approx 80 \mu\text{m}^2$) for each cardiomyocyte, and a fluorescence background corresponding to an area without cells was removed. For comparative purposes, the fluorescence intensity minus background was normalized according to the initial fluorescence value.

Results are expressed as means \pm s.e.m of seven experiments. For each experiment, we calculated the response observed by averaging the fluorescence changes obtained from all of the cardiomyocytes contained in three fields (at least 50–75 cells).

2.6. Detection of reactive oxygen species in cardiomyocytes

To evaluate ROS formation, cardiomyocytes were loaded either with 5 μM of the non-fluorescent probe 2,7-dichlorodihydrofluorescein-diacetate (DCFH-DA; Invitrogen, Illkirch, France) for 30 min in the dark or with 1 μM of the fluorogenic dye MitoSOX. DCFH-DA is cleaved by cellular esterases and is oxidized by ROS (mainly H_2O_2 and $\cdot\text{OH}$) and yields the fluorescent product DCF. MitoSOX is selectively targeted to the mitochondria where it is oxidized by superoxide and exhibits red fluorescence. After incubation, DCF fluorescence was measured using 460 to 490 nm excitation and 510 nm emission filters. MitoSOX fluorescence was excited at 520 to 550 nm and recorded at 580 nm. Photographs were taken using an inverted fluorescence microscope (Olympus IX-81, Rungis, France). Measurement of DCF or MitoSOX fluorescence intensity was performed for each image using a digital epifluorescence imaging software (Cell P; Olympus, Rungis, France). Fluorescence was integrated over a region of interest ($\approx 80 \mu\text{m}^2$) for each cardiomyocyte, and a fluorescence background corresponding to an area without cells was removed.

The results are expressed as means \pm s.e.m of 5-7 experiments. For each experiment, we calculated the response observed by averaging the fluorescence changes obtained from all of the cardiomyocytes contained in three fields (at least 50–75 cells).

2.7. Experimental protocol

To determine the optimal toxic concentration of doxorubicin in cells, isolated cardiomyocytes were continuously exposed to increasing concentrations of doxorubicin *i.e.*, from 0.25 to 20 μM for 18 hours and the results were compared to control cells (non exposed to doxorubicin).

In order to analyse the effect of doxorubicin on mPTP opening, ROS production and consequent impact on contractile dysfunction and cell viability, cardiomyocytes were pre-treated with pharmacological agents targeting these processes: 1 μ M cyclosporin A (CsA) (Assaly et al., 2012) or an antioxidant mixture containing 5 μ M resveratrol and 5 mM N-acetylcystein (Sigma, Saint Quentin Fallavier, France) (Andersson et al., 2011; Horstkotte et al., 2011) before addition of 20 μ M doxorubicin. The protective effect of TSPO ligands on the deleterious effects of doxorubicin was determined by pretreating cardiomyocytes with either increasing concentrations of 4'-chlorodiazepam (Sigma, Saint Quentin Fallavier, France) or TRO40303 (synthesized by Synchem, Dijon, France) or fixed concentrations of the drugs (1 μ M for 4'-chlorodiazepam and 3 μ M for TRO40303) before exposure to 20 μ M doxorubicin. These last concentrations were chosen because they were the lowest efficient concentrations in the concentration-dependent experiments and also because they showed cardioprotective effects in previous studies (Obame et al., 2007; Schaller et al., 2010).

2.8. Data analysis

All data were expressed as means \pm s.e.m. Comparisons of the data between different groups were performed by one way ANOVA followed by Sheffe with significant differences taken at $p < 0.05$.

3. Results

3.1. Doxorubicin induces contractile dysfunction and cell death in isolated adult rat cardiomyocytes

To determine the concentration-dependent effects of doxorubicin on contractile dysfunction and cardiomyocyte death, cells were first exposed to increasing concentrations of doxorubicin (from 0.25 to 20 μM) for 18 hours.

As shown in Table 1, the average sarcomere length of cells used in this study was not significantly different among groups. In this model, treatments with 1 and 5 μM doxorubicin caused a paradoxical increase in cardiomyocyte contraction (Fig 1A). At concentrations higher than 5 μM , contractility as well as velocities of contraction and relaxation (Fig 1A, B and C) were significantly decreased. This was associated with a decrease in cytosolic calcium concentration (Fig 1D). This deleterious effect was confirmed by the study of the cellular contraction/relaxation cycle. Doxorubicin (20 μM) prolonged the cellular contraction/relaxation cycle duration by 36.5% (0.20 ± 0.01 vs. 0.30 ± 0.03 ms for control and doxorubicin, respectively; $p < 0.05$; Fig 1C) as shown by the time necessary to return to the basal value. As shown in figure 1E, doxorubicin reduced cell viability in a concentration-dependent manner ($p < 0.05$ from 5 to 20 μM).

In order to work with an optimal window of improvement, the concentration of 20 μM was used in the next experiments to study the effect of TSPO ligands and control compounds, as it induced the most significant deleterious effect.

3.2. Doxorubicin-induced contractile dysfunction and cell death are closely associated with ROS production and mPTP opening.

As ROS generation was suggested to be a major cause of doxorubicin-induced cell death (Davies et al., 1986; Mimnaugh et al., 1985; Simunek et al., 2009; Wallace, 2003), we monitored the production of ROS in cardiomyocytes exposed to increasing concentrations of doxorubicin for 18 hours. The study confirms that doxorubicin enhances ROS production in a concentration-dependent manner (Fig 2A) as observed with the fluorescent probe DCF that is commonly utilized to detect generalized ROS production. The increase in ROS production was also found with MitoSOX that specifically revealed the production of superoxide in mitochondria (Fig 2B). Preincubation of cardiomyocytes with a mixture of antioxidant agents (resveratrol (5 μ M) and N-acetylcysteine (5 mM)) prevented the increase in ROS production induced by 20 μ M doxorubicin (Fig 2B). Antioxidant treatment also prevented the doxorubicin-induced decrease in intracellular Ca^{2+} as measured by the fura-2 ratio (Fig 2C), mPTP opening (Fig 2D) and preserved contractile function. Indeed, antioxidants restored cell shortening (Fig 2E and 2G) and improved velocities of contraction and relaxation (Fig 2F) but had no significant effect on the duration of the cellular contraction/relaxation cycle (0.30 ± 0.03 vs. 0.25 ± 0.02 ms for doxorubicin and doxorubicin + antioxidants, respectively, Fig 2G). Antioxidant treatment also improved cardiomyocyte viability as it counteracted the increase in propidium iodide fluorescence intensity induced by doxorubicin (Fig 2H). Antioxidant pretreatment alone had no significant effect on any of these parameters.

In parallel to ROS generation, doxorubicin treatment dose-dependently induced mPTP opening monitored by a loss of mitochondrial calcein fluorescence (Fig 3A). This was confirmed in the presence of the specific mPTP inhibitor, cyclosporin A, which was able to blunt this effect induced by 20 μ M doxorubicin (Fig 3B), although cyclosporin A had no effect on ROS production (Fig 3C) or decreases in intracellular calcium levels (Fig 3D) induced by doxorubicin. Cyclosporin A treatment maintained cell shortening to its control level (Fig 3E and 3G) and prevented the doxorubicin-induced prolongation of the time

necessary for the sarcomere length to return to the basal value (0.30 ± 0.03 vs. 0.21 ± 0.02 ms for doxorubicin and doxorubicin + cyclosporin A, respectively; $p < 0.05$; Fig 3G) but had no effect on contraction and relaxation velocities (Fig 3F). Cyclosporin A also improved survival as it counteracted the increase in propidium iodide fluorescence intensity induced by doxorubicin (Fig 3H). Moreover, it should be noted that pretreatment with cyclosporin A had no proper effect on any of these parameters.

3.3. 4'-Chlorodiazepam and TRO40303 prevention of doxorubicin-induced contractile dysfunction and cell death involves inhibition of ROS production and mPTP opening.

In order to see whether TSPO ligands might prevent doxorubicin-induced contractile dysfunction and cell death, cardiomyocytes were pre-incubated for 30 min with increasing concentrations of 4'-chlorodiazepam or TRO40303 (Schaller et al., 2010) before doxorubicin treatment (20 μ M for 18 hours). Both 4'-chlorodiazepam and TRO40303 prevented doxorubicin-induced alterations in cell contraction (Fig 4A, 4B, 4C, 4D), mPTP opening (Fig. 5A) and cell death (5B) in a concentration-dependent manner.

It should be noted that these drugs did not show any proper effect on cell contraction, as, they had no significant effect on cardiomyocyte contractile performance compared to controls (Fig 4A) and did not modify the mean cell sarcomere length (Table 1). At the same concentrations, 4'-chlorodiazepam and TRO40303 had no significant effect on the duration of the cellular contraction/relaxation cycle altered by doxorubicin (0.30 ± 0.03 vs. 0.24 ± 0.01 ms for doxorubicin and doxorubicin + 4'-chlorodiazepam, respectively and 0.30 ± 0.03 vs. 0.25 ± 0.02 ms for doxorubicin and doxorubicin + TRO40303, respectively; Fig 4C and 4D) but prevented the decrease in fura-2 ratio induced by doxorubicin, maintaining calcium levels similar to those observed in control cells (Fig 5C). In the same way, 4'-chlorodiazepam and TRO40303 attenuated ROS production (Fig. 5D).

In order to further investigate the action mechanism of TSPO ligands and more particularly to see whether the protection from TSPO ligands overlaps with cyclosporin A, additional experiments were performed with cardiomyocytes isolated from CypD KO mice. As well as observed with cardiomyocytes issued from rat hearts, doxorubicin induced mPTP opening in a concentration-dependent manner in cardiomyocytes isolated from wild type and KO mice but the effect was more pronounced in WT mice (Fig. 6A). This was associated with an enhanced production of ROS at the mitochondrial level as demonstrated by the increase in MitoSOX fluorescence (Fig. 6B) and a decrease in myocytes contractility (Fig. 6 C). TRO40303 inhibited mPTP opening in KO mice, reduced ROS production and improved the contractile performance of the myocytes whereas cyclosporin A did not (Fig. 6A, B and C).

4. Discussion

The major findings of the present study are as follows: 1) TSPO ligands prevent doxorubicin-induced contractile dysfunction and inhibit cell death, 2) these effects are associated with a decrease in ROS and mPTP opening and 3) both were coupled with normalization of cytosolic calcium dysfunction induced by doxorubicin.

In the present study we used an acute model of doxorubicin-induced cardiotoxicity. Clinically, doxorubicin-induced cardiotoxicity can be characterized by acute myocardial injury occurring immediately after an initial treatment. Alternatively, chronic cardiotoxicity is characterized by congestive heart failure, ventricular dysfunction and arrhythmia and may occur years to decades after anthracycline treatment ([Lipshultz et al., 1991](#); [Schwartz et al., 1987](#); [Steinherz et al., 1991](#)). It should be reminded that the long term effects of the drug are the consequence of its acute toxicity that further progresses with repeated exposure. Thus preventing the acute cardiotoxicity of the drug is an important approach to develop

cardioprotective therapies against doxorubicin toxicity. The paradoxical positive inotropic effect that we observed in freshly isolated cardiomyocytes cultured with low concentrations of doxorubicin (1 and 5 μM) might reflect the increases in both systolic and diastolic functions and electrocardiographic changes, observed in patients 1 to 4 hours after doxorubicin treatment (Brown et al., 1989). Timolati *et al.* (Timolati et al., 2006) have shown that treatment with 1 μM doxorubicin for 18 hours caused partial degradation of sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) without modification of the sarcolemmal reticulum calcium content. It has been suggested that such a positive inotropic effect of doxorubicin is linked to an acute ROS production leading to opening of L-type calcium channels and subsequent increased in cardiac contractility (Campbell et al., 1996; Thollon et al., 1995; Wang & Korth, 1995). In contrast, at concentrations higher than 5 μM , doxorubicin exhibits a well described negative inotropic effect associated with a decrease in calcium amplitude. This is in line with the present results and the data of Timolati *et al.* (Timolati et al., 2006) who showed that the anthracycline-induced negative inotropy mainly depends on the down-regulation of the SERCA pump associated with a decrease in 90% time of relaxation and a decrease in sarcolemmal reticulum calcium loading.

One of the proposed mechanisms for doxorubicin-induced cardiac dysfunction is oxidation of cellular components via ROS formation (Minotti et al., 2004). Indeed, the quinone moiety of doxorubicin is prone to the generation of oxygen radicals through enzymatic mechanism involving mitochondrial respiratory chain (Gutierrez PL, 2000). A number of studies has also suggested that doxorubicin mediated alteration in calcium homeostasis may be one of the possible mechanisms of its cardiotoxicity (Shadle et al. 2000). The present study confirms that ROS production is a crucial event occurring during doxorubicin treatment and is associated with induction of mPTP opening which plays a key role in the life and death of cells (Javadov et al., 2009; Leung & Halestrap, 2008; Zorov et al., 2009). The mPTP opens

under conditions of elevated mitochondrial calcium, especially when associated with oxidative stress, *i.e.*, exactly the conditions occurring during chronic doxorubicin cardiotoxicity (Cardoso et al., 2008; Tokarska-Schlattner et al., 2006; Wallace, 2007). Pharmacological strategies developed to inhibit mPTP opening have demonstrated their protective efficacy (Morin et al., 2009; Ovize et al., 2010) more particularly during ischemia/reperfusion. Using 4'-chlorodiazepam and TRO40303 as TSPO ligands it has been recently reported that TSPO is an interesting target to reduce myocardial infarct size in animal models of ischemia/reperfusion (Obame et al., 2007; Schaller et al., 2010). In the present work, we report for the first time to our knowledge that TSPO is also a relevant target to prevent doxorubicin toxicity and that the mechanism of cardioprotection from this drug afforded by TSPO ligands is linked to limitation of mitochondrial membrane permeabilization and ROS production. TSPO modulation could counteract the effects of H₂O₂- or ischemia/reperfusion-induced impairment of cardiac mitochondrial oxidative phosphorylation as well as apoptosis (Obame et al., 2007; Schaller et al., 2010; Leducq et al., 2003), indicating that TSPO modulation prevents ROS generation. Here, we demonstrate that 4'-chlorodiazepam and TRO40303 decrease the production of ROS during doxorubicin treatment, which may be one of the mechanisms by which these ligands decrease mPTP opening since similar protection was provided by antioxidant agents.

In addition, the data obtained with cardiomyocytes from CypD KO mice show that the protection of TSPO ligands does not overlap with cyclosporin A as these ligands inhibit ROS production and mPTP opening whereas cyclosporin A does not. This demonstrates that these ligands do not interact with CypD and reinforces the hypothesis of an inhibition of ROS production.

Interestingly, these effects were concomitant of a mechanical improvement in adult cardiomyocytes as pretreatment with TSPO ligands blunted the contractile dysfunction

induced by doxorubicin. The pattern of cardioprotection induced by these ligands was similar although their binding domains on TSPO are different. TRO40303 interacts specifically at the cholesterol binding site of the protein, while the TSPO benzodiazepine binding site is targeted by 4'-chlorodiazepam (Lacapere et al., 2001; Kalivendi et al., 2001). The fact that the decrease in calcium amplitude induced by doxorubicin was counteracted by TSPO ligands suggests that these agents could prevent perturbations in calcium homeostasis, which are known to interfere with muscle contraction and relaxation. This effect of TSPO ligands is most likely due to a decrease in ROS production rather than a direct inhibition of mPTP opening as antioxidant agents exhibit a similar effect whereas it was not obtained in the presence of cyclosporin A, as observed here with CypD KO mice. These findings are also corroborated by several studies demonstrating a close relationship between calcium homeostasis and ROS production since doxorubicin-induced ROS production is reduced by the calcium chelator BAPTA (Kalivendi et al., 2001; Kalivendi et al., 2005). In addition, antioxidant properties of calcium channel blockers have been observed in *in vitro* and *in vivo* studies (Umemoto et al., 2004). Recent data also suggested that 4'-chlorodiazepam is able to blunt the decrease in cell contractility and to prevent reperfusion arrhythmias in a model of ischemia-reperfusion through a mechanism involving inhibition of the mitochondrial inner membrane anion channel (IMAC) and recovery of the mitochondrial inner membrane potential (Akar et al., 2005). However, high concentrations of 4'-chlorodiazepam were required to observe this effect (32-100 μ M).

In conclusion, this study provides novel evidence that two TSPO ligands, 4'-chlorodiazepam and TRO40303, can significantly reduce the incidence of contractile dysfunction and cell death during doxorubicin treatment by inhibiting ROS production and mPTP opening. Modulation of TSPO may represent a new pharmacological strategy for

developing cytoprotective agents against cell death and contractile dysfunction induced by doxorubicin.

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Conflicts of interest: Alain Berdeaux has served as consultant and received honorarium from TROPHOS. Rebecca M. Pruss holds shares in TROPHOS.

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Legends for Figures

Fig. 1: Effect of doxorubicin on contractile function and cell death.

A and B: cardiomyocytes were cultured for 18 hours in the presence of increasing concentrations of doxorubicin (Dox), and then cell shortening (A) and maximal velocities of contraction and relaxation (B) were measured in 1Hz paced cells.

C: typical examples of cardiomyocyte contractions without or with 20 μ M Dox treatment.

D: cardiomyocytes were loaded with 5 μ M fura-2-AM, were excited at 360 nm and 380 nm, and the resulting fluorescence was measured at 510 nm.

E: cells were loaded with propidium iodide and cultured for 18 hours in the presence of increasing concentrations of Dox. Cell death was determined by evaluating propidium iodide fluorescence.

Each value is the mean \pm SEM of five independent experiments including 30-45 cells. *: $p < 0.05$ versus control (Ctl) cells.

Fig. 2: Doxorubicin-induced ROS production, mPTP opening, contractile dysfunction and cell death were prevented by antioxidant agents.

A: cardiomyocytes were cultured for 18 hours in the presence of doxorubicin (Dox), and then loaded with 5 μ M DCF-DA or 1 μ M MitoSOX. At the end of the incubation period, ROS production was evaluated by measuring DCF or MitoSOX fluorescence.

B-H: cardiomyocytes were pretreated with an antioxidant cocktail (resveratrol, 5 μ M + N-acetylcystein, 5 mM, AO) and then cultured for 18 hours in the presence of 20 μ M Dox.

B: cardiomyocytes were loaded with 5 μ M DCF-DA or 1 μ M MitoSOX and ROS production was evaluated by measuring DCF or MitoSOX fluorescence. C: cardiomyocytes were loaded with 5 μ M fura-2-AM, were excited at 360 nm and 380 nm, and the resulting fluorescence was measured at 510 nm. D: cardiomyocytes were loaded with 1 μ M calcein-AM

supplemented with 1 mM CoCl₂ and mPTP opening was evaluated by measuring calcein fluorescence. E and F: cell shortening (E) and maximal velocities of contraction and relaxation (F) were measured in 1 Hz paced cells. G: Typical examples of cardiomyocyte contractions without or with 20 μM Dox or Dox + AO treatment. H: Cells were loaded with propidium iodide and cell death was determined by evaluating propidium iodide fluorescence. Each value is the mean ± SEM of five independent experiments, including 30-45 cells. *: p<0.05 versus control (Ctl) cells; ‡: p<0.05 versus Dox.

Fig. 3: Cyclosporin A was able to prevent doxorubicin-induced mPTP opening without altering ROS production.

A: cardiomyocytes were cultured for 18 hours in the presence of increasing concentrations of doxorubicin (Dox), and then loaded with 1 μM calcein-AM supplemented with 1 mM CoCl₂. At the end of the incubation period, mPTP opening was evaluated by measuring calcein fluorescence.

B-H: cardiomyocytes were pretreated with cyclosporin A (CsA, 1 μM) and then cultured for 18 hours in the presence of 20 μM Dox. B: cells were loaded with 1 μM calcein-AM supplemented with 1 mM CoCl₂ and mPTP opening was evaluated by measuring calcein fluorescence. C: cells were loaded with 5 μM DCF-DA or 1 μM MitoSOX and ROS production was evaluated by measuring DCF and MitoSOX fluorescence. D: cells were loaded with 5 μM fura-2-AM, were excited at 360 nm and 380 nm and the resulting fluorescence was measured at 510 nm. E and F: cell shortening (E) and maximal velocities of contraction and relaxation (F) were measured in 1 Hz paced cells. G: Typical examples of cardiomyocyte contractions without or with 20 μM Dox or 20 μM Dox + 1 μM CsA.

H: cells were loaded with propidium iodide and cell death was determined by evaluating propidium iodide fluorescence.

Each value is the mean ± SEM of five independent experiments including 30-45 cells. *: p<0.05 versus control (Ctl) cells; ‡: p<0.05 versus Dox.

Fig. 4: Doxorubicin-induced contractile dysfunction was prevented by TSPO ligands.

A and B: cardiomyocytes were pre-treated with increasing concentrations of 4'-chlorodiazepam (CDZ) or TRO40303 (TRO) and cultured for 18 hours in the presence of

doxorubicin (Dox, 20 μ M). Then cell shortening (A) and maximal velocities of contraction and relaxation (B) were measured in 1 Hz paced cells.

C and D: typical examples of cardiomyocyte contractions without or with Dox (20 μ M), Dox + CDZ (1 μ M) or Dox + TRO (3 μ M) treatments.

Fig. 5: Doxorubicin-induced cell death, mPTP opening and ROS production were prevented by TSPO ligands.

A and B: cardiomyocytes pre-treated with increasing concentrations of 4'-chlorodiazepam (CDZ) or TRO40303 (TRO) and cultured for 18 hours in the presence of doxorubicin (Dox, 20 μ M). At the end of the incubation period, cells were loaded with 1 μ M calcein-AM supplemented with 1 mM CoCl_2 (A) or 5 μ M propidium iodide (B), and mPTP opening (A) and cell death (B) were evaluated by measuring calcein and propidium iodide fluorescence, respectively

C: cardiomyocytes were pre-treated with either 1 μ M CDZ or 3 μ M TRO and cultured for 18 hours in the presence of 20 μ M Dox. Then, cells were loaded with 5 μ M fura-2-AM, were excited at 360 nm and 380 nm, and the resulting fluorescence was measured at 510 nm.

D: cardiomyocytes were pre-treated with either 1 μ M CDZ or 3 μ M TRO and cultured for 18 hours in the presence of 20 μ M Dox. Then, cardiomyocytes were loaded with 5 μ M DCF-DA or 1 μ M MitoSOX and ROS production was determined by evaluating DCF and MitoSOX fluorescence.

Each value is the mean \pm SEM of five independent experiments including 30-45 cells. *: $p < 0.05$ versus control (Ctl) cells; ‡: $p < 0.05$ versus Dox.

Fig. 6: Comparison of the effects of cyclosporin A and of the TSPO ligand, TRO40303 on doxorubicin-induced mPTP opening, ROS production and contractile dysfunction in cardiomyocytes isolated from wild type and CypD KO mice.

A: cardiomyocytes pre-treated with 3 μ M TRO40303 (TRO) or 1 μ M cyclosporin A (CsA) and cultured for 18 hours in the presence of doxorubicin (Dox, 20 μ M). At the end of the incubation period, cells were loaded with 1 μ M calcein-AM supplemented with 1 mM CoCl_2 and mPTP opening was evaluated by measuring calcein fluorescence.

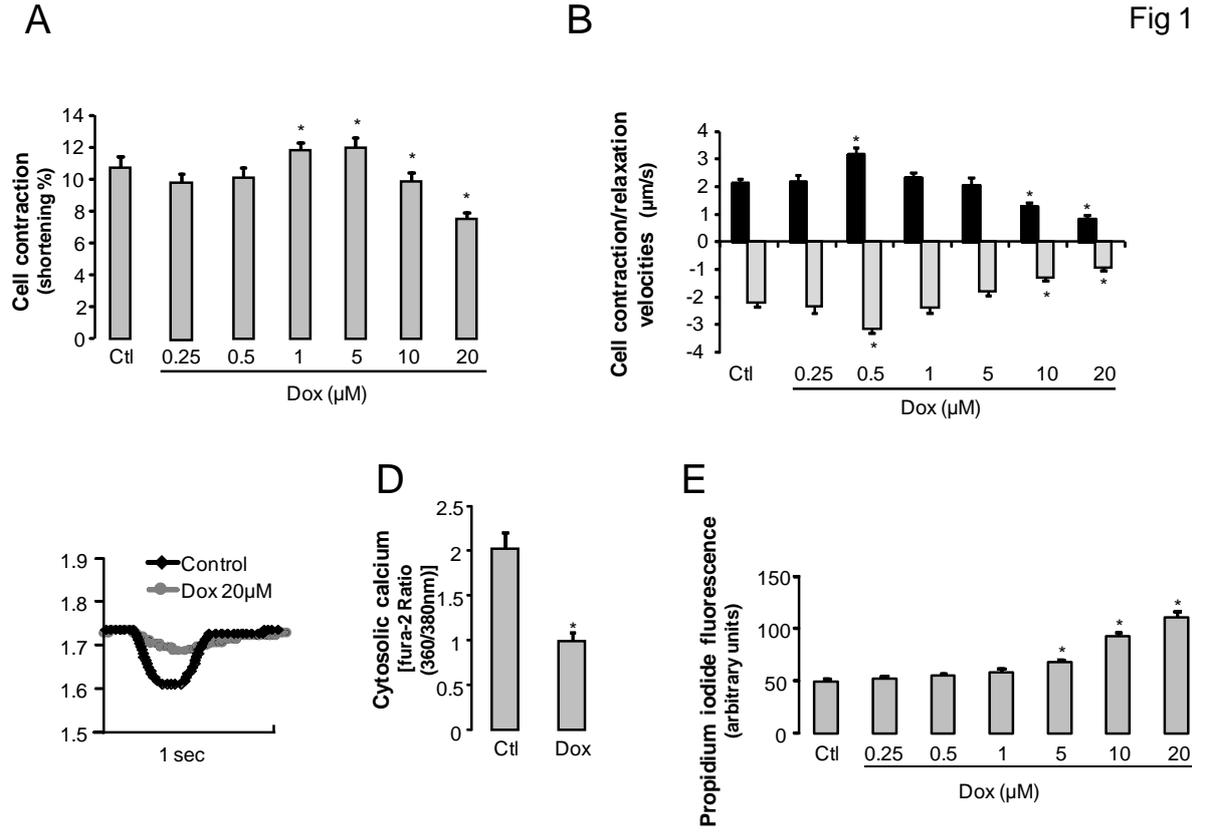
B: cardiomyocytes were pre-treated with either 3 μ M TRO or 1 μ M CsA and cultured for 18 hours in the presence of 20 μ M Dox. Then, cardiomyocytes were loaded with 1 μ M MitoSOX and ROS production was determined by evaluating MitoSOX fluorescence.

C: cardiomyocytes were pre-treated with either 3 μ M TRO or 1 μ M CsA and cultured for 18 hours in the presence of 20 μ M Dox and then cell shortening was measured in 1Hz paced cells.

Each value is the mean \pm SEM of five independent experiments including 30-45 cells.

*: $p < 0.05$ versus respective control (Ctl); ‡: $p < 0.05$ versus respective cells treated with Dox.

Fig 1



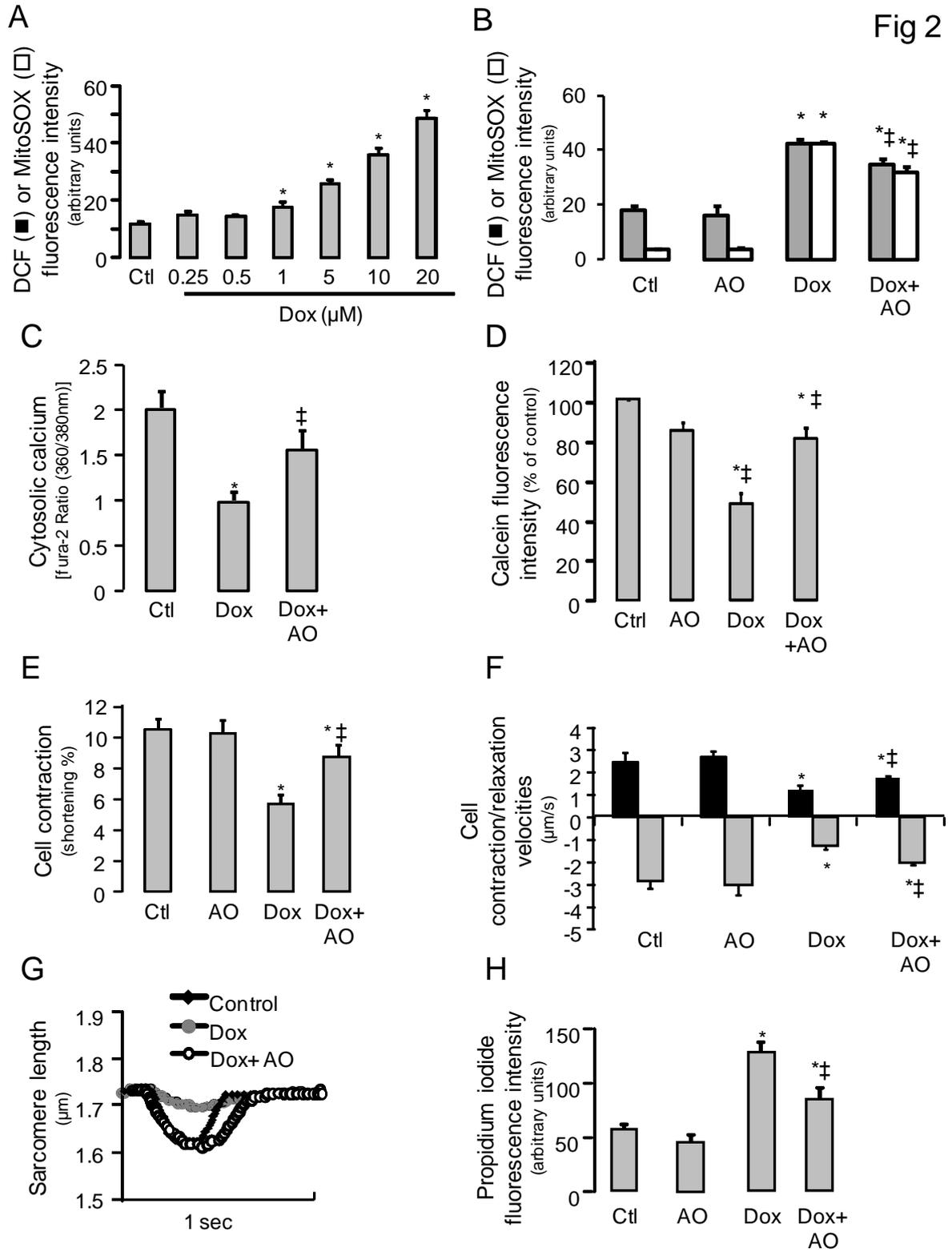


Fig 3

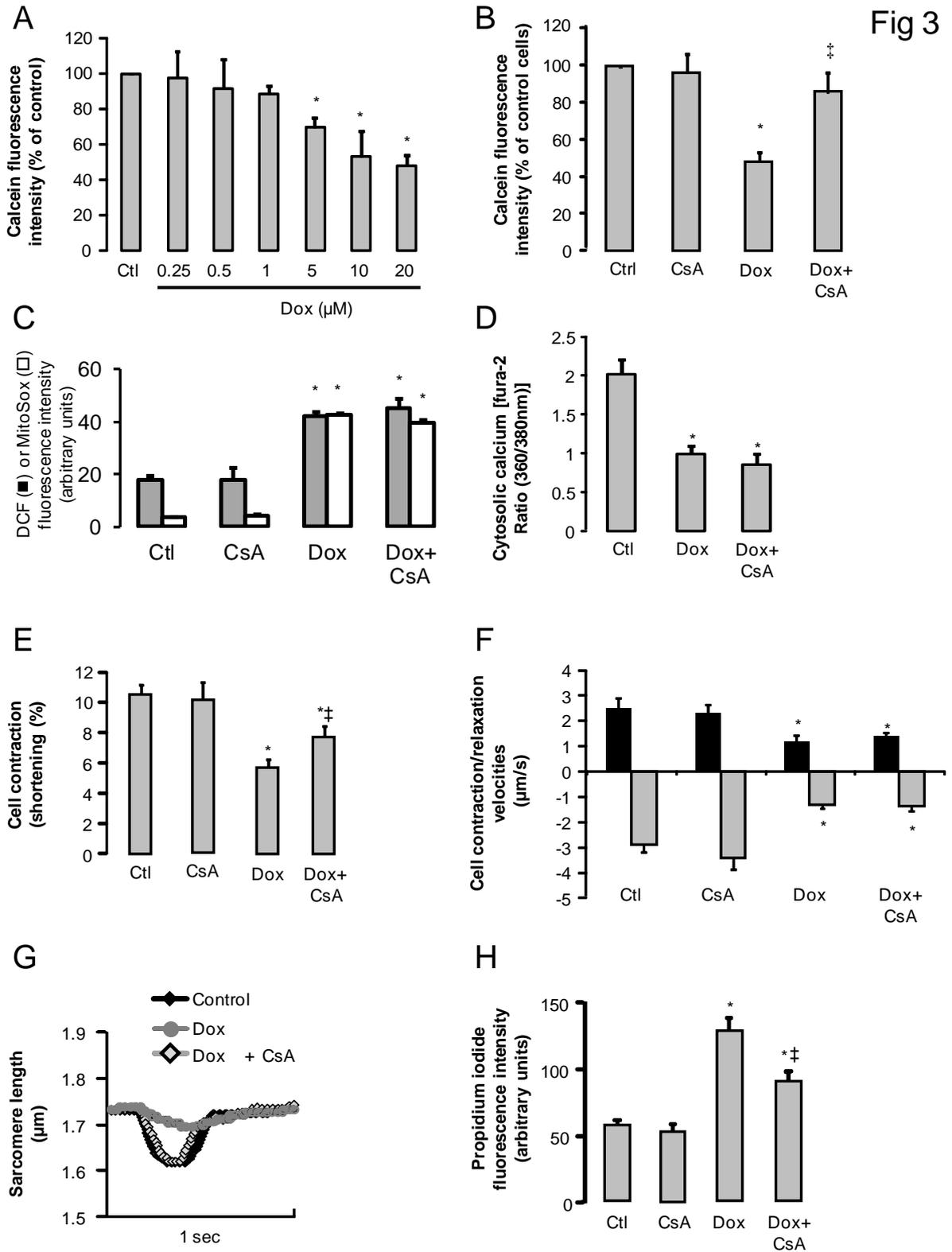


Fig 4

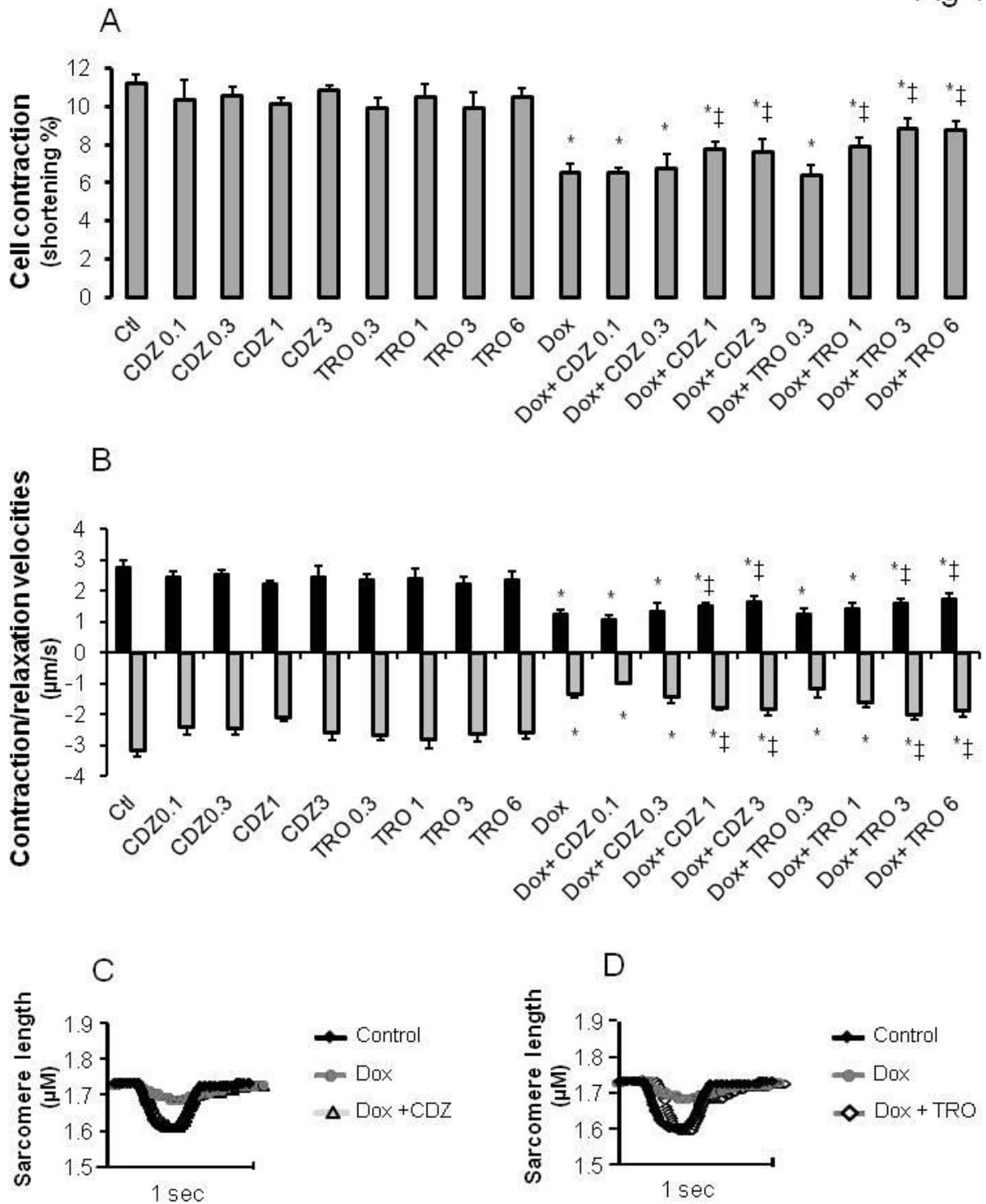


Fig 5

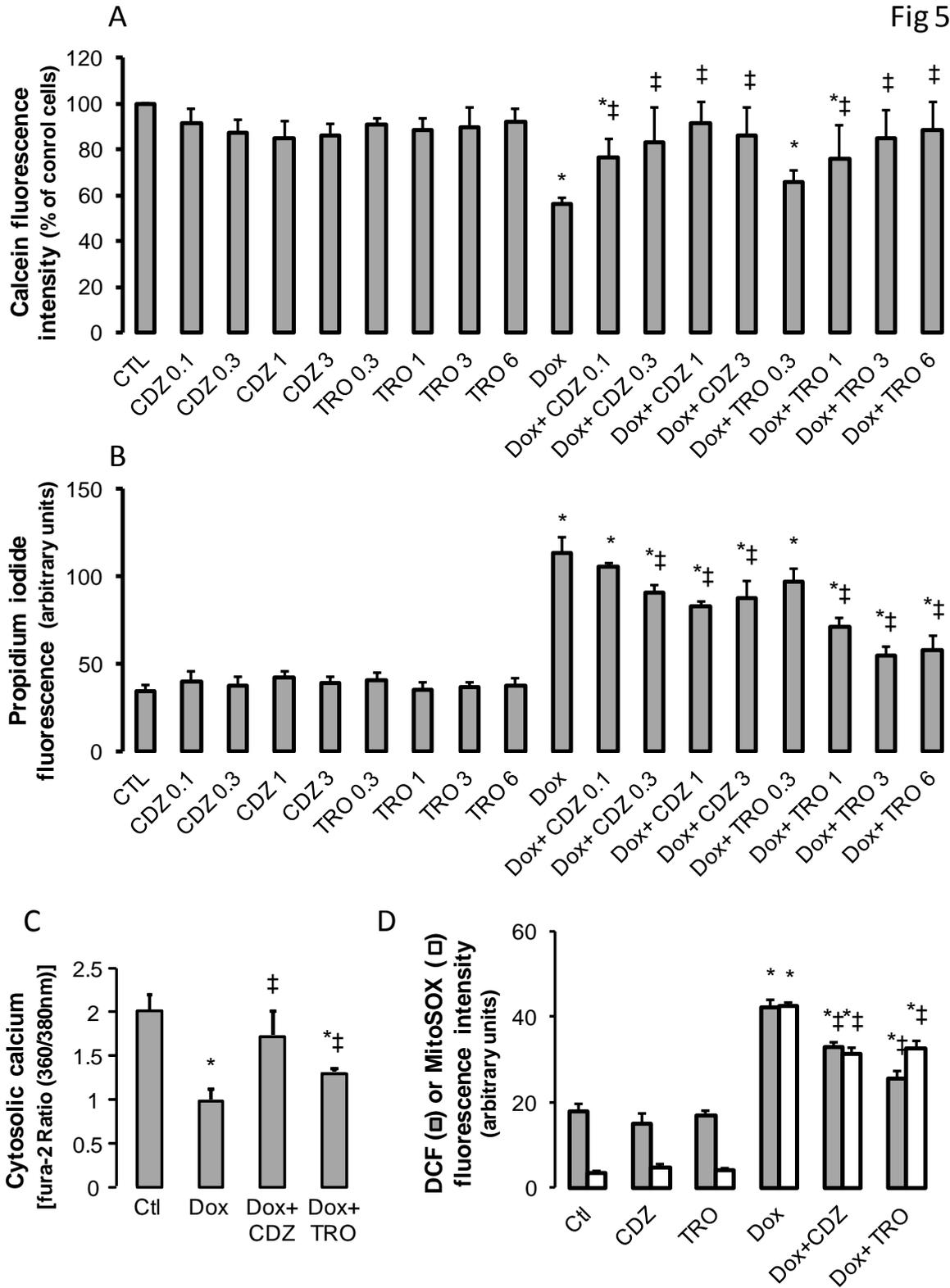


Fig 6

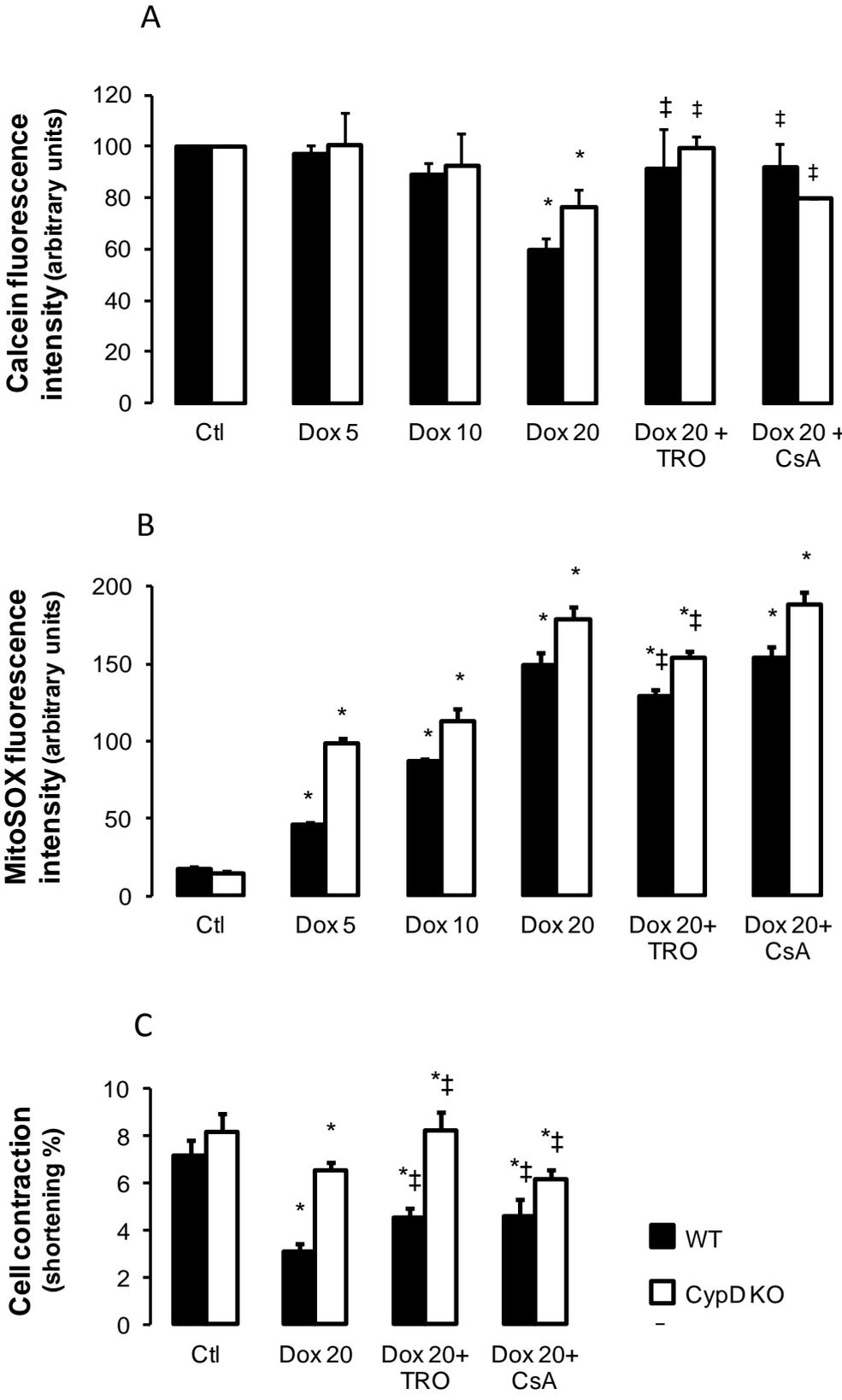


Table 1: sarcomere length

Group	n	sarcomere length (μm)
Control	34	1.68 \pm 0.01
CDZ	11	1.70 \pm 0.01
TRO40303	16	1.73 \pm 0.04
Cyclosporin A	14	1.70 \pm 0.02
Antioxidants	10	1.73 \pm 0.03
Doxorubicin	30	1.70 \pm 0.02
Doxorubicin + CDZ	11	1.70 \pm 0.02
Doxorubicin + TRO40303	20	1.71 \pm 0.02
Doxorubicin + Cyclosporin A	11	1.71 \pm 0.02
Doxorubicin + Antioxidants	15	1.70 \pm 0.03

Effect on sarcomere length of 4'-chlorodiazepam (CDZ, 1 μM), TRO40303 (3 μM), antioxidants (resveratrol 5 μM and N-acetylcystein 5 mM), Doxorubicin (20 μM) or cyclosporin A (1 μM)