

# Doxorubicin treatment in vivo activates caspase-12 mediated cardiac apoptosis in both male and female rats

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**Abstract** We investigated in vivo the chemotherapeutic anthracycline agents doxorubicin and its ability to activate mitochondrial-mediated, receptor-mediated and endoplasmic/sarcoplasmic reticulum-mediated apoptosis transduction pathways in cardiac tissue from male and female rats. We administered a single low dose of doxorubicin (10 mg/kg of body weight, i.p.) and then isolated mitochondrial and cytosolic proteins one and four days later from the heart. Caspase-3 protein content and caspase-3 activity were significantly increased after day four of doxorubicin treatment in both male and female rats. However, while males had DNA fragmentation at day one but not day four following doxorubicin administration, females showed no significant increase in DNA fragmentation at either time. Caspase-12, localized in the SR, is considered a central caspase, and its activation by cleavage via calpain indicates activation of the SR-mediated pathway of apoptosis. Cleaved caspase-12 content and calpain activity significantly increased after day four of doxorubicin treatment in both sexes. In the mitochondrial-mediated pathway, there were no significant treatment effects observed in cytosolic cytochrome *c* and cleaved (active) caspase-9 in either sex. In control rats (saline injection), glutathione peroxidase (GPX) activity and hydrogen peroxide ( $H_2O_2$ ) production were lower in females compared to males. Doxorubicin treatment did not significantly affect  $H_2O_2$ , GPX activity or ATP production in isolated mitochondria in either sex. Female rats produced significantly lower levels of  $H_2O_2$  production one day after doxorubicin treatment, whereas male rats produced significantly less mitochondrial  $H_2O_2$  four days after doxorubicin treatment. The receptor-mediated pathway (caspase-8 and c-FLIP) showed no evidence of being significantly activated by doxorubicin treatment. Hence, doxorubicin-induced apoptosis in vivo is mediated by the SR to a greater extent than other apoptotic pathways and should therefore be considered for targeted therapeutic interventions. Moreover, no major sex differences exist in apoptosis signaling transduction cascade due to doxorubicin treatment.

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**Keywords:** Calcium; Caspase; Sex difference; Hydrogen peroxide; Oxidative stress; Gender

## 1. Introduction

Doxorubicin causes dose-dependent acute and chronic cardiotoxicities [1] and irreversible myocardial damage that re-

sults in dilated cardiomyopathy with fatal congestive heart failure [1,2]. The mechanism of cardiomyopathy remains unclear, but reactive oxygen species (oxidants) are likely involved [3–6]. Furthermore, it is unknown whether sex differences exist in doxorubicin-induced cardiotoxicity and in doxorubicin-induced apoptosis and apoptosis signaling transduction cascades. Compelling evidence from several epidemiological and clinical studies indicates a substantially higher incidence of heart failure and cardiovascular disease in men compared with age-matched women [7–9], suggesting that sex differences in cardioprotection exist. The biochemical and molecular bases for these differences have been attributed in part to increased estrogen levels and increased telomerase activity [10,11], which could increase tissue regeneration capacity [12].

Mitochondrial derived oxidants probably play a key role in triggering doxorubicin toxicity in vitro [13,14]. Isolated heart mitochondria shuttle single electrons to doxorubicin, giving rise to oxygen radicals through the autooxidation of adriamycin semiquinones [15]. However, mitochondria have excellent adaptive defense systems which have evolved over millions of years to cope with sudden changes in oxidative challenges [16,17]. Recently, we found that the mitochondria of doxorubicin-treated (20 mg/kg) male rats show adaptive mechanisms, including increased antioxidant defenses and increased mitochondrial efficiencies, with minimal effects on ATP production [18]. This raises the possibility that hydrogen peroxide ( $H_2O_2$ ) released from the mitochondria may inactivate key proteins and enzymes present in nearby targets, which could include cardiac sarcoplasmic reticulum (SR). Protective antioxidant defense mechanisms of the SR may be incapable of coping with the sudden increases in  $H_2O_2$  produced by the mitochondria and effect apoptosis signaling [19].

The central signaling component of apoptosis is a proteolytic system involving a family of cysteine proteases called caspases (cysteine-dependent, aspartate-specific proteases) localized in various parts of the cell [20]. Interest in the control of apoptosis has grown significantly, since disturbed apoptosis may contribute to cardiomyopathies resulting from chronic use of drugs such as doxorubicin [21,22]. Depending on the dose of doxorubicin and model system used (i.e., cell culture or animal model), several pathways of apoptosis have been implicated in the induction of cell death [2,4,23,24], including the mitochondrial pathway, the extrinsic receptor-mediated pathway, and the endoplasmic/sarcoplasmic reticulum (ER/SR) pathways. In the mitochondrial pathway, mitochondrial dysfunction can lead to the release of mitochondrial cytochrome *c* [20,25,26] into the cytoplasm. Cytochrome *c*, along

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with Apaf-1, caspase-9, and dATP, may then form a complex called the apoptosome that activates caspase-3, a key cell death protease [27]. Activation of the extrinsic receptor-mediated pathway leads to activation of procaspase-8, which then cleaves and activates procaspase-3 to initiate the caspase cascade [28]. Doxorubicin treatment in vitro causes apoptosis via Fas-mediated activation of caspase-8 [29], but it is not known whether this occurs in vivo.

Less attention has been paid to the ER/SR-mediated pathway of apoptosis. The SR, which is a specialized form of the ER, provides maintenance of optimal calcium ( $\text{Ca}^{2+}$ ) levels for proper contractility in cardiac myocytes. Recently, it has been shown that increased intracellular  $\text{Ca}^{2+}$  concentrations significantly increase susceptibility to apoptosis via activation of caspase-12, localized on the cytoplasmic side of the SR [30,31]. Hence, acute and chronic oxidative stress by doxorubicin may induce apoptosis by SR-mediated caspase-12, in vivo, a possibility, which has not been investigated.

## 2. Methods

### 2.1. Animals and doxorubicin treatment

Six-month-old male and female Fisher 344 rats (National Institute of Aging colony, Harlan Sprague Dawley, Indianapolis, IN) were used in this study. The animals were housed individually in a temperature (18–22 °C) and light-controlled environment with a 12-h light/dark cycle and were provided with food and water ad libitum. Animals from both male ( $N = 24$ ) and female groups ( $N = 24$ ) were randomly assigned either to a control or to a doxorubicin-treated group. Doxorubicin hydrochloride (Sigma Chemical Co., St. Louis, MO) was dissolved in saline and administered by intraperitoneal injection at a dose of 10 mg/kg, while control animals were injected with a comparable volume of saline. At one day after injection (saline: 6 males and 6 females; doxorubicin: 6 males and 6 females) and four days after injection (saline: 6 males and 6 females; doxorubicin: 6 males and 6 females), animals were sacrificed by deep anesthesia with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight) followed by thoracotomy and removal of blood by cardiac puncture. The entire heart was then excised, rinsed in saline to remove any remaining blood, blotted dry, and weighed. All experimental procedures received approval from the University of Florida's Institute on Animal Care and Use Committee.

### 2.2. Mitochondrial function

Mitochondria were immediately isolated from the left ventricle (500 mg) of the heart as previously described [32]. Briefly, the tissue was minced on ice in 5 volumes of isolation buffer (0.225 M mannitol, 0.075 M sucrose, and 0.2% fatty acid-free bovine serum albumin, pH 7.4), homogenized on ice in a Potter–Elvehjem glass homogenizer and centrifuged for 10 min at  $700 \times g$  at 4 °C. The resulting supernatant was centrifuged again for 10 min at  $8000 \times g$  at 4 °C. The supernatant (cytosolic fraction) was aliquotted and stored at –80 °C. The mitochondrial pellet was resuspended in 5 ml of isolation buffer and centrifuged for 10 min at  $8000 \times g$ . The final mitochondrial pellet was resuspended in 1 ml of isolation buffer. Mitochondrial  $\text{O}_2$  consumption was determined as previously described [33]. ATP production was measured with firefly luciferase, which fluoresces in proportion to the presence of ATP, as described previously [34,35]. Mitochondrial  $\text{H}_2\text{O}_2$  production rate was measured enzymatically using homovanillic acid [36]. Protein concentrations were determined by Bradford assay [37].

### 2.3. Western blots analysis of caspase-3, caspase-8, caspase-9, caspase-12, XIAP, FLIP, Bcl-2, and bax

Cytosolic proteins were separated electrophoretically on a 4–20% precast polyacrylamide gel (Cambrex, Rockland, ME) and transferred onto a nitrocellulose membrane (Cambrex). The membranes were blocked overnight using a blocking solution containing 0.05% Tween and 5.0% dried milk, incubated for 90 min with primary antibody in blocking solution, rinsed in PBS buffer, incubated for 90 min in anti-

rabbit or mouse Ig horseradish peroxidase (Amersham Life Science, England) [1:1000] and rinsed again. The membranes were then developed using ECL Western blotting detection reagent (Amersham Pharmacia Biotech, England) and imaged using a Kodak Image Station 440CF (Eastman Kodak, Rochester, NY). To detect caspases, polyclonal antibodies (1:1000 dilution) were used against caspase-3 (full length), caspase-8 (full length), caspase-9 (cleaved) (all from Stressgen, Canada), caspase-12 (cleaved), and caspase-12 antibody (cleaved) (both from Oncogene Research Products, San Diego, CA). Inhibitors of apoptosis (XIAP, X-linked inhibitor of apoptosis; FLIP, FLICE-like inhibitory protein) were detected in the cytosolic fraction of the cardiomyocytes using a monoclonal antibody against XIAP (MBL, Watertown, MA, 1:500) and antiserum against FLIP (Alexis, San Diego, CA, 1:1000). Bcl-2 family proteins were detected using polyclonal antibodies against Bax (Ab-1; 1:200) and Bcl-2 (Ab-4; 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA).

### 2.4. Measurement of DNA fragmentation

DNA fragmentation was quantified by measuring the content of cytosolic mono- and oligo-nucleosomes (180 base pair nucleotides or multiples) using a Cell Death ELISA kit (Roche Molecular Biochemicals, Germany) that employs the sandwich enzyme immunoassay technique.

### 2.5. Caspase-3, calpain, glutathione peroxidase and cytochrome c assays

Caspase-3 activity was measured using the synthetic peptide *n*-acetyl-DEVD-AMC (BD PharMingen, San Diego, CA). Calpain activity was measured using a fluorometric assay kit that utilizes the unique ability of calpain to digest synthetic substrate Suc-LLVY AMC in the presence of the  $\text{Ca}^{2+}$  ion and the reducing agent TCEP (Oncogene). Selenium-dependent glutathione peroxidase (GPX) activity was assayed according to Nakamura et al. [38] using  $\text{H}_2\text{O}_2$  as the substrate. Cytosolic cytochrome *c* was quantified using an ELISA kit that employs the sandwich enzyme immunoassay technique (R&D Systems Minneapolis, MN). All assays were measured in triplicate.

### 2.6. Estrogen (estradiol) levels

Plasma estradiol concentration was measured using a radioimmunoassay (Yerkes Endocrine Laboratory, Atlanta, GA).

### 2.7. Statistical analysis

Two-way ANOVA was used for comparisons between groups. Bonferroni post-hoc testing was performed if significance was found. A *P*-value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Body weight and heart weight

The mean body weight of male rats was significantly greater than that of female rats ( $P < 0.05$ ; Table 1). Male heart weights were 36% greater than that of females ( $P < 0.001$ ), but female rats had a significantly higher heart weight-to-body weight ratio ( $P < 0.001$ ). Body weight was not different one day after doxorubicin treatment in male or female rats. However, there was a significant decrease (–10%) in body weight four days after doxorubicin treatment in male rats ( $P < 0.001$ ), but not in female rats (–7.7%;  $P > 0.05$ ), while the heart weights of male and female rats remained unaltered one day following doxorubicin treatment. In both sexes, there was a significant decrease in heart weight four days after doxorubicin treatment. Males showed a 15% decrease in heart weight ( $P < 0.001$ ) and females showed a slightly smaller decline (13%) ( $P < 0.05$ ). The heart weight-to-body weight ratio in male and female rats was not altered at either time point after doxorubicin treatment (Table 1).

### 3.2. Plasma estrogen levels in male and female rats

Plasma estrogen was only 20–30% higher in male than in female rats (Table 2;  $P < 0.05$ ) and there were no differences

Table 1

Body weight, heart weight, and heart weight-to-body weight ratio of male and female rats treated with doxorubicin or saline

	Male			Female		
	Control	Dox-day 1	Dox-day 4	Control	Dox-day 1	Dox-day 4
Body weight (g)	358.8 ± 31.0	357.2 ± 24.0	323.9 ± 25.0 <sup>a</sup>	197.6 ± 10.0 <sup>b</sup>	190.6 ± 14.0 <sup>b</sup>	176.0 ± 7.0 <sup>b</sup>
Heart weight (g)	0.81 ± 0.07	0.78 ± 0.05	0.69 ± 0.04 <sup>a</sup>	0.52 ± 0.03 <sup>b</sup>	0.51 ± 0.05 <sup>b</sup>	0.45 ± 0.01 <sup>a,b</sup>
HW/BW ratio	0.0022 ± 0.001	0.0021 ± 0.0008	0.0023 ± 0.0007	0.0026 ± 0.0001 <sup>b</sup>	0.0027 ± 0.0002 <sup>b</sup>	0.0026 ± 0.0009 <sup>b</sup>

Body weight, heart weight, and the heart weight-to-body weight ratio (HW/BW) from male ( $N = 24$ ) and female rats ( $N = 24$ ), which were randomly assigned either to a control (saline injected) or to a doxorubicin-treated group (10 mg/kg body weight). Dox; doxorubicin.

<sup>a</sup>Significantly different from saline injected rats ( $P < 0.05$ ).

<sup>b</sup>Significant sex difference ( $P < 0.05$ ).

Table 2

Plasma 17- $\beta$  estradiol levels of male and female rats treated with doxorubicin or saline

	Control	Dox-day 1	Dox-day 4
Male	5.7 ± 0.6	6.0 ± 1.5	6.1 ± 0.7
Female	8.4 ± 2.7 <sup>a</sup>	8.7 ± 2.4 <sup>a</sup>	7.0 ± 1.4 <sup>a</sup>

Plasma estrogen (estradiol; pg/ml) in male ( $N = 24$ ) and female rats ( $N = 24$ ) of randomly assigned control (saline injected) or doxorubicin-treated rats (10 mg/kg) (mean ± S.E.M.).

<sup>a</sup>Significant sex difference ( $P < 0.05$ ).

due to doxorubicin treatment. Furthermore, the variability between the individual female rats due to estrous cycle was approximately 15–25% (average S.E.M. of approximately 2.2 pg/ml) (Table 2).

### 3.3. Sarcoplasmic reticulum-mediated apoptosis determined by the activation of caspase-12 and calpain activity

Calpains and caspase-12 are two cysteine protease families that play important roles in regulating pathological cell death. There were no differences in cleaved caspase-12 content or calpain activity between male and female control rats (Fig. 1A) or at one day after doxorubicin treatment. However, four days after doxorubicin treatment there were significant increases in both cleaved caspase-12 content ( $P < 0.01$ ) and calpain activity ( $P < 0.05$ ) in both male and female rats (Fig. 1B). Calpain activation in cell culture is responsible for cleavage of procaspase-12 to generate active caspase-12 [39,40]. Hence, these data suggest that this novel apoptotic pathway involving calcium-mediated calpain activation and cross talk between calpain and caspase families exists in vivo.

### 3.4. Apoptosis, caspase-3 content and activity, and XIAP levels

To assess the incidence of apoptotic cell death, we determined the levels of mono- and oligo-nucleosomes contents, cleaved caspase-3 and caspase-3 activity in the isolated cytosolic fraction of the heart tissue. Apoptosis is characterized by the formation of mono- and oligo-nucleosome in the nucleus. Levels of mono- and oligo-nucleosome were similar in the male and female control animals (Fig. 2A). However, male rat hearts contained greater levels of mono- and oligo-nucleosome content one day after doxorubicin treatment ( $P < 0.01$ ) with levels returning to those seen in control animals by day four. In contrast, female rats showed no changes following doxorubicin administration, suggesting that female hearts were more resistant to apoptosis.

There were no differences in caspase-3 proteolytic enzyme activity between male and female control rats, or at one day after doxorubicin administration (Fig. 2B). However, four

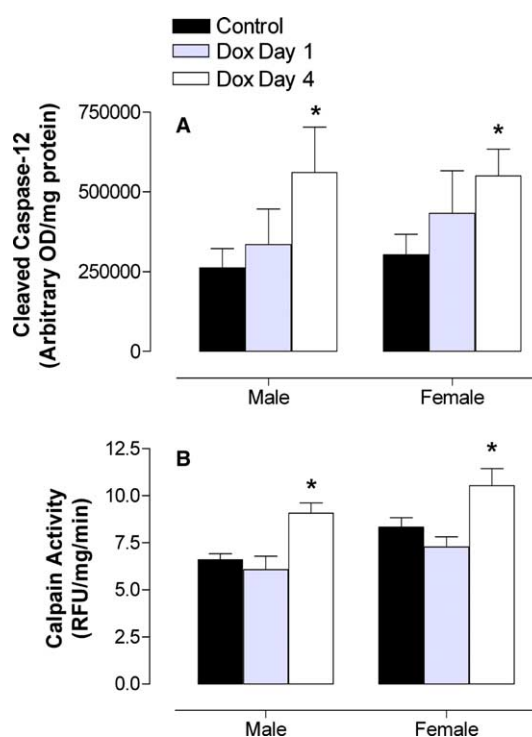


Fig. 1. Sarcoplasmic reticulum-mediated pathway. The effect of doxorubicin administration on the content of (A) cleaved caspase-12 and (B) cytosolic calpain activity. Animals from both male ( $N = 24$ ) and female groups ( $N = 24$ ) were randomly assigned either to a control or to a doxorubicin-treated group. Doxorubicin hydrochloride (Sigma) was dissolved in saline and administered by intraperitoneal injection at a dose of 10 mg/kg body weight, while control animals were injected with a comparable volume of saline (See Section 2). Values presented are means ± S.E.M. Significantly different from control animals (\* $P < 0.05$ ).

days after the administration of doxorubicin there were significant increases in caspase-3 activity in both male and female rats ( $P < 0.01$ ). This increase in activity was due to cleavage of zymogen (pro-caspase-3), because levels of the cleaved active caspase-3 were significantly increased after day four in both the male and female rats (Fig. 2C). No sex or treatment differences were found in the zymogen pro-caspase-3 or X-linked inhibitor apoptotic protein (XIAP), a repressor of caspase-3 activity (Table 3).

### 3.5. Mitochondrial-mediated pathway of apoptosis

Apoptosis in cardiac myocytes is often associated with the release of cytochrome *c* from the mitochondria and activation of caspase-9. Female control rats had significantly lower cy-

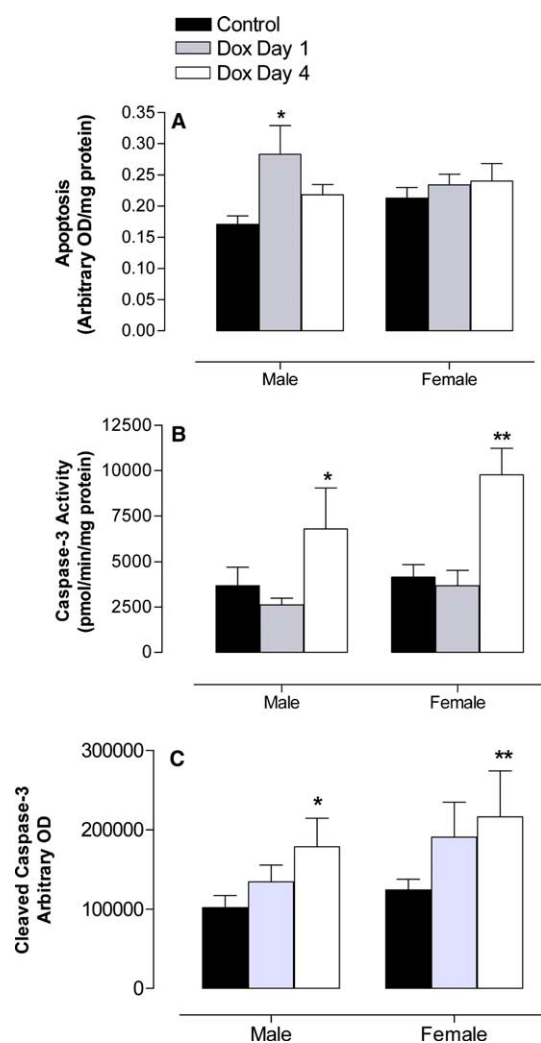


Fig. 2. DNA fragmentation and caspase-3 content and activity. The effect of doxorubicin administration (10 mg/kg) on (A) the amount of DNA fragmentation determined by the content of mono- and oligonucleosomes in the heart cytosol, (B) caspase-3 activity measured using fluorescence ELISA method, and (C) cleaved caspase-3 concentration determined by Western blot analysis. Values presented are means  $\pm$  S.E.M. Significantly different from control animals (\* $P < 0.05$ ) and (\*\* $P < 0.01$ ).

tosolic cytochrome *c* levels compared to male rats ( $P < 0.05$ ) (Fig. 3A). Doxorubicin did not affect cytosolic cytochrome *c* in male or female rats. Cleaved caspase-9 content was not different between male and female rats (Fig. 3B) and was not affected by doxorubicin treatment, suggesting no detectable increase in the activation of the mitochondrial-mediated pathway with this dose of doxorubicin.

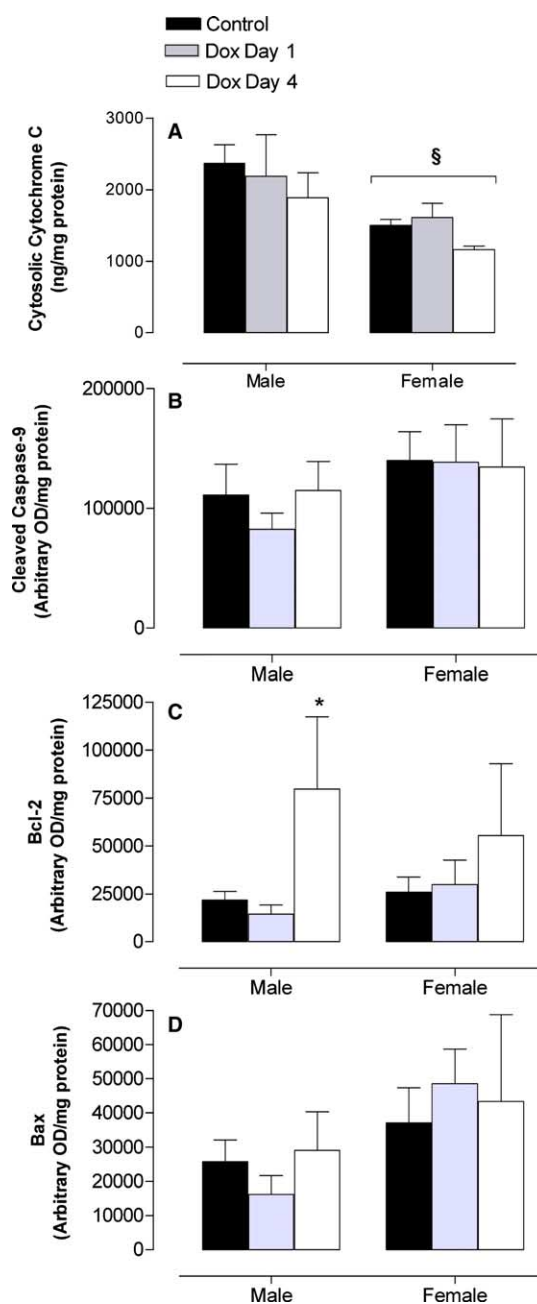


Fig. 3. Mitochondrial-mediated pathway. The effect of doxorubicin administration (10 mg/kg) on the mitochondrial-mediated pathway of apoptosis. (A) cytochrome *c* concentration in the cytosol, (B) cleaved caspase-9 content, (C) mitochondrial Bcl-2 content and (D) mitochondrial Bax content. Values presented are means  $\pm$  S.E.M. Significant sex difference (§ $P < 0.05$ ). Significantly different from control animals (\* $P < 0.05$ ).

Table 3  
The effects of doxorubicin administration on Pro-caspase-3 and XIAP levels

	Male			Female		
	Control	Dox-day 1	Dox-day 4	Control	Dox-day 1	Dox-day 4
Pro-Caspase-3	176 040 $\pm$ 113 210	182 680 $\pm$ 117 906	158 106 $\pm$ 100 880	154 092 $\pm$ 100 178	130 761 $\pm$ 98 793	161 284 $\pm$ 108 763
XIAP	375 257 $\pm$ 155 468	332 515 $\pm$ 135 272	341 874 $\pm$ 173 804	408 375 $\pm$ 164 343	394 945 $\pm$ 119 643	480 747 $\pm$ 235 776

XIAP, X-linked inhibitor of apoptosis. Male ( $N = 24$ ) and female rats ( $N = 24$ ) were randomly assigned to a control (saline injected) or to a doxorubicin-treated group (10 mg/kg body weight).

The levels of Bcl-2 (an anti-apoptotic protein) and Bax (a pro-apoptotic protein) were determined in the same isolated cardiac mitochondria (Fig. 3C and D). There were no differences in the levels of these proteins due to sex, but Bcl-2 was increased four days after doxorubicin treatment in the male and female rats (although the changes were only statistically significant in the male animals). Bax was not significantly different between sexes and was not affected by doxorubicin treatment.

### 3.6. Hydrogen peroxide production in isolated mitochondria and hydrogen peroxide scavenging capacity determined by levels of glutathione peroxidase

In order to measure the rate of reactive oxygen species production by heart mitochondria,  $H_2O_2$  formation was measured immediately after isolation of mitochondria in all groups (Fig. 4A). Basal  $H_2O_2$  production was significantly higher in males compared with females ( $P = 0.003$ ). Doxorubicin did not change  $H_2O_2$  production in the male rats after one day, whereas  $H_2O_2$  production was significantly decreased after day one in the female rats ( $P < 0.05$ ). Males did show a significant drop ( $P < 0.001$ ) in  $H_2O_2$  production after day four, whereas females remained at approximately the same level as at day one. These data show that female rats produce less  $H_2O_2$  and are able to downregulate the production of these oxidants one day after doxorubicin treatment, whereas reduced  $H_2O_2$  production was only seen in male rats four days after doxorubicin treatment. Male rats had significantly higher levels of GPX activity compared to female rats ( $P = 0.001$ ), which probably reflects the chronically higher  $H_2O_2$  production (Fig. 4B). There was no statistically significant effect of doxorubicin treatment on GPX activity.

### 3.7. Mitochondrial function

Mitochondrial oxygen consumption was measured during state 4 (no ADP) and state 3 respiration (0.5 mM ADP) in male and female rats (Table 4). There were no differences in state 4 and state 3 oxygen consumption measurements due to sex or doxorubicin treatment. However, there was a significant sex difference in ATP production (determined in state 3) among control rats, with female rats producing significantly less ATP per milligram of protein of isolated mitochondria ( $P = 0.0032$ ). However, ATP production in isolated mitochondria was not affected by doxorubicin treatment. The P/O ratio, which compares the amount of ATP that is phosphorylated with the amount of oxygen consumed, was also not affected by sex or doxorubicin treatment, suggesting that mitochondria isolated one day and four days after doxorubicin treatment were able to adapt and show little evidence of oxidative stress.

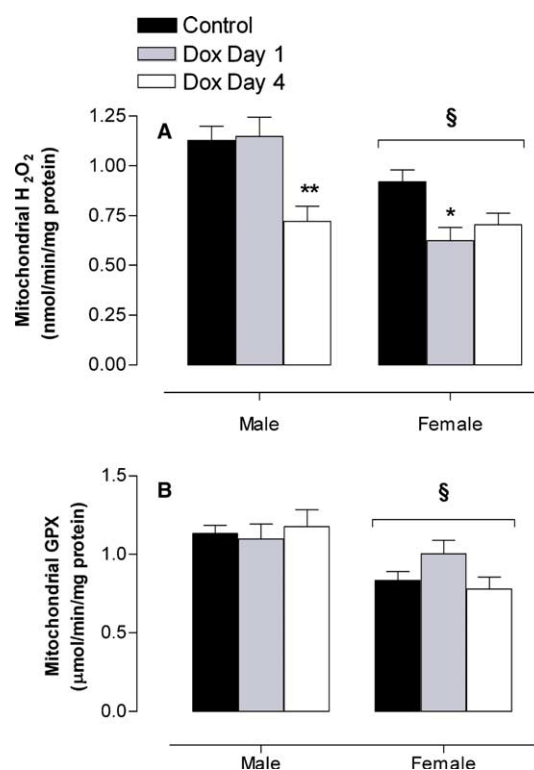


Fig. 4. Mitochondrial hydrogen peroxide production and glutathione peroxidase activity. The effect of doxorubicin administration on heart (A) mitochondrial hydrogen peroxide ( $H_2O_2$ ) production and (B) mitochondrial glutathione peroxidase activity (GPX). Mitochondria were isolated on day one and day four after the injection of doxorubicin (10 mg/ml) or an equal volume of saline in control animals. Values presented are means  $\pm$  S.E.M. Significantly different from the control group (\*\* $P < 0.001$ ) and (\* $P < 0.05$ ). Significant sex difference (§ $P < 0.05$ ).

### 3.8. Receptor-mediated pathway of apoptosis

Pro-caspase-8 content (Fig. 5A), cleaved caspase-8 (Fig. 5B) and cFLIP, an inhibitor of caspase-8 activation (Fig. 5C), were not different between male and female rats and were not affected by doxorubicin treatment.

## 4. Discussion

We found that a relatively low pharmacological dose of doxorubicin selectively affected the SR-mediated pathway of apoptosis by activating calpain, caspase-12, and caspase-3. The activation of caspase-3 appears to be largely due to SR-

Table 4

The effects of doxorubicin administration on the mitochondrial function of male and female rats treated with doxorubicin or saline

	Male			Female		
	Control	Dox-day 1	Dox-day 4	Control	Dox-day 1	Dox-day 4
State 4 $VO_2$	8.4 $\pm$ 1.4	3.0 $\pm$ 5.1	8.5 $\pm$ 2.5	9.4 $\pm$ 3.0	7.2 $\pm$ 4.8	8.7 $\pm$ 3.1
State 3 $VO_2$	42.2 $\pm$ 28.6	47.5 $\pm$ 26.1	34.6 $\pm$ 24.8	31.8 $\pm$ 22.5	25.9 $\pm$ 17.2	21.1 $\pm$ 6.7
ATP	31.8 $\pm$ 8.8	38.1 $\pm$ 6.8	33.3 $\pm$ 8.5	27.8 $\pm$ 8.7 <sup>a</sup>	26.8 $\pm$ 12.4 <sup>a</sup>	23.4 $\pm$ 6.3 <sup>a</sup>
P/O Ratio	1.2 $\pm$ 0.4	1.0 $\pm$ 0.5	1.5 $\pm$ 0.8	1.3 $\pm$ 0.5	0.9 $\pm$ 0.3	1.2 $\pm$ 0.3

State 4 and state 3, nmol oxygen consumption/mg protein/min and ATP production, nmol ATP/mg protein/min. Male ( $N = 24$ ) and female rats ( $N = 24$ ) were randomly assigned to a control (saline injected) or to a doxorubicin-treated group (10 mg/kg). Values presented are means  $\pm$  S.E.M.

<sup>a</sup>Significant gender difference. ( $P < 0.05$ ).



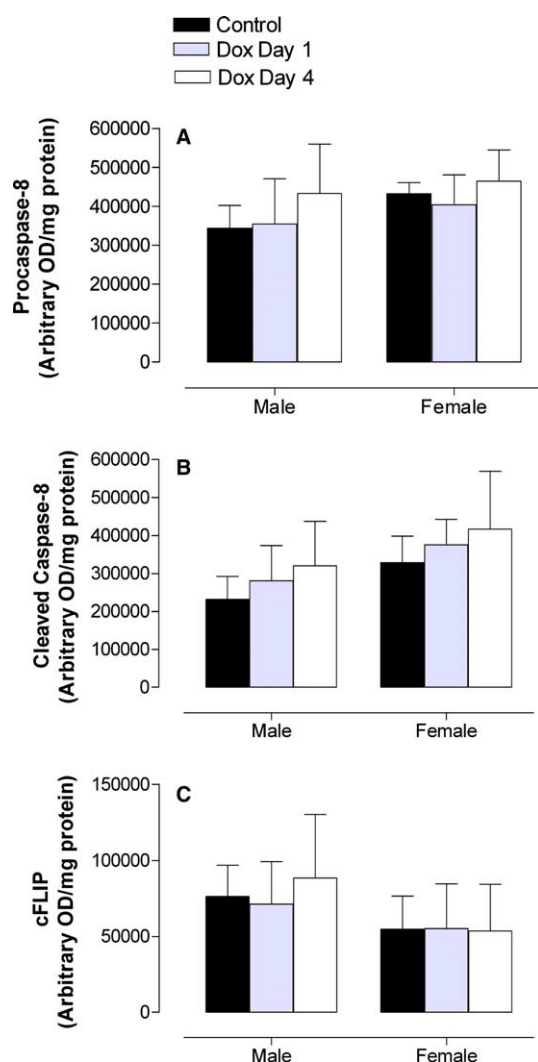


Fig. 5. Receptor-mediated pathway. The effect of doxorubicin administration (10 mg/kg) on receptor-mediated apoptosis. (A) Content of procaspase-8, (B) cleaved caspase-8 content and (C) cFLIP content. Values presented are means  $\pm$  S.E.M.

mediated stress and not due to the mitochondrial- or receptor-mediated pathways. The mitochondrial pathways may have been attenuated by: (1) decreased production of oxidants, (2) upregulation of Bcl-2 proteins, which may have prevented cytochrome *c* release and caspase-9 activation, or (3) maintenance of energy balance (ATP production). The receptor-mediated cell death pathway appears to play a minor role in doxorubicin toxicity, since there were no significant increases in caspase-8, suggesting that intrinsic pathways of apoptosis play a greater role.

We failed to detect any major sex differences in apoptosis signaling following doxorubicin treatment. However, we did find less DNA fragmentation after doxorubicin, lower basal cytosolic cytochrome *c* levels and lower basal levels of mitochondrial  $H_2O_2$  production in female rats. In our animals, plasma estrogen levels were in the pg/ml range, which, in comparison to the much larger concentrations of lipid and water soluble antioxidants found in the plasma [41], may have been too low to have any significant effect on attenuating oxidative stress.

In cardiac myocytes, the SR functions as an intracellular  $Ca^{2+}$  store for use in excitation-contraction coupling. The SR  $Ca^{2+}$  ATPase (SERCA) pumps  $Ca^{2+}$  into the SR terminal cisternae, leading to cardiac relaxation [42]. A prolonged disruption in SR function or SR stress stimulates activation of caspase-12 [31], which is ubiquitously expressed and, like all caspases, is synthesized as an inactive proenzyme consisting of a regulatory prodomain and two catalytic p20 and p10 subunits [31,43]. However, unlike other caspases, caspase-12 is remarkably specific to insults that elicit SR stress and is not proteolytically activated by other death stimuli [30]. We show that caspase-12 activity is increased four days after doxorubicin treatment. Although the molecular mechanisms by which caspase-12 mediates apoptosis are still unclear, cell culture studies suggest that it cleaves pro-caspase-9 without the involvement of cytochrome *c* and apoptosome formation [44]. In our study, we found that calpain activity was significantly increased four days after doxorubicin administration, but we did not detect any significant increases in cleaved caspase-9 *in vivo* in male and female rats. Calpain activation is responsible for cleavage of procaspase-12 to generate active caspase-12 [39,40] and may be the preferred pathway *in vivo*. Hence, our data suggest that this novel apoptotic pathway, which involves calcium-mediated calpain activation and cross talk between calpain and caspase families, exists *in vivo*.

Cytosolic levels of cytochrome *c*, caspase-9 content,  $H_2O_2$  production and ATP production showed little change after doxorubicin treatment, suggesting that the mitochondrial-mediated pathway was not significantly activated. Because the mitochondrial anti-apoptotic protein Bcl-2 was significantly increased in response to doxorubicin administration four days after treatment, this may have affected cytochrome *c* release. Moreover, because ATP production was not significantly affected by doxorubicin treatment, it is likely that little mitochondrial damage and oxidative stress occurred in the isolated mitochondria. This is in agreement with reports that mitochondrial DNA damage and damage to respiratory complexes need to be excessive (i.e., exceeding ~50% of its function) before significant decreases in ATP production are observed [45].

$H_2O_2$  production *in vitro* increases immediately after doxorubicin treatment [5,15], which is in contrast to our observation that  $H_2O_2$  production was unchanged or even decreased following doxorubicin administration *in vivo* (Fig. 6).  $H_2O_2$  is a stable oxidant and is able to diffuse out of the mitochondria and react with cytosolic and nuclear targets [46]. Indeed, the occurrence of a Cu/Zn-superoxide dismutase in the inter-membrane compartment may account for part of the  $H_2O_2$  released by mitochondria, contributing to a cytosolic steady-state level of this species in the cytosol [46]. Cytosolic  $H_2O_2$  can transiently target specific oxidative stress sensitive proteins on the SR. For example,  $H_2O_2$  could target calcium ATPase pumps, such as SERCA located on the surface of the SR [47], which is more prone to oxidation compared to other  $Ca^{2+}$  regulatory channel proteins [19]. Mitochondria themselves are very well equipped with antioxidant defense systems, such as MnSOD, GPX, GR and glutathione. Hence, this adaptation may explain why mitochondrial mediated apoptosis was not detected *in vivo*. The SR, however, may not have such elaborate defense systems, as it has evolved in an environment that is less affected by oxidative processes.

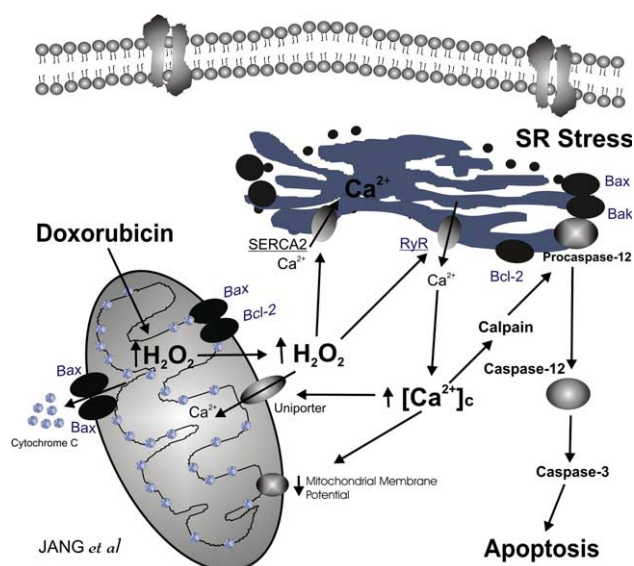


Fig. 6. Proposed scheme of SR mediated apoptosis. Hydrogen peroxide is a stable oxidant and diffuses out of the mitochondria and could react with cytosolic and nuclear targets. Hence, cytosolic H<sub>2</sub>O<sub>2</sub> can transiently target specific oxidative stress sensitive proteins on the SR. H<sub>2</sub>O<sub>2</sub> could target calcium ATPase pumps, such as SERCA located on the surface of the SRs and affect calcium homeostasis. The SR Ca<sup>2+</sup> ATPase (SERCA) pumps Ca<sup>2+</sup> into the SR terminal cisternae, leading to cardiac relaxation [42]. A prolonged disruption in SR function or SR stress stimulates activation of caspase-12, which is ubiquitously expressed and, like all caspases, is synthesized as an inactive proenzyme. Unlike other caspases, caspase-12 is remarkably specific to insults that elicit SR stress and is not proteolytically activated by other death stimuli. We show that caspase-12 activity is increased four days after doxorubicin treatment. The caspase-12-mediated SR apoptotic pathway may be a key pathway for doxorubicin toxicity in vivo, independent of the receptor- or mitochondria-mediated apoptotic pathways.

Doxorubicin selectively decreases cardiac specific SERCA2a mRNA level and can also bind to ryanodine receptors in SR [48,49]. Hence, oxidative damage to calcium regulatory channel proteins and decreases in gene expression may cause alterations in Ca<sup>2+</sup> homeostasis and subsequently increase intracellular calcium level. Sustained increase in intracellular Ca<sup>2+</sup> may activate Ca<sup>2+</sup> dependent protease calpain, which could then degrade key structural proteins such as titin [50] and other sarcomeric proteins, thereby impairing contractility. Furthermore, calpain can also cleave caspase-12 and other caspases to initiate a caspase cascade and apoptosis of cardiomyocytes [39,40]. These events will significantly reduce the number of functional cardiac cells and ultimately lead to cardiomyopathy.

Our study also suggests that the receptor-mediated apoptotic pathway is not affected by sex or this dose of doxorubicin treatment. Furthermore, no significant sex differences in the caspase-8 inhibitor, cFLIP, were detected, nor were levels significantly altered by doxorubicin treatment. It appears, therefore, that this pathway may not play an essential role in inducing apoptosis in vivo, although it remains possible that the dose injected in these rats did not induce a sufficient stress response to significantly activate this pathway. Therefore, caspase-12-mediated SR apoptotic pathway may be a key pathway for doxorubicin toxicity in vivo, independent of the receptor mediated apoptotic pathways, but may be sensitive to

mitochondrial H<sub>2</sub>O<sub>2</sub> production and altered calcium homeostasis.

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