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# Dexrazoxane pre-treatment protects skinned rat cardiac trabeculae against delayed doxorubicin-induced impairment of crossbridge kinetics

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**1** Dexrazoxane (DXR, ICRF-187) has been shown both in animal studies and clinical trials to provide a substantial cardioprotection when co-administered with anthracycline drugs like Doxorubicin (DOX). In a previous study, we showed that chronic DOX treatment in rats is associated with a clear impairment of the crossbridge kinetics and shift in myosin iso-enzymes.

**2** The present study was adopted to investigate whether the cardioprotective action of DXR involves preservation of the normal actin-myosin interaction. Rats were treated for 4 weeks with either DOX at a weekly dose of 2 mg $kg^{-1}$ (i.v.), or were pre-injected with DXR (40 mg $kg^{-1}$, i.v.) at a 20 : 1 dose ratio 30 min prior to the DOX infusion. Rats receiving saline or DXR alone were included in the experiments. Cardiac trabeculae were isolated 4 weeks after the last infusion and were skinned with detergent.

**3** Crossbridge turnover kinetics were studied after application of rapid length perturbations of varying amplitudes in $Ca^{2+}$-activated preparations. DXR treatment offered a significant protection against the DOX-induced impairment of the crossbridge kinetics in isolated cardiac trabeculae. Time constants describing transitions between different crossbridge states were restored to normal in both the quick release protocol and the slack-test. DXR prevented the shift from the 'high ATPase' $\alpha$-myosin heavy chain (MHC) isoform towards the 'low-ATPase' $\beta$-MHC isoform in the ventricles.

**4** We conclude that pre-administration of DXR in rats greatly reduces the deleterious effects of chronic DOX treatment on the trabecular actin–myosin crossbridge cycle. Preventing direct deleterious effects on the actin–myosin crossbridge system may provide a new target for preventing or reducing DOX-related cardiotoxicity and may enable patients to continue the treatment beyond currently imposed limits.

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**Keywords:** Doxorubicin; cardiomyopathy; dexrazoxane; cardioprotection; crossbridge kinetics

**Abbreviations:** BDM, 2,3 Butanedione monoxime; DOX, Doxorubicin; DXR, Dexrazoxane; MHC, myosin heavy chain; MOPS, 3-[Morpholino]propanesulphonic acid

## Introduction

Doxorubicin (DOX) is a highly effective chemotherapeutic agent that is widely used in the treatment of numerous solid tumours and haematological malignancies. However, DOX causes cumulative, dose-related, progressive myocardial damage that may compromise the contractile function of the heart. Prevention or reduction of this cardiotoxicity would enable DOX-responsive patients to continue the treatment beyond currently imposed limits.

We reported that long-term DOX treatment in rats causes a progressive attenuation of the isometric contractile performance of isolated skinned trabeculae (Bottone *et al*., 1998). We provided clear evidence that the underlying mechanism behind this negative inotropic action is an impairment of the crossbridge cycle in which both the rate of attachment and detachment of crossbridges is affected. This impairment is correlated with a shift from the $\alpha$-Myosin Heavy Chain (MHC) to the $\beta$-isoform, a shift related with a transition from the high ATP-ase to the low ATP-ase isoform (de Beer *et al*., 2000). This last observation opens the possibility to prevent cardiomyopathy by drugs blocking the transition to the low ATP-ase myosin iso-enzyme.

It is generally believed that DOX-induced cardiotoxicity is related to the production of oxygen free radicals (Doroshow, 1983). Several antioxidants have therefore been tested for their cardioprotective effects (Herman & Ferrans, 1981; Speyer *et al*., 1988; van Acker *et al*., 1993). Among them dexrazoxane (DXR or ICRF-187), a *bis*-dioxopiperazine compound that is hydrolyzed to form a chelating agent analogous to EDTA, showed to be the most promising. To explore a proof of principle that this drug counteracts the cardiotoxic effects on the molecular level of the actin myosin interaction we investigated the effect of DXR both on the

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mechanical performance and the myosin iso-enzyme expression. DXR has been found to ameliorate the cardiomyopathy associated with DOX in numerous preclinical studies (Herman & Ferrans, 1981; Imondi *et al*., 1996) and clinical trials (Speyer *et al*., 1988; 1990; Swain *et al*., 1997). The mechanism of action of DXR appears to be the prevention of free radical formation, probably through binding of iron (Hasinoff, 1994).

The present study was performed to investigate whether the cardio-protective properties of DXR involve the prevention of the deleterious effects of DOX on the actin–myosin interaction that we found in our earlier study (de Beer *et al*., 2000).

Crossbridge turnover kinetics in skinned cardiac preparations may be studied by application of rapid length perturbations of varying amplitudes and measuring the subsequent force adaptation. Small length changes ($\approx$0.5%; 0.01 $\mu$m sarcomere length) reveal mainly conformational changes in the strongly bound crossbridges (Huxley & Simmons, 1971), while larger length steps (5–10%; 0.1–0.2 $\mu$m sarcomere length) provide information on the rate of crossbridge cycling (Edman, 1977; Brenner, 1988). Trabeculae of the right ventricle of the rat were skinned by means of Triton X-100, thereby disrupting all membranous structures but leaving the contractile apparatus intact (Bottone *et al*., 1997). The results of the present study show that DXR provides substantial protection against the DOX-induced impairment of the crossbridge, while the shift in iso-enzymes was substantially reduced. Preventing actin–myosin contractile aberrations from occurring may provide a new pathway in precluding DOX-related cardiotoxicity.

## Methods

### *Animals and preparations*

Male Wistar rats were used in all experiments. Animals were given water and standard chow *ad libitum*, and were kept on a 12-h light/dark cycle. All experiments were approved by the University Experimental Animal Committee.

Animals were divided in four groups and received the following treatment once a week for a period of 4 consecutive weeks: group 1: DOX 2 mg kg$^{-1}$ week$^{-1}$; group 2: DXR 40 mg kg$^{-1}$ week$^{-1}$; group 3: DXR 40 mg kg$^{-1}$ week$^{-1}$+ DOX 2 mg kg$^{-1}$ week$^{-1}$; and group 4: saline control. Drugs were injected by slow i.v. injection into a tail vein. In rats that received both DXR and DOX, DXR was injected 30 min before DOX. Earlier experiments have shown that the DOX treatment scheme adopted in this study is suitable for studying DOX-related cardiotoxicity (Bottone *et al*., 1998).

At week 4 after the last infusion, rats were anaesthetized with Nembutal (60 mg kg$^{-1}$ body weight, i.p.). After tracheotomy, the thorax was opened and the aorta was cannulated. The heart was rapidly removed and connected to a Langendorff perfusion system. The Tyrode solution had the following composition (in mM): NaCl 130, KCl 4.7, $Na_2HPO_4$ 0.42, $NaHCO_3$ 20.2, glucose 10.1, $MgCl_2$ 1.0, $CaCl_2$ 2.0, and was continuously gassed with a mixture of 95% $O_2$ and 5% $CO_2$ to maintain pH at 7.4 at 30°C. 2,3 Butanedione monoxime (BDM, 15 mM) was added to the Tyrode solution to prevent contraction and muscle damage during dissection (Mulieri *et al*., 1989). Free running trabeculae ranging from 50 to 150 $\mu$m in diameter and 1 to 2 mm in length were dissected carefully from the right ventricle wall and skinned by exposure to Triton X-100 (1% v v$^{-1}$) for 30 min. Triton X-100 renders both the sarcolemma and inner membrane structures permeable for small ions and molecules. The skinning solution was removed by washing with relaxation solution. The composition of the various solutions used have been described in an earlier publication (Bottone *et al*., 1997). The basic composition of these solutions (in mM unless mentioned) is: relaxation solution MOPS 10, EGTA 7, phosphocreatine 10, MgATP 5, $Mg^{2+}$ 1, posphocreatinekinase 50 u ml$^{-1}$, ionic strength (set with KCl) 160, pH 7. The activation solution was achieved by adding $CaCl_2$ to the relaxation solution yielding a free [$Ca^{2+}$] concentration of 0.1 mM.

### *Measuring device*

The displacement generating system consisted of a coil moving in a permanent magnet, making step length changes completed within 1 ms, and has been described elsewhere in detail (Bottone *et al*., 1998). Isometric tension of the muscle preparations was measured with a Sensonor AE 801 force transducer (Horten, Norway). All tension signals and displacement signals were digitized by a computer with an AD-card (Keithley DAS 1602) for further analysis.

The preparation was mounted between the force transducer and a support connected to the coil of the displacement system with cyanoacrylate glue (Sicomet 77, Henkel, Germany). The glue was coloured with Berlin-red to check the interface of the glue and the functional preparation to obtain a precise measure for preparation length. Trabeculae were stabilized for 15 min in relaxation solution before the start of the experiments. Sarcomere length was measured by means of laser diffraction and was adjusted to 2.15 $\mu$m at the start of the experiments. Only preparations of which the sarcomere length remained constant throughout the experiments were used. In a number of experiments we followed the change in muscle length and the change in sarcomere length simultaneously during step releases. These experiments showed that the relative change in sarcomere length was equal to the change in muscle length which was accomplished by means of the stiff fixation of the muscle ends by the cyanoacrylate glue (de Winkel *et al*., 1995). Tension was calculated as force divided by cross-sectional area. The latter was calculated from the muscle dimensions by assuming an ellipsoid shape. All experiments were performed at 22°C and at pH 7.0.

### *Quick release*

The force adaptation to sudden length changes with amplitude of 0.01 $\mu$m of the sarcomere length was studied. The adaptation of the mechanical transient to a sudden length change reflects mainly conformational changes within a crossbridge while attached, together with processes like attachment and detachment of cycling crossbridges during transition to the new steady state (Huxley & Simmons, 1971).

The passive Youngs modulus, i.e. the stiffness normalized for the length and the diameter in relaxed preparations, was determined at the start of each experimental protocol. The

Youngs modulus is defined as $\Delta T/(\Delta l/l)$, where $\Delta T$ is the amplitude of the tension response, and $\Delta l/l$ is the relative change in length. Preparations were allowed to contract in activation solution at pCa = 4.0. At steady state contraction, tension transients resulting from quick releases were recorded. The resulting tension transients were fitted with a sum of three exponential functions, yielding the time constants $\tau_1$, $\tau_2$, and $\tau_3$, together with two extreme tension values $T_1$ and $T_2$ reflecting the adaptation of the preparation to its new length.

### *Slack-test*

The slack-test has been described in detail in an earlier report (de Beer *et al*., 2000) (see also Hancock *et al*., 1996; Janssen & de Tombe, 1997). In short, maximally activated trabeculae were slackened by imposing a series of six quick releases of increasing magnitude (5–10%; 0.1–0.2 $\mu$m sarcomere length) on the preparation. Following each step release, the muscle was returned to its initial length and isometric tension level. Adoption of this protocol allows for the collection within 60 s of a complete slack-test containing six releases during one single contraction. The time required to take up the imposed slack, i.e. the duration of the unloaded shortening was measured as the interval between the beginning of the length step and the onset of tension redevelopment. The slope of the relation between the magnitude of the release step and the duration of unloaded shortening corresponds to the velocity of unloaded shortening ($V_0$) (Edman, 1977). The subsequent redevelopment of force after the shortening of the preparation under zero load was fit by a mono-exponential function on the form $\text{Force} = a^*(1-e^{-t/\tau r})$, where $a$ is the redeveloped force, $t$ is the time, and $\tau_r$ is the time constant describing tension recovery (Brenner, 1988; Hancock *et al*., 1996).

### *Myosin heavy chain isoform analysis*

After isolation of trabeculae, right and left ventricles of both Dox-treated rats and control rats were frozen in liquid nitrogen and stored at −80°C. The freeze-dried samples were dissolved in a buffer containing (in mM): Tris (pH 6.8) 62.5, dithiothreitol 15, phenylmethyl-sulphonyl fluoride 0.1, leupeptin 0.5, 1% (w $v^{-1}$) SDS, 0.01% (w $v^{-1}$) bromophenol blue, and 15% (v $v^{-1}$) glycerol. Gel electrophoresis was performed as described previously (van der Velden *et al*., 1998a) using an acrylamide to bis-acrylamide ratio of 200 : 1 in the separating gel (12% total acrylamide; pH 9.3) and of 20 : 1 in the stacking gel (3.5% acrylamide; pH 6.8). A Protean II xi cell was used for electrophoresis (Bio-Rad, Hercules, California, U.S.A.). Samples (1 $\mu$g) were run at constant current (24 mA) for 5 h (approximately 1800-Volt h). Silver staining of the gels was performed as described in Giulian *et al*., 1983. Laser scanning densitometry was performed to identify differences in myosin isoform composition. Bands corresponding to contractile proteins were identified by Western immunoblotting using specific antibodies against rat α-MHC and β-MHC: mAb 249-SA4 equals anti-α-MHC and mAb 169-1-D5 equals anti-β-MHC in the rat (van der Velden *et al*., 1998b). The production of these antibodies has been described previously (de Groot *et al*., 1989).

### *Statistics*

Data values are given as mean ± s.e.mean for $n$ observations. In our statistical analysis, from each individual animal only one set of data of one trabecula in each protocol was incorporated. If more preparations of an individual animal were available, they showed similar results. An analysis of variance (ANOVA, two-tailed) was used to compare simultaneously differences between the four different groups (control preparations, DXR- and DOX-treated preparations). Differences with $P < 0.05$ were taken as significant.

## Results

### *Animals and preparations*

The mean starting body weight of all 22 rats used in the present study was 316 ± 3 g. Three out of the eight rats that were treated with DOX alone died during the post-treatment period. No treatment-related deaths occurred in the other groups. Table 1 summarizes the results of the different treatment schemes on body weight and heart weight at the moment of sacrifice. DOX treatment caused a significant reduction in body weight at the moment of sacrifice as compared to all other groups. DXR partially prevented DOX induced weight loss when administered just prior to the DOX infusion (see Table 1). The heart weight of DOX-treated rats was significantly reduced as compared to saline-treated rats at the moment of sacrifice (Table 1). DXR- and DOX + DXR-treated rats showed an intermediate heart weight. The relative heart weight (as percentage of body weight) of DOX-treated rats was significantly higher as compared to controls, whereas in the other groups the reduction in heart mass paralleled the decrease in body weight. The dimensions of the right ventricular trabeculae were not significantly different between the four experimental groups. The overall mean diameter was 112 ± 5 $\mu$m for all preparations in the present study ($n = 22$).

### *Maximal $Ca^{2+}$-activated tension*

The maximal tension of trabeculae of DOX-treated rats was significantly decreased as compared to the maximal tension of trabeculae of saline-treated rats (see Table 2A). The maximal tension of trabeculae of the DXR + DOX group and of the DXR group were not significantly altered as compared to control values (see Table 2A).

**Table 1** Body weight and heart weight at the moment of sacrifice, i.e. at 4 weeks post-treatment

| Group | *Terminal body weight (g)* | *Terminal heart weight (g)* | *Rel. heart weight (% of b.w.)* | n |
|---|---|---|---|---|
| Saline | 460 ± 11 | 1.56 ± 0.02 | 0.34 ± 0.01 | 5 |
| DOX | 195 ± 12[a] | 0.90 ± 0.10[a] | 0.50 ± 0.01[a] | 5 |
| DXR | 406 ± 4 | 1.46 ± 0.07 | 0.36 ± 0.02 | 4 |
| DXR + DOX | 356 ± 7[a] | 1.37 ± 0.06[a] | 0.38 ± 0.01 | 8 |

Initial starting weight of all four groups of rats together: 316 ± 3 g ($n = 22$). Data are means ± s.e.m for $n$ experiments. [a]$P < 0.05$ (ANOVA). The DOX data are significantly different from all other data, while in the DOX + DXR treated animals terminal body weight and heart weight were also significantly different from the saline and DXR groups.

**Table 2** Effect of long-term treatment with saline, DOX, DXR, and DXR+DOX on static and dynamic tension parameters in skinned cardiac trabeculae of rats

| | | *Saline* (n=5) | *DOX* (n=5) | *DXR* (n=4) | *DXR+DOX* (n=4) |
|---|---|---|---|---|---|
| A | $T_{max}$ | 80±8 | 52±2[a] | 82±10 | 75±4 |
| B | $\tau_1$ | 11±1 | 19±2[a] | 11±1 | 10±1 |
| | $\tau_2$ | 64±3 | 103±13[a] | 67±7 | 62±5 |
| | $\tau_3$ | 108±9 | 205±23[a] | 112±8 | 136±19 |
| C | $V_0$ | 8.8±0.3 | 6.1±0.4[a] | 8.6±0.5 | 7.7±0.4[a] |
| | $\tau_r$ | 66±5 | 112±6[a] | 75±10 | 80±7 |

The experiments were performed in week 4 after the last infusion. A: Maximal $Ca^{2+}$-activated tension $T_{max}$ (kN $m^{-2}$) of skinned trabeculae. B: Time constants ($\tau_1$, $\tau_2$, $\tau_3$, ms) describing tension recovery after a quick release step of 0.011 mm sarcomere length in maximally $Ca^{2+}$ activated preparations. C: Unloaded shortening velcity per sarcomere length ($V_0$, $\mu$m $s^{-1}$), and time constant of force redevelopment ($\tau_r$, ms) after the slack at a length step of 0.22 $\mu$m sarcomere length. Data are means+s.e.mean for *n* experiments. [a]$P<0.05$ (ANOVA). All DOX data are significantly different from the other groups and $V_0$ after the DOX+DXR treatment is significantly different from the other data.

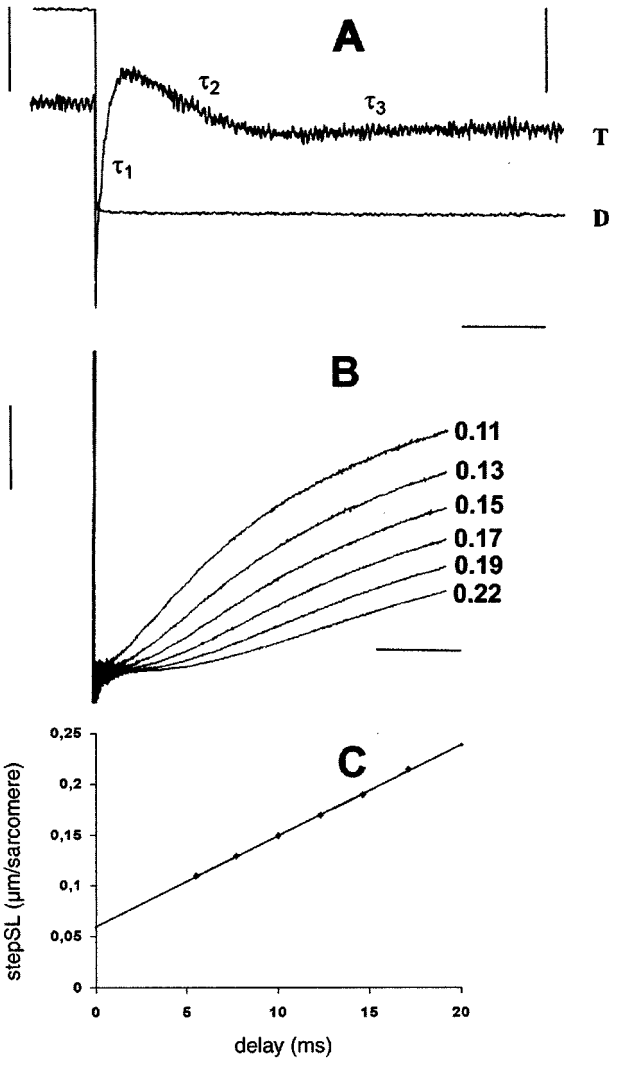


**Figure 1** Typical tension transients (T) resulting from a quick release experiment (A) or slack test (B). The length change in (A) is 0.011 $\mu$m sarcomere length. Length change Bar (Right side): 0.005 $\mu$m; Tension Bar (Left side) in (A): 25 $kNm^{-2}$; in (B): 20 $kNm^{-2}$. Time Bar in (A): 100 ms; in (B): 20 ms. In (A) the three time constants are indicated. Also the step D is indicated in (A). In (B) the magnitude of the steps in $\mu$m sarcomere length are given at the right site of the graphs. The duration of unloaded shortening measured in the Edman's slack test showed to be linearly proportional to the amplitude of the quick release step as shown in (C). The slope of this linear relation gives the maximal rate of shortening $V_0$ (In this particular experiment $V_0=8.95$ $\mu ms^{-1}$ and the intercept = 0.061 $\mu$m sarcomere length).

### *Quick release experiments*

In preparations that were maximally activated at pCa 4.0, the tension transients following step releases were fitted with a sum of three exponential functions, yielding the time constants $\tau_1$, $\tau_2$, and $\tau_3$. A typical recording is shown in Figure 1A. Dynamic experiments in relaxed preparations showed that the passive stiffness, as was measured by the Youngs modulus, did not differ from the control value of saline-treated rats (294±22 kN $m^{-2}$; $n=5$). This implies that the formation of connective tissue is negligible in the chronic treatment group. Table 2B summarises the results of DOX treatment on the three time constants in the quick release protocol. Chronic treatment of rats with DOX alone results in a significant increase of the three time constants describing the tension recovery as compared to saline-treated controls (Table 2B). Trabeculae of DOX+DXR treated rats and of DXR-treated rats showed time constants that were not significantly different from those of saline-treated animals (Table 2B).

### *Slack-test*

A family of typical recordings obtained with the slack test is shown in Figure 1B. Increasing the amplitude of the step from 0.11 till 0.22 $\mu$m sarcomere length results in a much longer time interval in which the fibre goes slack. During this interval the slack is taken up at zero load. The speed at which this zero load is taken up is defined as the maximal speed of shortening (Edman, 1977). The duration of unloaded shortening was linearly proportional to the amplitude of the quick release step, and the velocity of unloaded shortening ($V_0$) can be determined by the slope of this relation as shown in Figure 1C. Table 2C summarized the results obtained in the slack-test. The $V_0$ of saline-treated controls amounted 8.8±0.3 $\mu$m sarcomere length $s^{-1}$ ($n=5$). Compared to this value, $V_0$ of trabeculae of both DOX-treated rats and of DXR+DOX -treated rats was significantly lower (Table 2C). The time constant describing force redevelopment ($\tau_r$) was found to be dependent on the amplitude of the step and decreased with increasing step amplitudes. The average $\tau_r$ at a 0.22 $\mu$m length step in sarcomere length was taken as standard. In DOX-treated rats, $\tau_r$ was significantly higher as compared to values of saline-treated rats (Table 2C). The $\tau_r$ of DXR-treated rats and of DXR+DOX-treated rats was not significantly different from saline-treated controls.

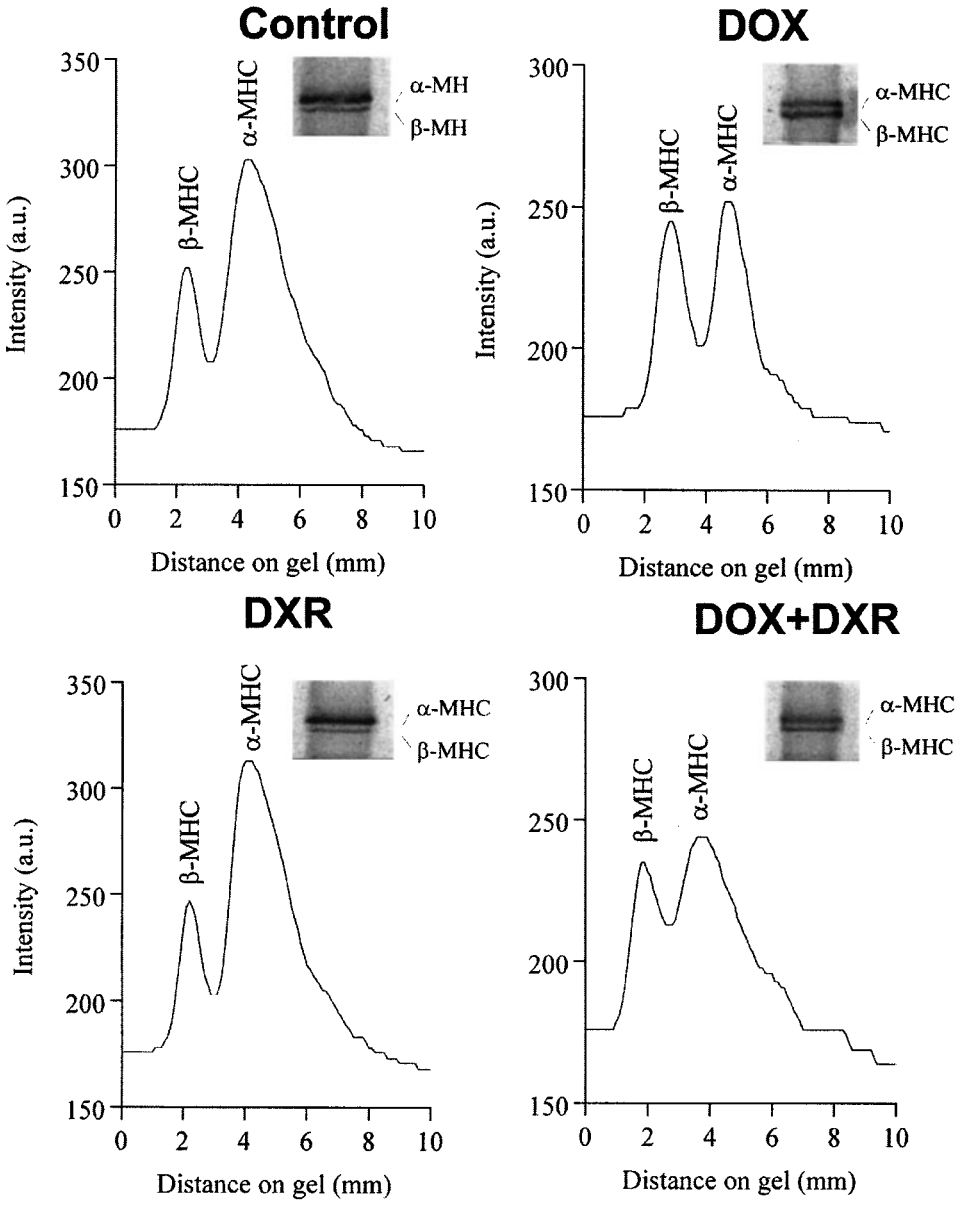


**Figure 2** Typical photographs of silver stained SDS-polyacrylamide gel of α- and β-MHC isoforms of ventricular tissue together with their laser scans. The four experiments are indicated: control; DOX; DXR; DOX + DXR.

**Table 3** Relative percentage of α-MHC and β-MHC heavy chain isoforms after different drug treatments

| *Group* | *% α-MHC* | *% β-MHC* |
|---|---|---|
| Saline ($n=5$) | 77±2 | 23±2 |
| DOX ($n=5$) | 56±2[a] | 44±2[a] |
| DXR ($n=4$) | 79±2 | 21±2 |
| DOX/DXR ($n=8$) | 74±5 | 26±5 |

Data are means±s.e.mean for $n$ experiments. [a]$P<0.05$ (ANOVA). Only DOX treatment results in a significant change in MHC-composition.

*Differences in myosin heavy chain isoform composition*

Typical silver-stained enlargements of the two bands visible in polyacrylamide gels of the electrophoretically separated proteins are shown in Figure 2. The fast α-MHC and slow β-MHC could be easily separated, and subsequent laser densitometric scans were used to quantify the two peaks. Chronic DOX treatment in rats significantly increased the ratio of β-MHC over α-MHC in ventricular tissue compared to controls (Table 3) However, in DXR pre-treated animals the shift in MHC-ratio was significantly less pronounced than in non-pre-treated animals and within the statistical limits equal to the control value.

## Discussion

It is well known that the development of a DOX-induced cardiomyopathy is a time-dependent process (Bottone *et al.*, 1998; Cirillo *et al.*, 2000). The time course strongly depends on the protocol to supply the drug (concentration, i.v. or i.p.,

etc). Because DOX induced-mortality in our protocol after 4 weeks is high, we assumed that the rats were in a late stage of heart failure.

The present study clearly shows that DXR when co-administered with DOX provides protection against DOX-induced impairment of the crossbridge cycle in skinned rat trabeculae. In an earlier report we showed that chronic DOX treatment in rats leads to a decrease of the isometric tension level in skinned trabeculae (Bottone *et al*., 1998). In this respect it is noteworthy that we provided evidence that, in our experimental model, the impairment of the isometric tension response is drug-related and cannot be ascribed to the loss of body weight associated with DOX treatment (Bottone *et al*., 1998). Moreover, we found compelling evidence that the impaired contraction was caused by an overall lower crossbridge cycling rate involving both the attachment and detachment processes within the crossbridge cycle (de Beer *et al*., 2000) associated with a shift in myosin iso-enzyme composition. Two dynamic perturbation protocols were applied in this study which differed in their step length amplitudes. The quick release protocol was adopted to study conformational changes in the strongly bound crossbridge head. The slack-test was used to study crossbridge turnover rates during the redevelopment of a steady-state tension level. DXR pre-treatment offered complete protection against alterations in the time constants governing adaptational processes in the quick release protocol. The DOX-related effect on $V_0$ and $\tau_r$, indicators for the crossbridge turnover rate, was almost completely absent in the DXR + DOX group. The values describing transition times between crossbridge states in the present study are in close range with those reported by others (de Winkel *et al*., 1995; Janssen & de Tombe, 1997), and with those of a previous study by our laboratory (de Beer *et al*., 2000).

The mechanism that is responsible for the DOX-induced impairment of the crossbridge cycle is not clear. A shift from the fast myosin isoform ($V_1$) to the slower isoform ($V_3$) has been associated with this impairment (de Beer *et al*., 2000), and may have accounted for the observed contractile impairment in our study. Especially the increase in the time constant of force redevelopment in the DOX-treated animals implies that the cycling rate is slowed down by a factor of two. From the quick release experiments we learnt that the rate of detachment ($1/\tau_2$) is lowered. Together with the decrease in tension, this implies that also the rate of attachment is decreased and that in a twitch contraction as found in the heart muscle the number of strongly bound states will be decreased. The hypothesis that the MHC-shift underlies the impairment in tension is supported by the observation that DXR almost prevented the shift from the fast ATP-ase $\alpha$-MHC to the slow $\beta$-isoform and the decrease in the maximal tension. The question remains whether this is a causal adaptation process. Generally a shift to a low ATP-ase activity myosin isotype is interpreted in an increase in economy of the contraction process (Swynghedauw, 1986). However such a shift implies at the same moment that the contraction process is slowed down and the range for adaptation of contractile performance is limited with chronic DOX treatment.

One of the intriguing questions is whether this process is mediated by free radicals. To investigate this question we incubated our skinned muscle fibres with either a superoxide generating mixture or HOCL. The main effect of addition of these radicals on the isometric tension was a decrease in isometric tension, a result similar to that found in the literature (data not shown; Miller & Macfarlane, 1995). However, in our experimental set-up DOX acutely increases tension (Bottone *et al*., 1997). This suggests that the acute effects of DOX on the contractile machinery of skinned fibres may not be mediated by free radical formation. Long term exposure of cardiac muscle to oxygen free radicals *in vivo* was not feasible and it remains to be determined whether the chronic effects of DOX are mediated by oxygen free radicals.

A clear dose-response relationship for protection against DOX-induced cardiomyopathy by DXR has been reported. In the present study a DXR : DOX ratio of 20 : 1 was chosen, since this ratio has been found to give a significant cardioprotection in rats (Imondi *et al*., 1996).

Although DXR provided a substantial cardioprotection against DOX-related cardiotoxicity in the present study, we still detected a slight impairment of the crossbridge cycle in the DXR + DOX group together with a marked reduction in body weight and heart weight as compared to saline-treated controls. The inability of DXR to provide complete cardioprotection may be related to pharmacokinetic differences between the two drugs. In humans, the terminal half-lives of DOX and DXR are 39.5 and 4.16 h respectively (Hochster *et al*., 1992). In rats also, the terminal half-life of DXR is considerably shorter than that of DOX (Baldwin *et al*., 1988). This suggests that plasma levels following a single dose of DXR may not be high enough over a sufficient period to provide an adequate protection against a high bolus of DOX. Second, free radical production by DOX may be generated by both iron-dependent (Gianni *et al*., 1983) and iron-independent mechanisms (Hasinoff, 1990). Since DXR probably acts as a metal ion chelator (Hasinoff, 1994), it would be expected to be effective in blocking the formation of free radicals formed through iron-dependent mechanisms but not those formed enzymatically. This implies that even in the presence of DXR, DOX is still able to cause cardiac injury by the formation of free radicals. With respect to its specificity it is noteworthy that we did not observe a protective effect of DXR against another type of cardiomyopathy, the crotaline myopathy (data not shown) (Lee-de Groot *et al*., 1998).

Does the used animal model reflect the human situation? A decrease in ventricular myofibrillar ATPase activity in failing human hearts has been reported (Swynghedauw, 1986). Using a quantitative reverse transcription Polymerase Chain Reaction, it was shown that substantial levels of $\alpha$-myosin heavy chain mRNA ($\sim$35%) are present in non-failing ventricular myocardium (Lowes *et al*., 1997; Nakao *et al*., 1997). In two types of myocardial failure, the $\alpha$-myosin heavy chain expression was markedly down-regulated and the $\beta$-myosin heavy chain exhibited reciprocal up-regulation. Recently Miyata *et al*. (2000) detected about 10% $\alpha$-MHC protein in non-failing human ventricles, while no $\alpha$-MHC or mRNA were observed in end-stage failing human hearts. Based on these recent data, both in the rat and in humans cardiac failure is associated with a shift in MHC-isoforms, indicating that the same process is involved. Therefore, it is likely that genes coding for myosin heavy chain isoforms are candidates as molecular base of both DOX-induced and other types of myocardial failure in humans. In this respect it is noteworthy

that DXR protects against DOX-induced cardiomyopathy, but not against crotaline-induced heart failure.

In summary, chronic treatment with DOX results in a clear overall lower crossbridge cycling rate by impairment of both the attachment and detachment rate of crossbridges. Pretreatment with DXR offered a substantial cardioprotection against the DOX-induced cardiotoxic effect on the crossbridge cycling rate and in the shift in myosin isotype. It shows the principle possibility that preventing or reducing actin-myosin contractile aberrations may provide a new pathway in reducing DOX-related cardiotoxicity, thereby enabling DOX-responsive patients to continue the treatment beyond currently imposed maximal cumulative doses.

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