ISOLATED MOUSE ATRIUM AS A MODEL TO STUDY ANTHRACYCLINE CARDIOTOXICITY: THE ROLE OF THE β-ADRENOCEPTOR SYSTEM AND REACTIVE OXYGEN SPECIES

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ABSTRACT

Cancer chemotherapy with anthracyclines, of which doxorubicin (DX²) is the main representative, is limited by cardiomyopathy developing in animals and patients after cumulative dosing. The toxicity is probably related to free radical formation by the anthracycline as well as its metabolites with concomitant O₂⁻ and ·OH generation resulting in lipid peroxidation and subsequent membrane damage. An in vitro model is required to investigate the individual contribution of each metabolite to cardiotoxicity. For in vivo studies, the species of choice is the mouse because it lacks the DX-induced nephrotic syndrome seen for instance in rats and rabbits. Thus, isolated mouse heart muscle was chosen as an in vitro model.

To characterize the model, we used l-isoprenaline/dl-propranolol and metacholine/atropine to measure the β-adrenergic and the muscarinic responses of (spontaneously beating) right and (paced) left atrium. Dose response curves (n=4) were highly reproducible: pD₂,iso = 8.0 ± 0.3 (left) and 8.5 ± 0.4 (right); pD₂,met = 6.7 ± 0.1 (left) and 6.2 ± 0.3 (right). Propranolol as well as atropine behaved as competitive antagonists, with pA₂-values of 8.4 ± 0.2/8.5 ± 0.2 (l/r) and 9.1 ± 0.1/9.1 ± 0.2 (l/r), respectively. These values corresponded to those obtained with other organ preparations.

We tested the effect of DX in two ways: a) by measuring the direct inotropic and chronotropic effect during 60 minutes of incubation with 10-100 µM DX in the organ bath, and b) by determining the remaining β-adrenergic response to l-isoprenaline after the incubation period. Both variables turned out to be equally affected. For paced left atria an IC₅₀ (causing 50% depression of contractile force) of 35 µM was determined. Right atria stopped beating at concentrations above 50 µM, thus hampering IC₅₀ determination.

The results indicate that anthracyclines exert an effect not related to receptor integrity, but directly to the functionality of heart muscle. To check whether radical stress can be involved in the observed negative inotropic effect, incubations with xanthine/xanthine oxidase (to produce reactive oxygen species) were performed. A pronounced negative effect on mouse atrial contraction was indeed observed. However, initially a positive inotropic effect accompanied by an increased resting tension were seen.

It can be concluded that mouse atrium can be used as a model to compare anthracyclines and their metabolites with regard to their acute cardiotoxic effects.

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²) Abbreviations used: DX: doxorubicin; iso: l-isoprenaline; met: metacholine; X: xanthine; XO: xanthine oxidase
INTRODUCTION

Doxorubicin (DX), an anthracycline, is a highly effective anticancer drug. Its clinical use, however, is limited by the acute and late toxic effects such as myelodepression and cardiomyopathy (Van Hoff et al., 1979, Dresdale et al., 1983). In vivo studies of DX-induced cardiotoxicity in animal models might be influenced by other effects such as nephrotoxicity (Jaenke and Fajarda, 1977). The mechanism of DX-induced cardiotoxicity has remained elusive, but several hypotheses have been postulated including formation of a toxic metabolite (Olson et al., 1988), generation of oxygen free radicals (Llesuy et al., 1985, Lown et al., 1984), membrane lipid damage (Mimnaugh et al., 1985), alterations in mitochondrial enzyme activities (Eckenhoff and Somlyo, 1989) and involvement of myocardial β-adrenoceptors (Politi et al., 1985, Rasmussen et al., 1989). Via a single-electron reduction of doxorubicin its semiquinone free radical can be formed (Bachur et al., 1977). In the presence of molecular oxygen, this semiquinone free radical is able to form reactive oxygen radicals through redox-cycling (Doroshow, 1983), possibly resulting in the initiation of membrane lipid peroxidation.

Toxic effects of anthracycline treatment, such as nephrotoxicity, are quite different in various animal species (Jaenke and Fajarda, 1977). Rats develop a drug related nephropathy, and thus it seemed possible that in this species anthracycline induced cardiotoxicity may be partly related to concomitant nephrotoxicity (Van der Vijgh et al., 1987, Julicher et al., 1988). In contrast to rats, mice do not develop a nephrotic syndrome during treatment with anthracyclines (Van der Vijgh et al., 1988), hence the mouse is the species of choice for in vivo studies. Furthermore, there are indications that mouse heart microsomes are more sensitive to DX than rat heart microsomes. This is illustrated by measuring the extent of lipid peroxidation, being larger in microsomes of mice as compared to those of rats (Mimnaugh et al., 1983). Because of the absence of the above mentioned nephrotoxicity, we chose isolated mouse heart muscle as an in vitro model to determine the cardiotoxicity of DX in comparison with its metabolites and analogs.
The aim of the present investigation was to study possible effects of DX on the cardiac
β-adrenoceptor function in vitro. Therefore, we determined the response of isolated mouse
atria to l-isoprenaline before and after incubation with DX. To characterize our in vitro
cardiac model we determined the pD₂ value of l-isoprenaline and metacholine as well as the
pA₂ value of the antagonists dl-propranolol and atropine. To elucidate the vulnerability of
isolated mouse atria for free oxygen radicals, incubations with xanthine/xanthine oxidase
were performed and the registered effects were compared with the response of isolated rat
atria on oxidative stress under the same experimental conditions.

MATERIALS AND METHODS

Animals. Male Balb/c mice aged 6-8 weeks (18-22 g), and male Wistar rats 160-180 g,
were obtained from C.P.B. Harlan Olec (Zeist, The Netherlands) and used within 4 weeks.
Chemicals. l-Isoprenaline hydrochloride, dl-propranolol hydrochloride, metacholine and
xanthine oxidase (grade III) were purchased from Sigma (St.Louis, USA); atropine sulphate
and xanthine from Merck (Darmstadt, Fed. Rep. Germany); doxorubicin hydrochloride was
kindly supplied by Farmitalia Carlo Erba (Milan, Italy).

Atrial preparations. Animals were sacrificed by decapitation, and the entire heart was
quickly removed. To arrest contraction, the heart was put in a calcium free phosphate buffer
(pH 7.4) containing (mM) NaCl (136.9); KCl (2.68); KH₂PO₄ (1.47); Na₂HPO₄ (7.19).
Atria were separated from ventricles and placed in a 20 ml organ bath containing
Krebs-solution (ambient temperature, which is then gradually warmed to 37°C) of the
following composition (mM) NaCl (117.5); KCl (5.6); MgSO₄ (1.18); CaCl₂ (2.5);
NaH₂PO₄ (1.28); NaHCO₃ (25); glucose (11.1); pH 7.4 gassed with 5% CO₂ in O₂.
Following a 30 min stabilizing period to each preparation a preload tension of 0.40 g was
applied. The isolated left atria were stimulated to contract with square wave impulses of 3
msec duration and a voltage of 1.5 times the threshold value. The impulses were generated
by Grass S88 stimulators at a fixed frequency of 4 Hz. The muscle contractions were
recorded isometrically by means of a Grass FT.03 force transducer and a Grass model 79 Polygraph. For right atria the frequency (Hz), and for left atria the amplitude of the recorder signal was used as the effect parameter.

Experiments. After an equilibration period of 30 min with intermittent washing, two cumulative dose response curves with agonist were recorded, of which the first was included in the stabilizing period. Between curves and thereafter a wash-out period of 30 min followed, during which the bath fluid was exchanged four times. Next, a 25 min pre-incubation with the antagonist took place and a dose response curve of the agonist was recorded in the same bath. The pD₂ values of the agonist and the pA₂ values of the antagonists were evaluated according to Van Rossum (1963).

In the experiments with DX the isolated atria were allowed to stabilize for 1 hour during which the bath fluid was replaced every 15 minutes. After the stabilisation period DX was added to the fluid (bath concentrations 10-100 μM) and the effect on the atria was monitored for 60 min. To study the effect of DX on β-adrenoceptor function, dose response curves with isoprenaline were constructed first in the absence of DX (as above) and next after incubation for 60 min with DX (10-100 μM).

Oxygen radicals were generated by adding xanthine oxidase (10 units/l) to the organ bath, which contained Krebs buffer supplemented with 2 mM xanthine.

RESULTS

Characterization

Because of the small dimensions of mouse atria as compared to those from rat (1 vs. 50 mg wet weight), and since the influence of preload tension on contractile properties has been demonstrated for other muscle preparations (Kenakin, 1987), we decided to test the effect of preload tensions. Initial tensions ranging from 0.2-1.2 g were applied to the muscle preparations and evaluated with regard to basal contraction development as well as the β-adrenergic response to isoprenaline (data not shown). A preload of 0.4 g appeared to be
optimal. Higher preloads resulted in reduced isoprenaline $\Delta \text{E}_{\text{max}}$ values, caused by overstretching, while at lower preloads the actual tension sometimes dropped to zero due to reequilibration of the muscle. Using 0.4 g, the tension drop upon muscle equilibration took about 10 min and never exceeded 0.2 g (as calculated from the recorder baseline).

Table I - Muscarinic and $\beta$-adrenergic responses of isolated mouse atria

<table>
<thead>
<tr>
<th></th>
<th>Isoprenaline</th>
<th></th>
<th>Metacholine</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>pD$_2$</td>
<td>n</td>
<td>pA$_2$</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Propranolol</td>
<td>Atropine</td>
</tr>
<tr>
<td>Atria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>8.0 ± 0.3</td>
<td>63</td>
<td>8.4 ± 0.2</td>
<td>8</td>
</tr>
<tr>
<td>Right</td>
<td>8.5 ± 0.4</td>
<td>55</td>
<td>8.5 ± 0.2</td>
<td>4</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. Basal beating frequency of the isolated right atria was 400 ± 20 beats/min (n=36); basal contractile force of paced left atria was 178 ± 65 mg (n=37).

The response of mouse atria to stimulation of the $\beta$-adrenergic and the muscarinic receptor system is shown in Table I. The pD$_2$ values are reproducible and comparable to those obtained with other animal species. It should be mentioned, however, that right and left atria responded to stimulation to different extents: right atria displayed a higher sensitivity to isoprenaline, while left atria were more sensitive to metacholine. In both cases, the difference was 0.5 log unit, indicating a shift in concentration by a factor of 3. No differences were observed in the pD$_2$ values obtained in the presence of propranolol and atropine as antagonists of isoprenaline and metacholine, respectively. As concluded from Hill-coefficients, which did not significantly differ from unity, competitive antagonism was seen in both cases. The resulting pA$_2$ values are also shown in Table I.

Effect of Doxorubicin

The effect of DX on the contractile properties of mouse atria was evaluated in two ways, viz. directly, by determining the effect on atrial chronotropy and inotropy, and
indirectly by comparing the maximal β-adrenergic response to isoprenaline before and after a 1 hour incubation with DX.

Fig. 1 - Time-course of the effect of 100 µM DX on the average contractile force developed by mouse paced left atria (n=4). Contractile force at the time of DX addition (indicated by the arrow) is set at 100%.

Fig. 2 - Inotropic and chronotropic effects of DX (10-100 µM) on isolated mouse left (O) and right (■) atria, as determined by measuring contractile force and beating frequency (n≥4). Basal force and frequency, see Table 1.

Fig. 1 displays the contractile force developed by electrically stimulated left atria,
measured at regular time intervals during stabilization and after the addition of 100 μM DX to the organ bath. Via a steep initial decline after about 30 min a plateau is reached at 30% of the basal contraction. The plateau values obtained with DX concentrations ranging from 10-100 μM are plotted in Fig. 2. For left atria, the estimated IC₅₀ (the concentration at which the contractile force is reduced by 50%) is 35 μM. With respect to right atria, IC₅₀ determination is hampered by the cessation of beating at DX concentrations above 50 μM.

Fig. 3 - Mean isoprenaline dose response curves obtained with isolated mouse left (A) and right (B) atria, before (□) and after (■) 1 hour incubation with DX (still present in the organ bath). [DX] is 50 μM for right (n=4) and 100 μM for left atria (n=3).
Isoprenaline dose response curves were constructed before and after 60 min incubation with DX (DX still present in the organ bath). The results are graphically presented in Fig. 3. Comparison of the average curves revealed a decrease in $\Delta E_{\text{max}}$ following incubation with DX. For left atria (Fig. 3A), this was accompanied by a shift of the curve to the left (pD$_2$ increases from 7.7 to 8.3). With right atria, no shift was observed (Fig. 3B). To exclude any time effect, a time-matched control experiment (n=2) was performed, in which only the vehicle (0.9% NaCl) instead of DX was added to the organ bath. In this case no effect on the isoprenaline dose response curve was seen. Resulting pD$_2$ and $\Delta E_{\text{max}}$ values are grouped in Table II. When the decrease in $\Delta E_{\text{max}}$ was compared to the decrease in basal contraction (Fig. 2), i.e. the indirect vs. the direct effect of DX, the latter was even more pronounced. Therefore, other (lower) DX concentrations were not investigated in combination with isoprenaline.

**Table II** - The effect of DX on the β-adrenergic response of isolated mouse atria to isoprenaline

<table>
<thead>
<tr>
<th>Atrium</th>
<th>n</th>
<th>pD$_2$ (- DX)</th>
<th>pD$_2$ (+ DX*)</th>
<th>$\Delta E_{\text{max}}$ (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>3</td>
<td>7.7 ± 0.2</td>
<td>8.3 ± 0.1</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>Right</td>
<td>4</td>
<td>8.6 ± 0.3</td>
<td>8.3 ± 0.3</td>
<td>65 ± 4</td>
</tr>
</tbody>
</table>

Values are mean ± S.D.

*) With left atria [DX] = 100 μM, with right atria [DX] = 50 μM.

**Effect of Oxygen Radicals**

In order to get more insight into the response of mouse atria to radical inducing agents other than DX, experiments were performed with xanthine/xanthine oxidase (X/XO) to monitor the effect of reactive oxygen species (O$_2^{-}$, H$_2$O$_2$ and ·OH) on contractile properties. When mouse paced left atria (n=8) were incubated with X/XO, a positive inotropic effect was seen, followed by a decline down to basal values (n=4, Fig. 4B) or a cessation of beating (n=4, Fig. 4A). Concomitantly, in all cases a rising baseline was
observed, indicating an increased resting tension.

Fig. 4 - Representative contractions of electrically stimulated (4 Hz) mouse (A,B) and rat (C) left atrium after addition of xanthine oxidase (10 U/l) at t=0 min (indicated by the arrow on the left) to the organ bath (containing Krebs buffer + 2 mM xanthine). After 60 min the incubation medium was replaced by fresh Krebs buffer (right arrow). Typical experiments are shown in which both A and B represent 4 mouse atria and C represents 4 rat atria.

When the incubation medium was replaced by fresh Krebs buffer after 1 hour, none of the 8 atria responded to stimulation anymore (Fig. 4A,B). Rat left atria tested under the same conditions, showed a small positive inotropic effect, which remained present after buffer exchange (Fig. 4C).

All 8 mouse atria that had stopped contracting, resumed beating after 1-2 subsequent washings. After X/XO treatment with subsequent washing, isoprenaline dose response curves were constructed. The resulting curve was shifted to the right as compared to the one obtained before X/XO addition, expressed in a pD₂ shift from 8.1 ± 0.1 to 7.2 ± 0.2 (n=6). As to the maximal isoprenaline response, a clear depression from 100 to 44 ± 12% was observed. In Fig. 5 the mean percentages of basal contraction for the 4 mouse left atria of Fig. 4B and the 4 rat left atria are plotted versus time. Within a few minutes after washing
(at 60 min), the mouse atria stopped contracting, thus showing a behaviour clearly different from the rat atria.

Fig. 5 - Time course of the effect of xanthine/xanthine oxidase (2 mM/10 U/l) on the contractile force developed by mouse (Q, n=4) and rat (■, n=4) left atria. Values are means ± S.D. of the measured contraction as compared to initial values.

**DISCUSSION**

In literature, very few data are available on the use of isolated mouse heart muscle as an *in vitro* testing model (Penn, 1970, Barovsky and Gross, 1981, Borda *et al.*, 1985, Musgrave *et al.*, 1987, Wong, 1987). Our decision to use isolated heart muscle from mice instead of the well-documented rats or guinea-pigs, as motivated in the "Introduction", compelled us to investigate this model thoroughly with respect to receptor responses. Initially, parallel experiments were performed with right ventricle (strips as well as the whole ventricle), but preparation difficulties negatively influenced the reproducibility. Therefore, only right and left atria were investigated further. Dose response curves obtained were highly reproducible and the calculated pD$_2$ and pA$_2$ values are in agreement with literature data obtained with other organ preparations (Bowman and Rand, 1980).
The effect of DX on the isoprenaline $\Delta E_{\text{max}}$ was paralleled by a decrease in basal contractile response (with 100 $\mu$M DX left atrium showed decreases of 56% and 70%, respectively), indicating that DX does not exert its inhibitory effect via disturbance of the receptor integrity, but more directly affects the functionality of the muscle cells. Parallel experiments with rat left atria, performed under identical conditions as with mouse atria, showed values of $61 \pm 10\%$ and $56 \pm 9\%$ for the decrease in $\Delta E_{\text{max}}$ and basal contraction, respectively. These observations seem in contrast with data from Politi et al. (1985) obtained with guinea pig atria, who suggested a competitive inhibition of chronotropic and inotropic response to noradrenaline up to 100 $\mu$M DX. However, in these experiments atria were allowed to incubate with DX for only 30 min before noradrenaline was added. When the incubation time of right atria was 1 hour, 100 $\mu$M DX decreased noradrenaline-induced $\Delta E_{\text{max}}$ by 32 % and the basal rate by 19% (Politi et al., 1985). Höflling and Bolte (1981) measured the direct effect of DX in papillary muscles of guinea pigs and rats, and found a decrease of 18% and 52%, respectively, at [DX] = 170 $\mu$M. In another report, Höflling et al. (1982) mentioned a lower $\Delta E_{\text{max}}$ upon dobutamine (a positive inotropic agent) stimulation of papillary muscle from DX pretreated rats (58% vs. 96% of resting tension). No difference in basal contractile response of papillary muscle from untreated and DX-pretreated rats was observed. In comparing toxicities of DX and its major metabolite doxorubicinol, Olson et al. (1988) focussed on contractility of rabbit papillary muscle. In their model, using the rate of force development ($dS/dt$) as contractility index, no effect of DX was seen up to 350 $\mu$M, although this parameter seems more sensitive than measuring the force $S$.

The influence of DX on the positive chronotropic action of isoprenaline was studied in rat right atria by Rasmussen et al. (1989). A decrease in atrial rate of less than 10% was found (100 $\mu$M DX), while $\Delta E_{\text{max}}$ was unaffected and the isoprenaline dose response curve shifted to the right, implicating competitive antagonism. Combination of these data with our own results obtained with rat and mice atria strongly suggests the higher sensitivity of
mouse atria to DX. A possible explanation might be found in a higher metabolic rate generally present in mice. Thus, a possible effect of a xenobiotic like DX on the cellular energy pools might become critical at an earlier timepoint.

The experiments with X/XO indicate a much more pronounced sensitivity of mouse atrium for in situ generation of superoxide anion radicals as seen with rat heart muscle. This is not only reflected by the direct effect of oxygen radicals, but also by the irreversibly decreased pD₂ of isoprenaline as a result of incubating with X/XO during 1 hour. The latter observation might be explained by a decrease in receptor density. The substantial depression of ΔEₘₐₓ implies that either the stimulus transfer and muscle contraction process is inhibited at some stage, or the amount of available receptor molecules (the "receptor reserve" (Kenakin, 1987)) becomes inadequate.

The slow initial increase in contractile force and in some cases beating frequency, might be caused by a release of endogenous catecholamines from the granulae in the nerve endings. Similarly, when rats were injected with the neurotoxin 6-hydroxydopamine, which is also capable to form oxygen radicals, an 80% depletion of the noradrenaline content of rat brain tissue was observed within one hour (Feenstra et al., 1990). This might also explain the sudden decrease in contractility (both frequency and tension) upon replacement of the X/XO containing Krebs buffer by fresh buffer, since the catecholamines that are washed out of the tissue are then removed from the organ bath. If we compare our findings, especially those with rat atria, with the results obtained by Gupta and Singal (1987), who measured the contractile force of perfused rat hearts under essentially the same conditions, their much more pronounced effect of X/XO can not easily be explained. A possible reason might be the relatively high stimulating frequency (7 Hz) they used in their experiments. Also, since heart perfusion instead of incubation was performed, their observed decrease in contractile force might predominantly be caused by an effect on the coronary blood vessels.

Having observed the rightward shift of the isoprenaline dose response curve,
interpretation of the shift to the left seen (with left atria) after DX incubation becomes rather complicated. It seems clear that if reactive oxygen species play a role they can not be the sole cause of DX induced cardiotoxicity. As already mentioned by Samokyszyn et al. (1988) and recently by Minotti (1989), semiquinone free radicals formed upon one-electron oxidation of DX are more effective than $O_2^-$ in releasing $Fe^{3+}$ from the iron storage protein ferritin, thereby inducing major cellular disturbances. When cellular $Ca^{2+}$-homeostasis is affected as well, contractile properties will be greatly influenced. Höfling and Bolte (1981) found a decreased effect of DX on the contractile force developed by rat papillary muscle with a higher $[Ca^{2+}]$ in the organ bath (5 mM instead of the usual 2.5 mM). This is probably (partly) due to a positive inotropic effect of $Ca^{2+}$ itself.

Conclusively, isolated mouse atria respond to incubation with DX in the same manner as rat atria, but display a higher sensitivity. The negative inotropic effect of DX on paced left atria is paralleled by a negative chronotropic effect on spontaneously beating right atria, but the latter parameter is prone to larger standard deviations. Especially left atria may thus serve as a useful in vitro model to measure the acute cardiotoxic effects of various anthracyclines. Because mice do not develop an anthracycline induced nephrotic syndrome, experiments with atria from mice pretreated with DX can be included in further studies.

REFERENCES


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