Human Carbonyl Reductase Overexpression in the Heart Advances the Development of Doxorubicin-induced Cardiotoxicity in Transgenic Mice[1]

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ABSTRACT

Doxorubicinol (dxol) is the major metabolite formed in the hearts of cancer patients being treated with the widely used chemotherapeutic agent, doxorubicin (dox). The well-documented cardiomyopathy associated with dox treatment has been studied *in vitro* and *ex vivo* providing evidence that the C-13 hydroxy metabolite, dxol, might play a key role in the development of dox-induced cardiotoxicity. In this report, we have developed transgenic mice with heart-specific expression of human carbonyl reductase (HCBR), an enzyme that metabolizes dox to dxol. Dox was rapidly converted to dxol in the hearts of the transgenic expressers, which led to advanced development of both acute and chronic cardiotoxicity. Acute cardiotoxicity was evident by a 60% increase in serum creatine kinase activity and a 5-fold increase in cardiac damage measured by electron microscopy. Myofibril degeneration was the major damage observed in acute dox toxicity. Electrocardiograph telemetry, survival data, and electron microscopy were monitored during chronic dox-induced cardiotoxicity. HCBR expressers developed cardiotoxicity 6–7 weeks before the nonexpressers. The HCBR expressers survived for 5 weeks compared with 12 weeks for the controls. Electrocardiograph profiles and necropsies showed the cause of death to be the development of cardiomyopathies leading to congestive heart failure. Levels of dxol were four times higher in the HCBR expresser hearts than in the nonexpressers. Electron microscopy data showed swelling and major structural damage of the mitochondria in the HCBR expressers. These data demonstrate that the C-13 hydroxy metabolite of dox advances the development of dox-induced cardiotoxicity in an *in vivo* system and suggest that heart carbonyl reductase activity may contribute to dox-induced cardiotoxicity in humans.

INTRODUCTION

Anthracycline chemotherapy that uses dox[3] is limited by the onset of a dosage- and time-dependent cardiotoxicity. Both acute and chronic cardiomyopathies leading to congestive heart failure can develop. However, the development of chronic cardiotoxicity is much more clinically important (1). Chronic cardiotoxicity can develop many years after treatment. Children and younger adults treated with anthracyclines are exposed to a lifetime risk of developing serious cardiomyopathy (2). Because cancer patients are not usually monitored for more than 5–7 years, the number of these patients developing late-onset cardiomyopathies can be expected to increase substantially in the future (2, 3).

Previous reports on *in vitro* studies have implicated the C-13 hydroxy metabolite, dxol, as a major component in the development

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[3] The abbreviations used are: dox, doxorubicin; dxol, doxorubicinol; HCBR, human CBR; CBR, carbonyl reductase; αMHC, α myosin heavy chain; ECG, electrocardiograph/electrocardiogram; EM, electron microscopy; HPCL, high-performance liquid chromatography.

of cardiotoxicity (4, 5). These data contrast with tumor cell killing in which the C-13 hydroxy metabolite is less toxic against tumor cells examined in tissue culture systems (6–9). Several ubiquitous enzymes such as CBR(s) and aldehyde reductase(s) metabolize dox to dxol, implicating these enzymes in the development of cardiotoxicity (10, 11). HCBR is a cytosolic enzyme that reduces dox to dxol in the presence of the cofactor, NADPH (11, 12). In this report, we developed a transgenic mouse model using a mouse heart-specific promoter (13) to express HCBR in the hearts of transgenic mice and measured morphological and ECG changes during the development of acute and chronic dox-induced cardiotoxicity.

MATERIALS AND METHODS

Heart-specific Vector. The mouse heart-specific expression vector, pαMHC, was kindly supplied by Dr. Jeffrey Robbins (Children's Hospital Medical Center, Cincinnati, OH; Ref. 13). The coding region of the HCBR cDNA was amplified from the plasmid pHCR 311 (14) using the PCR and synthetic primers containing a *Sal*I linker at the 5′ ends. The modified HCBR cDNA was cloned into the *Sal*I site of pαMHC. DNA sequencing on an ABI Model 373 fluorescent DNA sequencer verified the sequence and orientation. The expression vector containing the HCBR cDNA was digested with *Not*I to linearize the DNA. The fragment containing the heart-specific promoter and HCBR cDNA was purified on a 10–40% linear sucrose gradient, was concentrated, and was stored in TE buffer [10 mM Tris (pH 7.50) and 0.1 mM EDTA] at −20°C. The final concentration was adjusted to 2.5 μg/ml.

Transgenic Mice. All of the animal procedures were approved by the American Association of Accreditation of Laboratory Animal Care and were certified by an institutional animal care committee. B6C3F1 mice were obtained from Taconic (Germantown, N.Y.) and were used to produce the transgenic lines. Standard procedures were used to produce transgenic mice in the transgenic core facility at the City of Hope (Duarte, CA; Ref. 15). Positive HCBR founder transgenic mice were identified by in-gel Southern analyses on genomic DNA isolated from 0.3-cm tail biopsy and/or by PCR analyses (16) using primers to the αMHC promoter region and to the HCBR coding sequence. The HCBR cDNA was used as a probe for Southern and Northern analyses.

Enzyme Assays. Ten to 20 mg of tissue were homogenized in 1 ml of 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA and were centrifuged at $16,000 \times g$ for 10 min at 4°C. The supernatant was used for enzyme assays. Protein concentration was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA) using BSA as the protein standard.

Creatine Kinase Activity. Animals were sedated using approved veterinary procedures. The thoracic cavity was opened and blood was drawn from the left ventricle. Blood samples were kept on ice until all of the samples were collected, then were centrifuged at $16,000 \times g$ for 5 min at 4°C. Serum was collected and stored at −20°C until assayed. Creatine kinase was determined using a CK 10 Kit (Sigma, St. Louis, MO).

CBR Activity. CBR activity was determined from tissue extracts by measuring the reduction of cytochrome *c* at 550 nm at 25°C using a Uvikon 930 spectrophotometer (Kontron Instruments/Research Instruments International, San Diego, CA; Ref. 17). Extract-containing enzyme was added to buffer consisting of 0.1 M potassium phosphate (pH 6), 0.2 mM menadione, 1.5 mM cytochrome *c*, and 0.2 mM NADPH. The CBR inhibitor, rutin (20 μM), was added to the reaction mixture 5 min prior to starting the reaction with NADPH. The amount of enzyme activity attributed to CRB is reported as rutin-inhibitable activity.
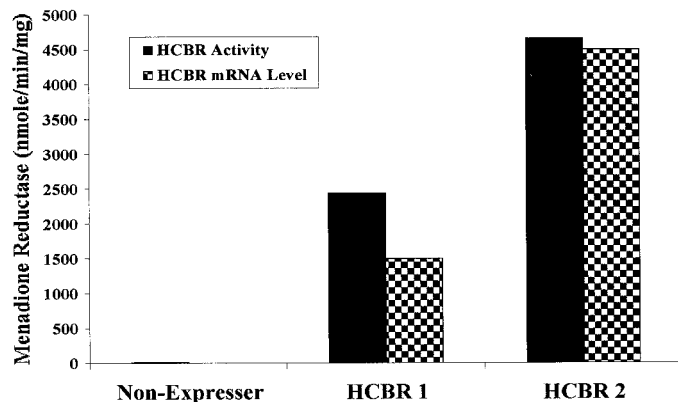
Fig. 1. CBR heart activity and HCBR mRNA levels in expresser lines HCBR1 and HCBR2.

dxol Formation. Dxol was measured by incubating cell extracts (10–100 μg) at 37°C in 0.1 M potassium phosphate (pH 6), 0.65 mM dox, and 0.5 mM NADPH (17, 18). The reaction was stopped and extracted with 0.6 ml of chloroform:isopropanol (1:1). After centrifugation, 0.5 ml of the organic layer was transferred to a new tube, and the sample was dried in a Speed-Vac concentrator. The dried sample was dissolved in HPLC mobile phase buffer and analyzed by HPLC using a phenyl column (19).

EM. Mice, 8–12 weeks old, were injected i.p. once with 15 mg/kg dox (Sigma) for acute dox treatment and i.p. with 5 mg/kg/wk for chronic dox treatment. After 96 h (acute treatment) or 4 weeks (chronic treatment) the animals were anesthetized, the hearts were perfused with NaCl solution (0.8%) and then fixative (4% paraformaldehyde/2% gluteraldehyde in 0.1 M cacodylate buffer) via the right atrium. Portions of the left ventricle were cut into 1-mm^2 cubes and were washed three times in cacodylate buffer. Samples were post-fixed at 4°C for 1 h in 1% OsO$_4$, washed 3 × in buffer, dehydrated in alcohols, and embedded in Eponate. One-μm sections were stained in 5% uranyl acetate, followed with Sato's Lead stain. The samples were viewed and photographed with a Philips CM 10 transmission electron microscope. All of the samples were coded and scored in a blind fashion. One representative picture was taken for every 10–15 fields, and five pictures were taken for each heart.

HCBR Polyclonal Antibody. Polyclonal HCBR antibody was produced in rabbits using purified HCBR protein isolated from *Escherichia coli* expressing a HCBR cDNA.

Immunohistochemical Staining. Fresh tissue was excised from mice, fixed in 10% buffered formalin overnight, and embedded in paraffin (20). Polyclonal HCBR antibody was used with dilutions of 1:2000–1:5000. The antibody-antigen complex was detected by a modified ABC method (Vantana Medical Systems Inc.) according to the manufacturer's directions with the chromagen, 3′3-diaminobenzidine.

ECG Telemetry. ECGs were measured telemetrically in conscious, freely moving mice by monitoring signals from surgically implanted transmitters (model ETA-F20; Data Sciences, St. Paul, MN). Six implantable telemetric biopotential transmitters, receivers, and analogue ECG adapters were used to collect data into a MacLab charts program. Data were collected three times a week using a MacLab/4 MKIII analogue digital instrument and a Macintosh G3 series PowerBook computer.

RESULTS

Heart-specific CBR Activity. CBR is a ubiquitous NADPH-dependent, cytoplasmic enzyme that displays both aldo-keto reductase and quinone reductase activities. Acting as an aldo-keto reductase, it reduces the C-13 methyl ketone side chain of dox, forming the major metabolite dxol, and changes the pharmacological properties of the drug (11, 17, 21, 22). CBR does not reduce the quinone group of dox, but it does metabolize the quinone, menadione (23). Both activities are inhibited by rutin.

Three transgenic founders (HCBR1, HCBR2, HCBR3) expressing heart-specific HCBR were produced and were used to generate heterozygote and homozygote transgenic lines. HCBR1 and HCBR2 heart extracts displayed heart-specific menadione reductase activities of 2500 nmol/min/mg protein and 4700 nmol/min/mg protein, respectively, corresponding to a 250- and 470-fold increase, respectively, over nonexpresser controls (Fig. 1). A corresponding increase in HCBR mRNA was also observed in the HCBR expressers (Fig. 1). Table 1 shows organ-specific CBR activity in HCBR3. There was a 700-fold increase in heart-specific CBR activity in transgenic expresser line HCBR3 and low activity in the other organs. Transgenic lines HCBR1 and HCBR2 showed similar CBR activities in other organs (data not shown). No significant increase in CBR activity was observed in any other organs other than the heart. Heterozygotes with heart-specific HCBR activity up to 7,000 nmol/min/mg appeared normal. Homozygotes were produced with heart CBR activities from 5,000 to 10,000 nmol/min/mg. Transgenics expressing HCBR >9,000 nmol/min/mg developed severe cardiomyopathies. Hearts were enlarged, and the mice did not reproduce. Heterozygotes with HCBR expression levels ~5,000 nmol/min/mg (HCBR2) were used in experiments.

Immunohistochemical Staining. Mouse hearts were prepared as described in "Materials and Methods. Fig. 2*A* shows immunohistochemical staining of HCBR in the hearts of HCBR2 expressers. HCBR protein expression was myocyte-specific and was expressed abundantly and uniformly in all of the myocytes. Epithelial cells were not stained (Fig. 2*A*, *arrows*). The myocyte staining pattern was representative for all of the HCBR expressers including the homozygotes. Fig. 2*B* shows that the nonexpresser hearts stained negative for HCBR protein.

Intercellular dox Metabolism. The percentage of dxol formation over 48 h was determined in several organs of HCBR expressers and wild-type mice (*n* = 4 for each group) after a single injection of dox (10 mg/kg, i.p.). Tissue was collected and profused, and drug was extracted for HPLC analyses at the indicated times (Fig. 3; Table 2). In the heart, HCBR expressers converted 82% of dox to dxol within 0.5 h after injection compared with 5% conversion in the nonexpressers (Fig. 3).

The percentage of dxol in the hearts of nonexpresser mice was equal to the expressers after 24 h. Presumably, the longer time for conversion in the hearts of nonexpressers was attributable to the low endogenous CBR activity. Table 2 shows the percentage of dxol in liver, lung, spleen, and quadricep. A maximum of 22% dxol was observed in the liver after 12–24 h. Kidney dxol percentages were also measured. However, we were not able to obtain accurate measurements because of an interfering peak. The metabolism of dox to dxol in the kidney appeared similar to that in the liver. The percentage of dxol in other organs was low and similar in the HCBR expressers and nonexpressers (Table 2).

Table 1 *Tissue-specific CBR activity in transgenic mice*

Tissue	Nonexpresser[a]	HCBR expresser[a]
Heart	9	7742
Lung	22	56
Liver	8	14
Kidney	0	34
Spleen	3	0
Quadricep	0	48
Brain	0	4
Ovary	nd[b]	6
Testes	0	0

[a] Rutin-inhibitable menadione reductase activity (nmol/min/mg).
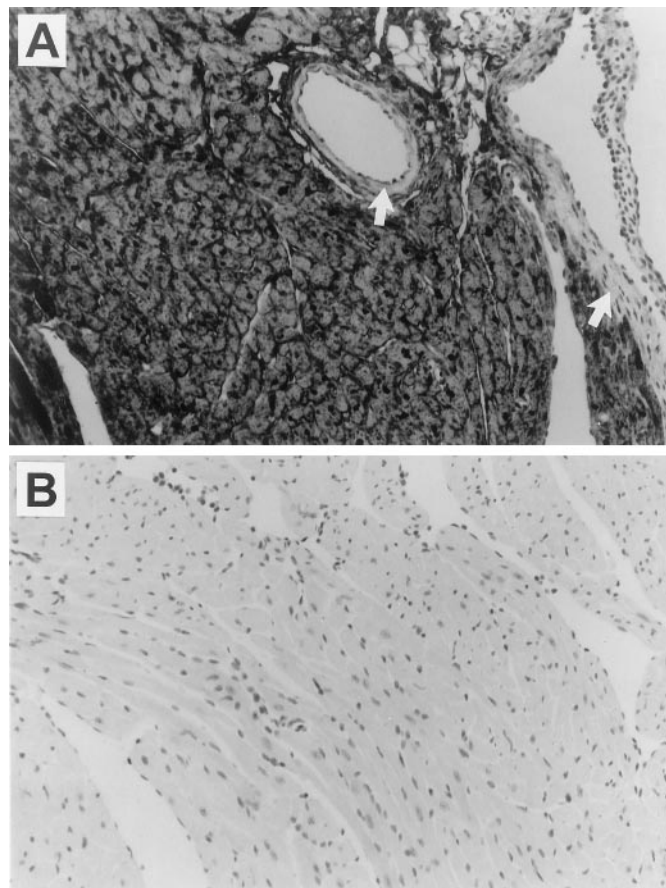[b] nd, not determined.

Fig. 2. Immunohistochemical staining of paraffin-imbedded heart tissue with rabbit anti-HCBR polyclonal antibody. *A*, HCBR3 expresser; *B*, nonexpresser.
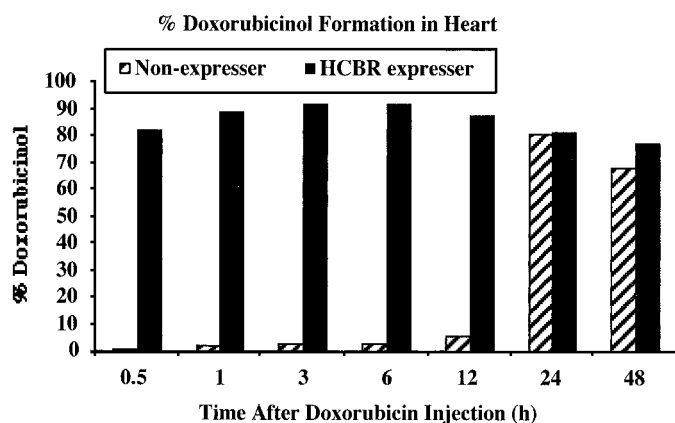

Fig. 3. Percentage of dxol formation in hearts of HCBR expressers and nonexpressers over 48 h.

Total dox + dxol Levels in the Hearts of dox-treated Mice. The amount of heart dox + dxol was determined for the mice shown in Fig. 3. Drug levels were averaged for hours 0.5–12, which included 20 hearts each for the HCBR expressers and nonexpressers. The HCBR expressers contained an average of 2124 ng/g heart tissue compared with 517 ng/g heart tissue for the nonexpressers. These results show that dxol concentrations rapidly increased in the HCBR expresser hearts and were four times higher than in the nonexpressers over the 12-h time period. Although the total drug concentration was four times higher in the HCBR expressers over 12 h, it decreased 5-fold by 48 h to a level similar to that of the nonexpressers.

Effect of Acute dox Treatment on Serum Creatine Kinase Levels. HCBR expresser and nonexpresser mice ($n = 5$) were given a single injection of dox (10 mg/kg, i.p.). Serum was collected at 48 h and analyzed for serum creatine kinase activity. HCBR expressers displayed a 60% higher level of serum creatine kinase activity compared with the nonexpressers (Table 3). These data suggest that HCBR expressers are more sensitive to heart or muscle damage than nonexpressers.

EM Studies in dox-induced Acute Cardiotoxicity. HCBR expressers and nonexpressers were divided into two groups of five each. HCBR expressers and nonexpressers ($n = 5$) were given a single injection of dox (15 mg/kg, i.p.). Controls (minus dox) were injected with NaCl solution (0.8%). The hearts were collected 96 h later and were prepared for EM analyses of the left ventricle as described in "Materials and Methods." Samples were coded, and photographs were taken and scored independently by two people (B. G., G. E.) using a scale of 0–3 with 0.5 intervals. The results of scoring more than 100 photos representing 1000–1500 fields are shown in Fig. 4. HCBR expressers displayed increased cardiac damage, which was five times higher than the cardiac damage found in nonexpressers.

Electron micrographs of representative dox-induced cardiac damage are shown in Fig. 5. Fig. 5*A* shows a dox-treated HCBR expresser exhibiting extensive myofibril degeneration. A HCBR-untreated control is shown in Fig. 5*B*. Fig. 5*C* shows increased vacuolization with some myofibril degeneration in the dox-treated nonexpresser. An untreated nonexpresser control is shown in Fig. 5*D*.

Chronic dox-induced Cardiotoxicity Measured by ECG Telemetry in Freely Moving Mice. HCBR expressers and nonexpressers ($n = 3$) were surgically implanted with ECG biopotential transmitters.

Table 2 *Percentages of dxol in tissue after dox injection*[a]

Time (h)	Quadricep		Spleen		Lung		Liver	
	HCBR	WT[b]	HCBR	WT	HCBR	WT	HCBR	WT
0.5	0	0	0.25	0.5	0	0	1.7	6.4
1	0	0	0.25	0.4	0.5	0.2	3.9	8.0
3	1.7	0	0.4	1.2	0	0.7	7.9	9.7
6	3.4	2.5	2.5	0.5	1.7	0.6	9.1	13.6
12	3.2	2.2	0.5	0.9	3.3	1.4	9.0	22.0
24	4.5	4.0	0.8	0.6	2.3	5.0	7.9	21.0
48	4.2	3.0	0.7	1.1	8.0	3.0	10.0	7.9

[a] dox (10 mg/kg) i.p.; average four hearts per point.
[b] WT, wild type.

Table 3 *Serum creatine kinase activity 48 h after dox (10 mg/kg, i.p.)*

Sample	CPK[a] (units/ml)[b]
HCBR expressers	540 ± 87
Nonexpressers	334 ± 179

[a] CPK, creatine phosphokinase.
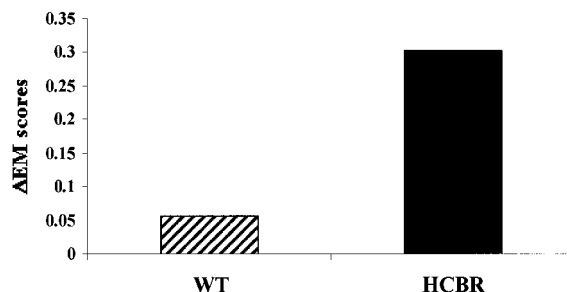[b] Average of five mice/sample ± SD ($P < 0.025$).

Fig. 4. Dox-induced acute cardiac damage after 96 h (15 mg/kg, i.p.). Shows difference from non-dox-treated controls. *WT*, wild type.
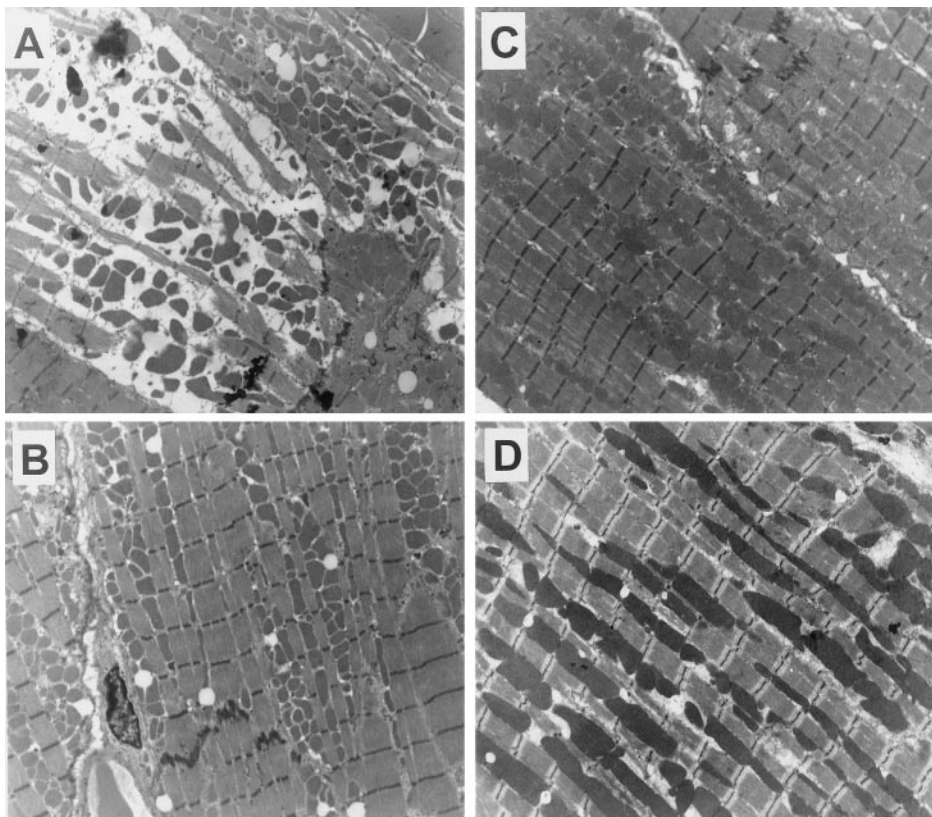
Fig. 5. Electron micrographs of dox-injected (15 mg/kg, i.p.) and NaCl- (0.8%) injected HCBR expressers and nonexpressers after 96 h. Left ventricle samples. *A*, HCBR + dox; *B*, HCBR + NaCl (0.8%); *C*, nonexpresser + dox; *D*, nonexpresser + NaCl (0.8%).

After 2 weeks of recovery, a chronic schedule of dox treatment (5 mg/kg, i.p.) was given once per week. ECG telemetry readings were taken three times per week. Fig. 6 shows the survival data for the HCBR expressers and the nonexpressers. The HCBR expressers died within 5 weeks, whereas the nonexpressers survived for 12 weeks.

ECG Telemetry Profiles. Measurement of ECG telemetry in freely moving mice can detect developing dox-induced cardiotoxicity (24). Fig. 7*A* shows representative ECG profiles collected each week during chronic dox treatment of a HCBR expresser. The largest peak represents depolarization of the ventricular myocardium resulting in heart contraction. Immediately following the main peak is the T wave (arrow), which represents repolarization of the heart. A broadening and decreasing T wave correlates with the development of dox-induced cardiotoxicity in mice (24, 25). Fig. 7*A* shows that the T wave started to broaden by week 2 and was almost undetectable by week 3. In addition to the change in the T wave, the main peak dropped to 50% of its initial value. The main peaks for the other two HCBR expressers

dropped by 80%. These data indicate the development of severe cardiomyopathy.

The HCBR expressers gradually lost weight during dox treatment and then gained 2–5 grams a few days before death. Labored breathing was also observed with the weight gain. Necropsy examination showed fluid in the thoracic cavity along with discolored atria. The data show that the mice died of congestive heart failure.

Fig. 7*B* shows ECG profiles in a nonexpresser mouse at 3-week intervals. The data show that the T peak began to broaden by week 6–8 and continued to broaden with the development of cardiotoxicity. The nonexpresser mice survived for 12 weeks, 2.5 times longer than the HCBR expressers.

There were differences in the development of chronic cardiotoxicity between the HCBR expressers and nonexpressers. The main ECG peak in the control mice did not decrease as it did in the HCBR mice. The onset of cardiotoxicity was slower in the controls, and the mice showed a steady decline in their health as observed by their inactivity and coat texture. Necropsy examination showed enlarged hearts. There was not a build-up of fluid in the thoracic cavity before death as was observed in the HCBR expressers.

Mitochondrial Damage in Chronically Treated Mice. HCBR expressers and nonexpressers ($n = 4$) were treated chronically with dox (5 mg/kg, i.p.) for 4 weeks. Hearts were processed for EM analysis as described earlier. The dox-treated HCBR expressers displayed more extensive mitochondrial damage (Fig. 8*A*, *arrow*) compared with the dox-treated nonexpressers (Fig. 8*B*). These data were in contrast to the data from acutely treated animals in which the major type of damage was myofibril degeneration as shown in Fig. 5*A*.
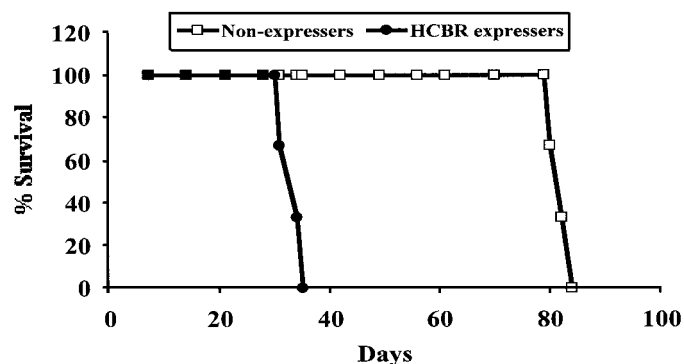
DISCUSSION

Dox was discovered over 30 years ago and is still one of the most widely used and effective anticancer drugs for the treatment of many

Fig. 6. Survival data of chronic dox-treated (5 mg/kg/wk, i.p.) HCBR expressers and nonexpressers.
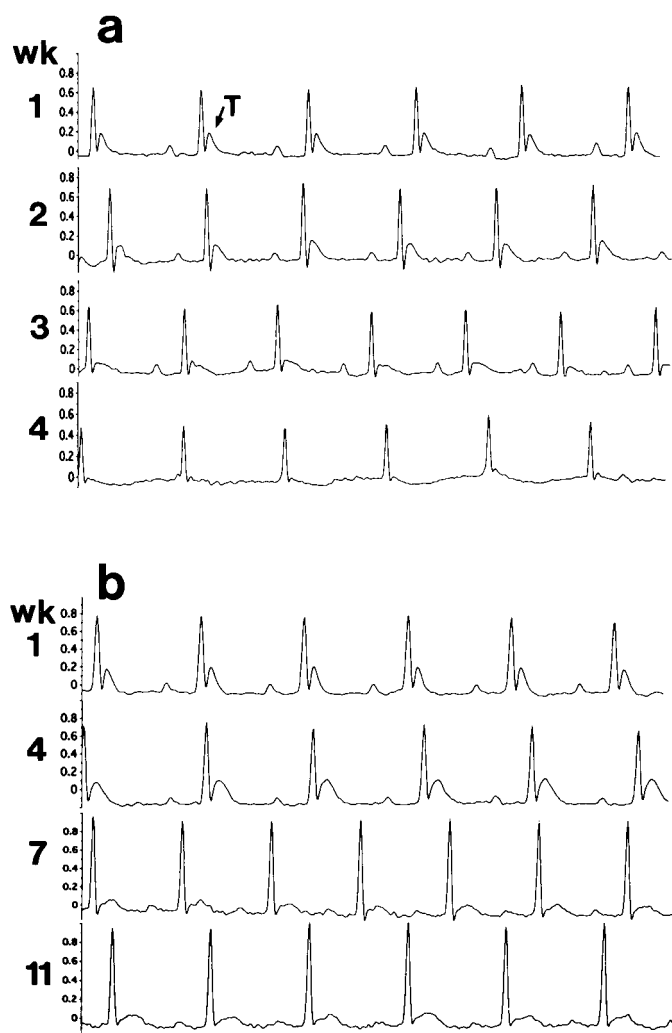
Fig. 7. ECG profiles of chronically treated HCBR expressers and nonexpressers. Same mice as shown in Fig. 7. *a*, HCBR expressers; *b*, nonexpresser.

Dxol levels preferentially build up in the heart (30, 33), which suggests increased involvement of the C-13 hydroxy metabolite. Dxol build-up could be caused by lower transport through heart membranes, which is attributable to the more polar dxol molecule.

The HCBR transgenic mouse model allows one to exclude the potential membrane transport problems of using dxol, which may lead to lower drug concentrations in the heart (34). Both acute and chronic dox schedules led to enhanced cardiotoxicity in the HCBR expressers; dxol levels increased rapidly and remained four times higher than the dox levels in the nonexpressers over 12 h. Total drug levels dropped 5-fold by 48 h and were similar in the expressers and the nonexpressers.

HCBR expressers showed five times more acute dox-induced cardiac damage as judged by EM along with a 60% increase in serum creatine kinase activity above that in nonexpressers. A single acute dose of dox caused heart damage 96 h after injection, which was mostly represented by myofibril degeneration. Chronic dox treatment (5 mg/kg/wk) showed mitochondrial swelling and degeneration after 4 weeks. The differences between acute and chronic cardiotoxicity could be attributable to different intercellular targets of dox and dxol or else to the higher concentrations of dxol. Transcriptional factors, heart-specific proteins, energy metabolism, and mitochondrial enzymes are sensitive to low concentrations of dox (35–38). Dxol levels
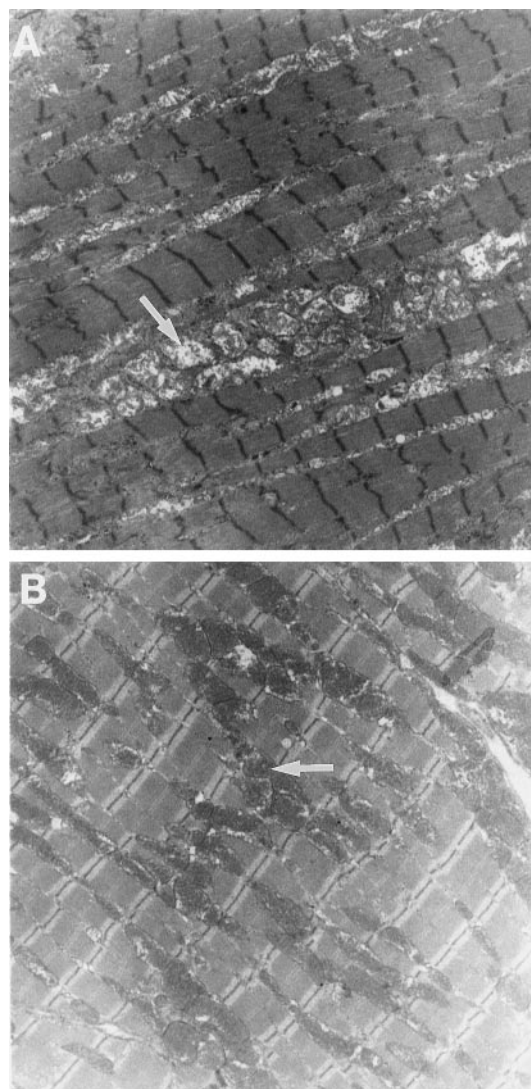
Fig. 8. Chronic 4-week dox treatment of HCBR expressers (*A*) and nonexpressers (*B*). Samples from left ventricle.

tumors. Cardiotoxicity is the main limiting side effect and confers a lifetime risk to patients. Previous reports have suggested that oxygen free radicals and drug metabolites play a major role in the development of cardiotoxicity (2, 4, 26). In this report, we describe an *in vivo* heart-specific transgenic mouse model capable of increasing the intracellular dox metabolite, dxol, with a corresponding increase in acute and chronic cardiotoxicity.

Dxol is the major metabolite of dox found in tissue (27). It is formed by an enzymatic two-electron reduction of the C-13 keto group by cytoplasmic, NADPH-dependent aldo-keto reductases and short-chain dehydrogenases (10, 17, 21, 22, 28, 29).

Dxol seems to be more cardiotoxic than the parent drug. It inhibits many of the ion channel pumps. The calcium pump of the sarcoplasmic reticulum, the Na/Ca pump of the sarcolemma, and the proton pump of the mitochondria are inhibited by dxol but not by similar concentrations of dox (5, 9). There is also evidence that dox and dxol may act together at different sites to generate cardiotoxicity (30). Lowering the level of dxol *in vivo* by inhibiting aldo-keto reductases with phenobarbital leads to less toxicity as measured by decreased creatine kinase levels (31). Dxol also interacts with *cis*-aconitate and inhibits aconitase activity. This interaction causes the delocalization of iron from the active center of aconitase with reoxidation of dxol to dox (32). This mechanism irreversibly inactivates aconitase and interferes with the aconitase-iron regulatory protein-1 function.

in the hearts of the HCBR expressers were four times the levels of dox in the nonexpressers. The effect of this difference in metabolite concentration on mechanisms involved in cardiotoxicity is not known, but it appears to significantly advance the development of cardiotoxicity.

Dox-induced free radicals have been implicated in developing cardiotoxicity (39–45). The C-13 hydroxy metabolite of dox can generate free radicals (46, 47). NADH dehydrogenase from heart mitochondria and heart sarcosomes containing NADPH cytochrome P450 reductase reduce dxol, generating superoxide anion. (47). Dxol appears to produce 50% of the free radicals produced by dox (47). The levels of dxol in the expresser hearts were four times the levels of dox in the nonexpresser hearts, providing a potential for a 2-fold increase in free radical formation. Enzymes that protect against the toxic effect of superoxide anion, such as the mitochondrial manganese superoxide dismutase, provide protection against dox-induced cardiotoxicity (48, 49).

Our data show more mitochondrial damage in the dox-treated HCBR expressers. Recent data suggest that several components of the mitochondrial electron transport chain, capable of generating superoxide anion, are involved in dox-induced cardiotoxicity. Dox-mediated inactivation of the NADH-dehydrogenase (complex I) in transgenic mice is protected by overexpression of manganese superoxide (50). In yeast, cytochrome oxidase (complex IV), a source of superoxide anion, is necessary for dox-induced toxicity. Overexpression of mitochondrial manganese superoxide reduces the toxicity (48).

In summary, we developed an *in vivo* transgenic mouse model system and showed that overexpression of HCBR in the heart leads to earlier development of dox-induced cardiotoxicity. The data show that the C-13 hydroxy metabolite of dox played a major role in cardiotoxicity and implicate CBR as a contributor to dox-induced human cardiotoxicity. The actual mechanisms involved were not determined but involve mitochondrial damage and leave open a role for direct drug metabolite interaction and free radical damage or combinations of both.

ACKNOWLEDGMENTS

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