Differential cardioprotective/cardiotoxic effects mediated by β-adrenergic receptor subtypes

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Bernstein, Daniel, Giovanni Fajardo, Mingming Zhao, Takashi Urashima, Jennifer Powers, Gerald Berry, and Brian K. Kobilka. Differential cardioprotective/cardiotoxic effects mediated by β-adrenergic receptor subtypes. Am J Physiol Heart Circ Physiol 289: H2441–H2449, 2005. First published July 22, 2005; doi:10.1152/ajpheart.00005.2005.—Recent data suggest that β-adrenergic receptor subtypes couple differentially to signaling pathways regulating cardiac function vs. cardiac remodeling. To dissect the roles of β1- vs. β2-receptors in the pathogenesis of cardiomyopathy, doxorubicin was administered to β1, β2, and β1/β2 knockout (−/−) and wild-type mice. Expression and activation of MAPKs were measured. Wild-type and β1−/− mice showed no acute cardiovascular effects, whereas β2−/− mice all died within 30 min. The additional deletion of the β1-receptor (β1/β2−/−) totally rescued this toxicity. β2−/− mice developed decreased contractile function, hypotension, QTc prolongation, and ST segment changes and a 20-fold increase in p38 MAPK activity not seen in the other genotypes. The MAPK inhibitor SB-203580 rescued β2−/− mice from this acute toxicity. The enhanced toxicity in β2−/− mice was also recapitulated in wild-type mice with the β2-selective antagonist ICI-118,551, although the effect of the β1-deletion was not recapitulated using the β1-selective antagonist metoprolol or the nonselective β-antagonist propranolol. These data suggest that β2-adrenergic receptors play a cardioprotective role in the pathogenesis of cardiomyopathy, whereas β1-adrenergic receptors mediate at least some of the acute cardiotoxicity of anthracyclines. Differential activation of MAPK isofoms, previously shown in vitro to regulate β2-agonist as well as doxorubicin cardiotoxicity, appears to play a role in mediating the differential effects of these β-adrenergic receptor subtypes in vivo.

Cardiomyopathy; anthracycline; adrenergic receptor; cell signaling; β-blocker; mitogen-activated protein kinase

β-ADRENERGIC RECEPTORS (β-ARs) are members of the superfamily of seven-transmembrane G protein-coupled receptors (30), three subtypes of which (β1, β2, β3) have been identified in the heart (6, 11, 13, 14, 25–27). Classically, β-ARs were regarded primarily as regulators of cardiac function (inotropy, lusitropy, and chronotropy). Recent evidence suggests that β-ARs also play a role as regulators of cardiac remodeling in response to stress. In vitro, stimulation of cardiomyocytes with β-agonists induces apoptosis (36). In vivo, chronic stimulation of the myocardium with β-agonists (29) or conditions associated with chronically increased sympathetic drive (5) leads to the development of dilated cardiomyopathy. Many of the adverse effects of increased sympathetic stimulation are blocked by β-antagonists, both in vitro and in vivo, and β-blocker therapy has become standard in the treatment of patients with dilated cardiomyopathy (4, 8, 12, 34).

There is increasing evidence to support a differential role for β1- vs. β2-AR subtypes in regulating both cardiac function and alterations in cardiac structure. In vitro, the β2-receptor has been shown to functionally couple to both the stimulatory (G1) and inhibitory (Gi) proteins (33) and to play a role in cardiac remodeling through activation of MAPK pathways (10, 22). β1-ARs have been linked to proapoptotic pathways (3, 9, 36), whereas a dual modulation has been suggested for β2-ARs (36). However, previous studies have been performed mostly in vitro; several have utilized cardiomyocytes transfected to express “nonphysiological” levels of signaling molecules of interest; the pharmacological blockers used have only relative specificity for each β-receptor subtype; and studies performed on rat vs. mouse and neonatal vs. adult myocytes have often yielded conflicting results. Confirmation of this dual role for β-AR subtypes has been more difficult in the intact circulation.

The anthracycline group of anticancer agents have been widely utilized as a model of toxic cardiomyopathy in both mice and rats (15, 19, 24, 31). Their cardiovascular effects are mediated by their ability to generate reactive oxygen species (H2O2 and ·OH), resulting in peroxidation of membrane lipids and mitochondrial DNA (18). Previous studies have demonstrated a role for MAPK pathways in this toxicity (35) as well as alterations in β-AR signaling (24).

The purpose of the current study was to determine the role of β1- and β2-AR subtypes in the pathogenesis of dilated cardiomyopathy in vivo. Utilizing β-AR knockout mice and a model of anthracycline toxic cardiomyopathy, we present evidence suggesting that β1-ARs mediate cardiotoxicity, whereas β2-ARs mediate cardioprotection. This subtype-specific toxicity could have significant implications in the design of more subtype-selective β-adrenergic antagonists for the treatment of patients with heart failure.

METHODS

Generation of β-AR Knockout Mice

Homozygous β1-AR and β2-AR knockouts were generated using a positive-negative selection strategy to effect homologous recombination in embryonic stem cells, as previously described (6, 25, 28). Crosses were then carried out between homozygous β1−/− and homozygous β2−/− mice to generate compound heterozygotes, which were in turn intercrossed to generate mice homozygous for the double knockout (β1/β2−/−) (28). Mice were genotyped for both β1-AR and β2-AR disruptions using the polymerase chain reaction. All proce-
dures on animals were approved by the Stanford Administrative Panel on Laboratory Animal Care.

Model of Doxorubicin Cardiotoxicity

Mice were injected with a single dose of 15 mg/kg of doxorubicin (NovaPlus, Bedford, OH) via the dorsal tail vein. Genotypes included β1−/−, β2−/−, β1/β2−/−, and wild-type mice. All mice studied were males and were 3 mo old. β1−/− and β2−/− mice were on a congenic FVB background. Experiments with β1−/− and β2−/− mice were compared with wild-type FVB littermates. β1/β2−/− mice were on a mixed FVB/C57/129/DBA background, as attempts to breed these mice on a congenic background have not been successful. Because of this, all experiments with β1/β2−/− mice were compared with wild-type littermates of the same mixed strain.

Hemodynamic Monitoring

Electrocardiography. Unanesthetized ECG recordings were obtained with an implantable telemetric unit (PhysioTel, Data Sciences, St. Paul, MN) in four wild-type and four β2−/− mice. ECG recordings were also performed during light anesthesia in six additional β2−/− mice performed in conjunction with echocardiograms (see below). Mice were anesthetized with 2% inhaled isoflurane, and an incision was made over the back. Two smaller incisions were made on the opposite sides of the pectoral region for suturing of the leads, which were tunnelled subcutaneously cranially and sutured into the pectoral muscles. The 3.5-g wireless radiofrequency transmitter was inserted and anchored in place. Skin incisions were sutured, and a warming lamp maintained body temperature for recovery.

Animals were allowed to recover for a minimum of 3 days after surgery. ECG signals were recorded from the telemetric unit using a receiver mounted under the cage (DataSciences International), digitized at a sampling rate of 1 kHz and fed into a microcomputer-based data acquisition system (MacLab System, AD Instruments, Milford, MA).

Blood pressure. Mice were anesthetized with inhaled isoflurane (3%) induced in a closed chamber and maintained via nose cone (1.5–2%) throughout surgery. With the mouse supine, a 1-cm midline neck incision was made from just below the mandible to the thoracic inlet. Under a dissecting microscope (Nikon USA, Melville, NY), the left carotid artery was identified and a stretched Intramedic PE-10 polyethylene catheter (Clay Adams, Parsippany, NJ) was advanced to an approximate depth of 1 cm and secured in place with 4-0 surgical silk suture (Ethicon, Somerville, NJ). The catheter was then flushed with heparinized saline (100 U/ml saline), sealed with cyanocrylate glue, tunneled to the back of the neck, and tucked into a subcutaneous pocket. Intravenous ampicillin (100 mg/kg) was administered after skin closure. The mice were then allowed to recover with food and water available ad libitum for 24 h before study. Two wild-type and two β2−/− mice had awake blood pressure measurements performed.

Echocardiography. Six β2−/− mice that had previously undergone implantation of ECG telemetry units were anesthetized with xylazine (250 mg/kg) and had carotid arterial catheters placed. Echocardiographic studies were performed using a Siemens Sequoia system with a 15-MHz linear array transducer (Siemens, Mountain View, CA). M-mode echocardiographic measurements were obtained from the parasternal short axis view, just below the level of the mitral valve. Baseline measurements included left ventricular internal dimension at end-diastole (LVIDd) and left ventricular internal dimension in systole (LVIDs). Left ventricular fractional shortening (%FS) was calculated. Measurements of mean blood pressure, ECG, and echocardiographic parameters were made at baseline and at 1-min intervals after administration of 15 mg/kg doxorubicin via tail vein. Echocardiograms were also performed in three wild-type mice after 15 mg/kg doxorubicin.

MAPK Activation and Inhibition

Expression and activation of the three major MAPK family members, p38, p42/44, and JNK, were quantified using anti-MAPK and anti-phospho-MAPK antibodies specific to each kinase by Western immunoblotting. Heart tissue was harvested and homogenized in lysis buffer (final concentration: 80 mM NaCl, 20 mM HEPES, 0.05% Triton X, 1 mM DTT, 0.5% sodium deoxycholate, 20 mM β-glycerophosphate, 50 mM Na2VO4, 4 μg/ml leupeptin, 1.0 mM EDTA, 10 μg/ml benzamidine, 2 μg/ml aprotinin, and 0.1 mM PMSF) on ice. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. Collected supernatant was assayed to quantify protein concentration. AT2-2 fusion was then loaded on 10% SDS gel and probed with a 1:100 dilution of anti-p38 kinase (Santa Cruz, CA). p38 phosphorylation was visualized by Western blotting. A gel was loaded with heart homogenate samples from each mouse, and samples were separated by 10% SDS-PAGE. The phosphorylated substrate AT2-2 was separated by SDS-PAGE. Blots were probed with anti-phospho-AT2-2 primary antibody and then horseradish peroxidase-conjugated secondary antibody and visualized by autoradiography.

There are two isoforms of p38 (α and β) expressed in the myocardium (32). To determine which isoform was activated in our experiments, we used both p38 (N-20:sc-728 Santa Cruz Biotechnology, Santa Cruz, CA), which is significantly more specific for the α-p38 isoform (although there is some cross-reactivity), and p38β (E-20:sc-6187 Santa Cruz Biotechnology), which is specific for the β-p38 isoform.

Further investigation of the role of p38 activation in the enhanced doxorubicin toxicity in β2−/− mice was achieved by using the p38-selective inhibitor SB-203580 (Calbiochem, La Jolla, CA). These studies were performed using the 50% lethal dose (LD50) of doxorubicin in β2−/− mice, determined by previous experiments to be 8 mg/kg. Sixteen β2−/− mice were treated with 8 mg/kg iv doxorubicin alone and 15 β2−/− mice received 1 mg/kg SB-203580 ip 30 min before administration of 8 mg/kg iv doxorubicin. The dose of SB-203580 was chosen after dose-ranging studies performed in myocardium demonstrating a near total reduction in p38 activation after 1 mg/kg of SB-203580 (see Fig. 6A). Assessment of the possible crossover inhibition of JNK by SB-203580 was performed using an immunoprecipitation (IP) kinase assay for JNK activity, similar to that described for p38, but using c-jun as the phosphorylated substrate. Further investigation of the role of JNK activation was achieved by using the JNK-selective inhibitor SP-600125 (Calbiochem, La Jolla, CA). SP-600125 was dissolved in vehicle, modified to reduce toxicity from that previously described (2, 16), containing 25% DMSO-15% cremophor-2.5% ethanol-57.5% saline. SP-600125 was administered 30 min before doxorubicin at an LD50 dose of 8 mg/kg. Eight β2−/− mice were treated with vehicle (100–150 μl iv) 30 min before intravenous doxorubicin, and nine β2−/− mice received 3 mg/kg SP-600125 iv 30 min before intravenous doxorubicin. This dose was
chosen based on a previous study showing inhibition of JNK activity in the central nervous system after intravenous administration (16). Surviving mice were killed 40 min after doxorubicin, and their hearts, as well as the hearts of those that died, were excised for assessment of JNK activity by IP kinase assay (Cell Signaling, Beverly, MA). Unlike the effect of SB-203580 on p38, the inhibition of JNK in myocardium was less consistent with SP-600125, possibly due to its poor solubility.

**Pathology**

Samples of left ventricular myocardium were obtained 30 min after administration of doxorubicin from all genotypes and placed in formalin and glutaraldehyde for both light microscopic (hematoxylin-eosin and trichrome stains) as well as electron microscopic analysis.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (R&D Systems, Minneapolis, MN) for detection of cardiomyocyte apoptosis was performed at 30 min in β2−/− and wild-type littermates receiving 15 mg/kg doxorubicin. Because it is possible that β2−/− mice receiving the full dose of 15 mg/kg did not survive long enough for the detection of apoptosis, additional studies were performed at 48 h in mice receiving 3.75 mg/kg doxorubicin. To further evaluate whether the apoptotic pathway played a role in the enhanced cardiotoxicity, an assessment of caspase 3 activity was performed at 30 min in mice receiving 15 mg/kg, at 6 h and 4 days in mice receiving 7.5 mg/kg, and at 4 days in mice receiving 3.75 mg/kg doxorubicin. Caspase 3 was assayed using the ApoTarget-Caspase-3/CPP32 kit (BioSource International, Camarillo, CA).

**β-Antagonists**

Mice were administered β-adrenergic antagonists intravenously 10 min before doxorubicin, including the β2-specific antagonist ICI-118,551 (500 μg/kg), the β1-selective antagonist metoprolol (2.5 mg/kg), and the nonselective β-antagonist propranolol (3 mg/kg, a dose previously demonstrated to block heart rate response to isoproterenol in wild-type mice). Additional mice received continuous propranolol (5 mg/kg) daily via a subcutaneous osmotic minipump (Alza, Palo Alto, CA) for 14 days before doxorubicin administration.

**Fig. 2.** Top: blood pressure (BP) and cardiac function changes in β2−/− mice after receiving doxorubicin (n = 6). Cardiac function, as measured by %fractional shortening (%FS), decreases linearly over time. This decrease is statistically significant by 6 min post-doxorubicin (*P < 0.05 vs. baseline by ANOVA). Mean blood pressure decreases slightly until 7 min, at which point it falls to ~50% of resting levels (*P < 0.05 vs. baseline by ANOVA), then stabilizes before falling again preterminally. The arrow shows the onset of p38 MAPK activation. Bottom: heart rate and corrected QT interval (QTc) changes in β2−/− mice after receiving doxorubicin (n = 6). Heart rate decreases slightly after administration of doxorubicin, reaching statistical significance at 12 min (*P < 0.05 vs. baseline by ANOVA). QTc increases by ~30% within 3 min of doxorubicin (1P < 0.05 by ANOVA).
Statistical Analysis

Comparisons of mortality between different genotypes or between different treatments (β-blockers, p38 inhibitor) were performed by χ² analysis. Where multiple comparisons were performed, Bonferroni’s correction was utilized. Comparisons of MAPK expression under different conditions or hemodynamics at various time points was performed by analysis of variance with Fisher’s protected least significant difference post hoc testing. Statistical significance was considered achieved when \( P < 0.05 \).

RESULTS

Mortality

Wild-type mice receiving 15 mg/kg of the anthracycline doxorubicin did not manifest acute adverse cardiovascular effects, and none of the animals died (Fig. 1). Results were similar in β1⁻/⁻ mice. In contrast, the mortality in β2⁻/⁻ mice was 100%, occurring within 20–30 min of doxorubicin administration. Surprisingly, the additional deletion of the β1-receptor (in β1/β2 double knockout mice) totally rescued the enhanced toxicity in the β2-knockout.

Hemodynamics and Pathology

In β2⁻/⁻ mice, systolic function (echocardiographic fractional shortening) and mean blood pressure fell nearly simultaneously, starting within 2 min of doxorubicin administration. The decrease in fractional shortening reached statistical significance \( (P < 0.05 \) by ANOVA) by 6 min and the decrease in blood pressure by 7 min, at which point it had fallen to \( \sim 50\% \)

Fig. 3. Representative ECG changes in a β2⁻/⁻ mouse after receiving doxorubicin. ST segment changes develop variably between 2 and 9 min, often following changes in fractional shortening and blood pressure. Preterminally, ECG changes progress to 2° and 3° heart block and eventually terminal bradycardia. A total of 10 mice were studied with ECGs, 4 while awake and 6 under light anesthesia in conjunction with blood pressure and echocardiographic measurements.
of baseline value (Fig. 2, top). Heart rate fell gradually within the first 2 min, reaching statistical significance by 12 min, then dropping again preterminally (Fig. 2, bottom). There were no acute changes in blood pressure or in left ventricular function in wild-type, β1−/−, or β1/β2−/− mice (data not shown).

In β2−/− mice, ECG changes developed either coincident with or subsequent to the decreases in contractility and blood pressure, ranging from 2 to 9 min after doxorubicin administration. These changes consisted initially of ST segment changes (Fig. 3), progressing to second- and third-degree heart block and eventually terminal severe bradycardia. Corrected QT interval (QTc), which has been shown to increase after doxorubicin administration, increased by ∼30% within 3 min of doxorubicin, then plateaued (Fig. 2, bottom). There were no significant ECG changes in wild-type, β1−/−, or β1/β2−/− mice (data not shown).

Because of the rapidity of decompensation in β2−/− mice, neither light nor electron microscopy demonstrated any significant ultrastructural changes in the myocardium. Importantly, there were no signs of acute ischemic damage, such as endothelial swelling in coronary arterioles. TUNEL staining of myocardium obtained from mice 30 min after 15 mg/kg doxorubicin failed to show an increase in the percentage of apoptotic nuclei compared with wild-type littermates and compared with mice that had not received doxorubicin. Electron microscopy and TUNEL staining performed 48 h after a sublethal dose of 4 mg/kg doxorubicin did not show any evidence of ultrastructural damage or apoptosis. Additionally, there was no

Fig. 4. Alterations in different MAPKs after doxorubicin. β2−/− mice demonstrate a 20-fold increase in p38 MAPK activation in contrast to smaller increases in other genotypes. p44 (ERK1), p42 (ERK2), and JNK are also differentially activated in β2−/− mice in response to doxorubicin (Dox), although less dramatically than for p38. *P < 0.005 vs. other genotypes by ANOVA. Cont, control.
evidence of caspase 3 activation at multiple time points after doxorubicin administration. Thus enhanced activation of pathways leading to programmed cell death is an unlikely mechanism of the enhanced cardiotoxicity in the β2−/− mice.

Role of Differential Activation of MAPKs

Activation of MAPK has been previously demonstrated in cultured cardiomyocytes exposed to anthracyclines, with ERK2 (p42 MAPK) thought to play an antiapoptotic role and p38 MAPK a proapoptotic role (35). Baseline expression of each MAPK (p38, p44/42, and JNK) was not altered in any of the knockout mice. However, baseline level of phospho-p38 was decreased in β2−/− mice compared with wild-type controls (0.065 ± 0.075 vs. 0.150 ± 0.075, ratio of phospho-p38/GAPDH, P < 0.05). None of the other genotypes showed alterations in phospho-p38 at baseline.

In wild-type mice treated with doxorubicin, there was a twofold increase in activation of p38, as assessed by level of phospho-p38. In contrast, in β2−/− mice, activation of p38 was increased 20-fold. The β1−/− mice showed a level of p38 activation that was not different from wild-type, whereas the level in β1/β2−/− was intermediate (Fig. 4). The marked activation of p38 in β2−/− mice was confirmed by IP-kinase assay (Fig. 5A), and the results were equivalent whether phospho-p38 was corrected for GAPDH or for total p38. Activation of p38 occurred within 1 min after administration of doxorubicin (Fig. 5B). Evaluation of p38 isoforms using isoform-specific antibodies showed that the dominant isoform activated in β2−/− mice with doxorubicin was α-p38, which has been shown to be the dominant form expressed in the heart and has been previously linked to myocyte death (32). There were also increases in activation of p44 (ERK1), p42 (ERK2), and JNK, primarily in β2−/− mice receiving doxorubicin, although to a much lesser extent than p38 (Fig. 4).

To examine whether p38 activation was persistently activated or was associated only with the rapid demise of the β2−/− mice, an additional group of three mice in each genotype was given a sublethal dose (3.75 mg/kg) and killed after 4 days. The level of phospho-p38 was still increased by ~7-fold over wild type, but only in the β2−/− mice (data not shown).

To examine whether p38 activation played a role in the enhanced cardiotoxicity encountered in the β2−/− mice, we administered the p38 inhibitor SB-203580, 1 mg/kg, to β2−/− mice 30 min before doxorubicin. We have shown this dose to significantly decrease p38 activation (Fig. 6A). When β2−/− mice were treated with the LD50 dose of 8 mg/kg doxorubicin, the survival was 63% (10/16); all mortalities still occurred within 22 min postdoxorubicin (Fig. 6B). However, when these mice were pretreated with SB-203580, the survival was 93% (14/15) (P < 0.05 by χ2 analysis). Although SB-203580 had been thought to be a specific inhibitor of p38, a previous report suggested that SB-203580 could also block JNK activation (7). Our studies have also determined that SB-203580 has some cross-reactivity with JNK (Fig. 6A) although the effect on p38 is significantly more dramatic. We attempted to eliminate the role of JNK by administering the JNK inhibitor SP-600125. Unlike SB-203580, SP-600125 did not rescue β2−/− mice from doxorubicin toxicity. Four of eight β2−/− mice receiving vehicle plus doxorubicin died vs. five of nine receiving SP-600125 plus doxorubicin [P = not significant (NS)]. However, the in vivo efficacy of SP-600125 in blocking JNK in the myocardium is less consistent than the activity of SB-203580 in blocking p38.

Effect of β-Blockade

Pharmacological blockade of the β2-AR in wild-type mice, either with the β2-specific antagonist ICI-118,551 or with the combined β1/β2-antagonist propranolol, recapitulated the effects of β2-AR gene disruption, markedly increasing mortality in response to doxorubicin (Fig. 7). p38 MAPK activity was increased eightfold in ICI-118,551-treated wild-type mice receiving doxorubicin compared with wild-type mice not treated with ICI-118,551. This activation of p38 MAPK is similar although less dramatic than the increase (20-fold) in β2−/− mice receiving doxorubicin.

In contrast to these results with β2-antagonists, pharmacological blockade of the β1-AR did not recapitulate the rescue effect we observed when the β1-AR was genetically deleted. When both β-ARs were genetically deleted (β1/β2−/−), 100% of mice survived; deletion of the β1-AR (β1−/−/mouse) also partially rescued mice from the combination of ICI-118,551 plus doxorubicin, with a 50% mortality rate vs. a 100%
mortality in wild types (P < 0.001 vs. β1−/− with no ICI-118,551 and P < 0.001 vs. β2−/−). In contrast, administration of the β1-selective antagonist metoprolol failed to rescue β2−/− mice from doxorubicin. This lack of effect of pharmacological blockade of the β1-AR is further evidenced by the effects of propranolol on wild-type or β2−/− mice, also failing to recapitulate the beneficial effects of genetically deleting the β1-AR. Administration of propranolol to mice lacking the β1-AR (both β1−/− and β1/β2−/−) did not substantially increase doxorubicin-induced mortality [12.5% in β1−/− (P = NS); 0% in β1/β2−/−]. Chronic administration of propranolol (5 mg·kg−1·day−1 for 14 days) also failed to rescue the β2−/− mice but had a markedly different effect on wild-type mice (20% mortality; P < 0.04 vs. wild type with no propranolol; P < 0.01 vs. β2−/− with propranolol) compared with acute propranolol (83% mortality).

**DISCUSSION**

The present study is the first comprehensive demonstration in vivo of a clear differential effect of β-receptor subtypes in mediating cardiotoxic vs. cardioprotective signaling in the heart. β2-ARs appear to modulate a cardioprotective role in anthracycline-induced cardiomyopathy. In contrast, β1-ARs appear to be responsible for at least some of the cardiotoxic effects. Previous studies in vivo have suggested a similar dual role for these β-receptor subtypes. Patterson et al. (23) showed enhanced cardiotoxicity in β2−/− mice after a chronic infusion of isoproterenol (23). Ahmet et al. (1) showed that a β2-AR-selective agonist exerted a beneficial effect on infarct size and ejection fraction in rats with ischemic cardiomyopathy.

The cardioprotective effect of the β2-AR was absent both in mice with genomic deletion of the β2-AR as well as mice with acute β2-AR blockade. In contrast, whereas genomic deletion of the β1-AR rescued the acute cardiac decapsulation in β2−/− mice, this effect of β1-ARs was not duplicated with acute β1-AR blockade. We speculated that this cardioprotective effect could require chronic β1-blockade (as would be present in the β1−/− mice); however, chronic β1-blockade also

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**Fig. 6.** A: SB-203580 (SB) decreased both p38 (top) and JNK (bottom) activation after doxorubicin although the effect on p38 was more dramatic (*P < 0.05 by ANOVA). B: the MAPK inhibitor SB-203580 markedly decreases doxorubicin cardiotoxicity in β2−/− mice receiving an LD₅₀ dose (*P < 0.05 by χ² analysis).

**Fig. 7.** Effect of pharmacological β-blockade on doxorubicin toxicity in β-AR knockout mice. Mice received the β2-AR specific antagonist ICI-118,551, the β1-specific antagonist metoprolol, or the nonspecific β-AR antagonist propranolol 10 min before doxorubicin administration. *P < 0.05 vs. control.
failed to rescue β2−/− mice from enhanced anthracycline toxicity, although the toxicity to wild-type mice was reduced. We speculate that genomic deletion of the β1-AR may result in disruption of a downstream cardiotoxic signaling pathway, as yet to be elucidated.

Previous studies in vitro have implicated MAPKs in doxorubicin cardiotoxicity. In cultured cardiomyocytes, daunomycin activates proapoptotic signaling mediated via p38 MAPK and antiapoptotic signaling mediated via p42 MAPK (35). Differential activation of MAPKs has also been described in vitro as a mechanism for the subtype-specific cardiotoxic/cardioprotective effects of β-receptor antagonists (36). We thus hypothesized that MAPK signaling represented a common pathway between these two cardiac stressors in vivo. Similar to in vitro, we found activation of multiple MAPK family members with doxorubicin. There was a dramatic (20-fold) activation of p38 in doxorubicin-treated β2−/− mice compared with a substantially lower activation in the other genotypes. This activation was of the α-p38 isoform, which has been implicated in cell death in myocytes in vitro (32). Doxorubicin also resulted in increased activity of p42, p44, and JNK in β2−/− mice, although the magnitude was not as great as p38. These data suggest that doxorubicin activates both pro- and antisurvival pathways in the myocardium of β2−/− mice, similar to results in vitro (35). The MAPK inhibitor, SB-203580, rescued β2−/− mice from doxorubicin toxicity, whereas rescue was not achieved with the JNK inhibitor SP-600125. This confirms a role for p38 in this enhanced toxicity.

The rapidity of the cardiotoxic response suggests that apoptosis is not the dominant mediator, and our studies failed to show evidence of TUNEL staining or caspase 3 activation. Attempts to define the pathology of this toxicity have been limited by the fact that none of the animals live long enough for the classical light or electron microscopic findings of doxorubicin cardiotoxicity to become manifest. However, our electron microscopy studies confirm the absence of ischemic damage (lack of endothelial swelling). The sequence of events leading to demise of the β2−/− mice is initiated by a decrease in contractile function (without cardiac dilation) accompanied by a decrease in blood pressure, with ECG changes occurring slightly later. There could be several additional mechanisms for the enhanced cardiotoxicity, including altered calcium fluxes, protein kinase C, Akt/GSK3β, and other regulators of cell death, mitochondrial energetics, or free radical processing. Compensatory up- or downregulation of other G protein-coupled receptors or components of their signaling pathways is also possible.

The acute cardiotoxicity we have described may be similar or quite different pathophysiologically from the chronic doxorubicin toxicity most commonly encountered in patients. Whether this enhanced acute toxicity is relevant to the clinical syndrome is not yet clear. Although most patients with doxorubicin cardiotoxicity manifest late heart failure, a minority suffer acute cardiotoxicity. More importantly, there is increasing recognition that so-called “late” clinical toxicity is almost always preceded by longstanding subclinical alterations in ventricular-vascular coupling (17, 21). Using echocardiographic indexes, up to 90% of children with anthracycline cardiomyopathy manifest some changes during their first year of therapy (20).

It is possible that our acute murine model does recapitulate the clinical syndrome, but with the “gain” of the toxicity markedly increased in terms of severity and time course. If this is the case, determining the mechanisms for this enhanced toxicity (and its rescue) may have important implications for patients receiving anthracyclines. Alternatively, our model may be mechanistically very different from the chronic clinical syndrome. In this case, we will still have shown, for the first time in an in vivo model, a marked difference in cardiotoxic/cardioprotective signaling mediated via different β-receptor subtypes. Although these differential effects have previously been demonstrated in cell culture, these studies have mainly used transsected cells expressing β-ARs at highly nonphysiological levels.

In patients with cardiomyopathy, state-of-the-art heart failure management includes the use of β-blocking agents, the most commonly used of which are nonspecific β-adrenergic blockers (e.g., carvedilol), although β1-selective antagonists have also been studied (e.g., metoprolol). Our results suggest the intriguing possibility that a combination of a β1-antagonist and a β2-agonist might further improve efficacy in heart failure management, similar to the findings by Ahmet et al. (1) in rats after myocardial infarction. These studies clearly support the need for further investigation of the subtype-specific cardiotoxic and cardioprotective effects of β-AR signaling.

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