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β2-Adrenergic Receptors Mediate Cardioprotection through Crosstalk with Mitochondrial Cell Death Pathways

Giovanni Fajardo, M.D.^{*}, Mingming Zhao, M.D.^{*}, Gerald Berry, M.D.[†], Lee-Jun Wong, Ph.D.[#], Daria Mochly-Rosen, Ph.D.[^], and Daniel Bernstein, M.D.^{*}

^{*}Department of Pediatrics (Cardiology) Stanford University, Stanford, CA

[†]Department of Pathology Stanford University, Stanford, CA

[^]Department of Chemical and Systems Biology Stanford University, Stanford, CA

[#]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX.

Abstract

Aims—β-adrenergic receptors (β-ARs) modulate cardiotoxicity/cardioprotection through crosstalk with multiple signaling pathways. We have previously shown that β2-ARs are cardioprotective during exposure to oxidative stress induced by doxorubicin (DOX). DOX cardiotoxicity is mediated in part through a Ca²⁺-dependent opening of the mitochondrial permeability transition (MPT), however the signals linking a cell surface receptor like the β2-AR to regulators of mitochondrial function are not clear. The objective of this study was to assess mechanisms of crosstalk between β2-ARs and mitochondrial cell death pathways.

Methods and Results—DOX administered to WT mice resulted in no acute mortality, however 85% of β2-/- mice died within 30 min. Several pro- and anti-survival pathways were altered. The pro-survival kinase, εPKC, was decreased by 64% in β2-/- after DOX vs WT (p<0.01); the εPKC activator ψεRACK partially rescued these mice (47% reduction in mortality). Activity of the pro-survival kinase Akt decreased by 76% in β2-/- after DOX vs WT (p<0.01). The α1-antagonist prazosin restored Akt activity to normal and also partially reversed the mortality (45%). Deletion of the β2-AR increased rate of Ca²⁺ release by 75% and peak [Ca²⁺]_i by 20% respectively in isolated cardiomyocytes; the Ca²⁺ channel blocker verapamil also partially rescued the β2-/- (26%). Mitochondrial architecture was disrupted and complex I and II activities decreased by 40.9% and 34.6% respectively after DOX only in β2-/- . The MPT blocker cyclosporine reduced DOX mortality by 41% and prazosin plus cyclosporine acted synergistically to decrease mortality by 85%.

Conclusion—β2-ARs activate pro-survival kinases and attenuate mitochondrial dysfunction during oxidative stress; absence of β2-ARs enhances cardiotoxicity via negative regulation of survival kinases and enhancement of intracellular Ca²⁺, thus predisposing the mitochondria to opening of the MPT.

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Contact Information: Daniel Bernstein, M.D. 750 Welch Road, Suite 325, Palo Alto, CA 94304 (650) 723-7913 danb@stanford.edu.

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Conflict of Interest

None.

Keywords

Adrenergic receptors; cardiomyopathy; mitochondria; signal transduction; protein kinases

1. Introduction

The two major cardiac β -adrenergic receptor (β -AR) subtypes, β_1 and β_2 , modulate cardiac signaling through both parallel and opposing pathways. Over the past decade, our understanding of the function of these key receptors has increased beyond their classical role in regulating inotropy and chronotropy, and now includes the complex regulation of cardiac remodeling. Stimulation of β_1 -ARs can induce apoptosis through PKA-dependent [1] and independent (e.g. CaMKII) pathways [2]. In contrast, stimulation of β_2 -ARs can be anti-apoptotic, mediated through G_i , $G_{\beta\gamma}$, phosphatidylinositol 3'-kinase and Akt [3]. In addition, β_2 -ARs can also signal through ERK via a GRK5/6- β -arrestin-dependent pathway, independent of G protein coupling [4].

We have previously shown that β_1 -ARs play a cardiotoxic and that β_2 -ARs play a cardioprotective role in oxidative stress, using as a model the anthracycline anti-cancer drug doxorubicin (DOX) [5, 6]. β_2 -AR knockout ($\beta_2^{-/-}$) mice receiving a single therapeutic-level dose of DOX (which has no acute effect on WT or $\beta_1^{-/-}$ mice) show markedly enhanced cardiotoxicity, with ECG, blood pressure and contractility changes within 2 min, and death within 30 min. Differential activation of MAPK isoforms was observed: p38 activity increased 20-fold only in the $\beta_2^{-/-}$ and p38 inhibition partly rescued this phenotype. However, the exact mechanisms mediating the extremely rapid demise of the $\beta_2^{-/-}$ mice and the additional pathways activated by β_2 -ARs to protect against anthracycline-induced oxidative stress are unknown.

In addition to the MAPKs, several other kinases play important roles in modulating cardiotoxicity/cardioprotection, including protein kinase C (PKC) and Akt. Of the 11 isoforms of PKC identified, opposing cardioprotective actions have been attributed to the novel subfamily members ϵ PKC and δ PKC. ϵ PKC attenuates, whereas δ PKC increases, ischemia/reperfusion injury [7]. Similar roles have been observed in both ethanol [8] and reactive oxygen species-induced cardioprotection [9], and may explain some of the cardioprotective effects of diazoxide [10]. Akt, a key regulator of cardiac growth and metabolism, also plays a central role in cardioprotection. Activation of Akt inhibits hypoxia-induced myocyte apoptosis [11] and plays a role in both ischemic preconditioning and postconditioning [12]. In DOX cardiotoxicity, Akt activation protects against myocyte apoptosis [13], attenuates heart failure [14] and may mediate the protective effects of dexrazoxane [15]. The interrelationship between these pro-survival kinases and β_2 -ARs in mediating protection against DOX cardiotoxicity is unknown.

A central intracellular target of DOX toxicity, and a point of convergence of signaling by the above kinases, is the mitochondria. The extremely rapid demise of the $\beta_2^{-/-}$ mice when exposed to DOX makes apoptosis an unlikely mechanism, and we have found no evidence of either apoptosis or ischemia. Rapid cell death in this time frame can be mediated by opening of the mitochondrial permeability transition (MPT) pore in the inner mitochondrial membrane, causing reversal of the F₀-F₁ ATPase and collapse of the mitochondrial membrane potential [16]. Previous evidence implicates mitochondrial dysfunction in DOX toxicity. DOX is redox cycled through mitochondrial complex I NADH dehydrogenase [17], increasing the formation of free radical intermediates. Mitochondria are also targets for the $[Ca^{2+}]_i$ dysregulation and bioenergetic failure that are hallmarks of DOX cardiotoxicity [17]. However, how signaling mediated through a cell surface G-protein coupled receptor

(GPCR), such as the β 2-AR, can so profoundly affect these mitochondrial cell death pathways is unknown.

In this study, we sought to determine the mechanisms by which a cell surface GPCR like the β 2-AR can crosstalk with mitochondrial cell death pathways to mediate cardiotoxicity/cardioprotection. We used DOX cardiotoxicity as a well established and highly reproducible model of oxidative stress. We hypothesized that absence of β 2-AR signaling would lead to subtle alterations in pro-survival kinase signaling and in $[Ca^{2+}]_i$ regulation. These alterations would be below the threshold for altering baseline cardiac physiology. However, when exposed to an oxidative stressor such as DOX, these alterations would combine to predispose to opening of the MPT and subsequent cell demise.

2. Materials and Methods

2.1 Doxorubicin Cardiotoxicity

DOX was used as a model of oxidative stress and has been well established in the mouse [6, 18, 19]. 3 month old male WT and β 2-/- mice, both on a congenic FVB background, were injected with a single dose of DOX (NovaPlus, Bedford, OH) (200–300 μ l) via the dorsal tail vein. Three different doses were administered in order to maximize the effect of the inhibitors tested: 8 mg/kg (LD₅₀ dose), 10 mg/kg and 15 mg/kg. These doses are within the normal range of dosing for patients with malignancies, based on human equivalent dosing models [20, 21]. Mortality was assessed at any time during the first 72 hours; however, all the mice that died did so at 20–30 min after DOX. Animal protocols were approved by the Stanford University Institutional Animal Care and Use Committee and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Drug Administration

Mice were administered PKC isozyme-specific regulatory peptides as previously described [22]. The ϵ PKC activator peptide ψ εRACK (20 nmol in 150 μ l) and the δ PKC inhibitor peptide δ V1-1 (20 nmol in 150 μ l) were each given i.p. 10 min before DOX. The α 1-AR blocker prazosin (0.1 mg/kg) was administered i.v. 10 min before DOX [23]. The L-type Ca^{2+} channel blocker verapamil (25 mg/kg) was given i.v. 30 min before DOX [24]. Cyclosporine (10 mg/kg), a blocker of the MPT, but also of the phosphatase calcineurin, was given i.v. 30 min before DOX [25]. FK-506 (0.5 mg/kg), a blocker of calcineurin but not the MPT [26] was given i.p. 30 min before DOX. The dose of DOX used in each case was the highest dose at which mortality could be decreased by the inhibitor, i.e. If an inhibitor decreased mortality at 10 mg/kg it was not tested at the lower 8 mg/kg (LD50) dose.

2.3 Levels and Translocation of PKC Isozymes

To isolate particulate and soluble fractions, whole-tissue lysates from mouse hearts were prepared as previously described [27]. The soluble and particulate fractions were separated by high-speed centrifugation. ϵ PKC and δ PKC levels and translocation were determined by SDS-PAGE, followed by Western blot analysis with anti-PKC, anti- ϵ PKC and anti- δ PKC antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), GAPDH was used as loading control (Advance Immunochemical, Long Beach, CA).

2.4 Akt levels, Phosphorylation and Activity

Akt levels and phosphorylation were measured by Western blotting using Akt and Phospho-Akt (Ser473) antibodies (Cell Signaling Technology, Danvers, MA). Immobilized Akt monoclonal antibodies (Cell Signaling Technology, Danvers, MA) were used to immunoprecipitate Akt from lysates, and an in vitro kinase assay was performed using

GSK-3 Fusion Protein as a substrate. Phosphorylation of GSK-3 was measured by Western blotting, using Phospho-GSK-3 α/β (Ser21/9) antibody.

2.5 Electron Microscopy

Hearts from WT and $\beta 2^{-/-}$ mice treated with and without DOX 15 mg/kg were collected 20 min after DOX; hearts from mice who survived 24 h after 15 mg/kg DOX treatment in the presence of a p38 inhibitor were also used for electron microscopy studies. Samples were processed at the Stanford Cell Science Imaging Facility. Heart samples were fixed in 2% glutaraldehyde (EMS, Hatfield, PA) and 4% formaldehyde (EMS). Samples were observed in the JEOL 1230 TEM at 80kV and photos taken using a Gatan Multiscan 791 digital camera (Gatan, Pleasanton, CA).

2.6 Electron Transport Chain Activity

The activities of complex I and complex II in WT and $\beta 2^{-/-}$ mice without and with DOX 15 mg/kg 20 min post treatment were assessed spectrophotometrically as previously reported [28].

2.7 Cardiomyocyte Isolation

Adult ventricular myocytes were isolated from $\beta 2^{-/-}$ mice and WT littermates based on previously published protocols, with modifications [29, 30]. Experiments were performed with freshly isolated myocytes resuspended in a HEPES-buffered solution.

2.8 Ca²⁺ Transient Measurements

Ca²⁺ transients were evaluated with a video-based sarcomere spacing acquisition system (SarcLen, IonOptix, Milton, MA). Rod-shaped myocytes with clear striation patterns and quiescent when unstimulated were chosen. Cells were electrically stimulated with suprathreshold voltage at 0.5 Hz and superfused with a HEPES-buffered solution. Background-corrected Fura 2 ratios were collected at 510 nm [31, 32].

2.9 Statistical Analysis

Data are expressed as mean $\pm$ SD. Unpaired t tests were used for comparisons between 2 groups, and ANOVA with Fisher's test was used for differences among >2 groups. Significance was attained with a $p < 0.05$.

3. Results

3.1 $\beta 2$ -ARs mediate cardioprotection through differential regulation of PKC isozymes

To gain insight into whether $\beta 2$ -ARs mediate their protective effects through crosstalk with specific PKC isozymes, levels of ϵ PKC and δ PKC and their translocation from soluble to particulate fractions were assessed at baseline and 20 min. after DOX (15 mg/kg) administration to WT and $\beta 2^{-/-}$ mice.

At baseline there was no difference in ϵ PKC levels in either soluble or particulate fraction between WT and $\beta 2^{-/-}$, however baseline δ PKC was increased slightly ($16.0 \pm 4.6\%$) in the particulate fraction in $\beta 2^{-/-}$ compared to WT (Figure 1A). 20 min after DOX, however, ϵ PKC significantly decreased in both particulate and soluble fractions by $67.3 \pm 12.3\%$ and $64.0 \pm 9.9\%$ respectively in $\beta 2^{-/-}$ whereas δ PKC increased by $22 \pm 5\%$ in the particulate fraction compared to WT (Figure 1B).

To examine the consequences of these PKC isozyme-specific alterations in mediating enhanced DOX cardiotoxicity in the $\beta 2^{-/-}$, the δ PKC inhibitor (δ V1-1) and the ϵ PKC

activator (ψ εRACK) were each given prior to DOX. When DOX was given at the LD₅₀ concentration of 8 mg/kg, the εPKC activator ψ εRACK decreased mortality by 47%, although it failed to rescue the acute cardiotoxicity when DOX was given at the higher doses of 15 mg/kg or 10 mg/kg, which induce 100% and 85% mortality in the β 2-/-, respectively. In contrast, there was no improvement in survival when mice were given the δPKC inhibitor δV1-1 (Figure 1C).

3.2 Akt is disregulated in β 2-/- mice after DOX

To determine whether β 2-ARs crosstalk with other pro-survival kinases in the regulation of cardiotoxicity/cardioprotection, Akt levels, phosphorylation and activity were assessed at baseline and 20 min. after 15 mg/kg DOX was given to WT and β 2-/- mice. There was no difference between WT and β 2-/- mice in Akt levels at baseline or after DOX (Figure 2A). Of interest, phosphorylation of Akt was lower in the β 2-/- compared to WT at baseline and only WT mice showed a decrease in phosphorylation of Akt after DOX, achieving levels similar to those in DOX β 2-/- treated mice (Figure 2B). At baseline, there was no difference in Akt activity in β 2-/- compared with WT. However, after DOX, Akt activity decreased by 76% in β 2-/- but not in WT (Figure 2C). As previous studies have shown that α 1-AR signaling can alter regulation of Akt (usually, but not always positively) [33, 34], we administered the α 1-AR blocker prazosin (0.1 mg/kg) 10 min before DOX and measured Akt activity. In the β 2-/-, prazosin restored Akt activity to normal as early as 20 min and for as long as 24 h after DOX. Prazosin alone did not alter Akt activity from baseline levels (Figure 2D). In β 2-/- mice prazosin reduced mortality from 85% to 47% after DOX (10 mg/kg) (Figure 2E).

3.3 β 2-ARs regulate mitochondrial function in DOX cardiotoxicity

To determine the role of mitochondrial dysfunction in acute DOX cardiotoxicity in the β 2-/-, we examined both structural and functional alterations. Because of the rapidity with which β 2-/- mice die after receiving DOX, neither light nor electron microscopy (EM) demonstrated any ultrastructural changes. There were also no signs on EM typical of acute ischemia, such as coronary endothelial swelling. This mortality is totally binary: even when given the LD₅₀ dose of DOX, those β 2-/- mice that die do so within 30 min; none survive longer. Those that survive do not manifest late acute toxicity up to 72 hrs after drug administration. We have previously shown that we can partially rescue these mice with the p38 inhibitor SB203580, which allowed us to repeat these pathologic examinations at 24 h, a time when non-treated β 2-/- do not survive. LV myocardium from β 2-/- mice treated with 15 mg/kg DOX for 24h in the presence of a p38 inhibitor exhibited mitochondria with disrupted, slit-like vacuolization of the cristae. In contrast, none of these changes were observed in WT mice treated with DOX (Figure 3).

Mitochondrial electron transport chain function was evaluated next. At baseline, there were no differences in complex I and II activities between β 2-/- and WT. However, after DOX, complex I activity decreased by 41% and complex II activity by 35% in the β 2-/. There was no change in these activities in the WT (Figure 4).

3.4 Deletion of β 2-ARs alters intracellular Ca^{2+} transients and predisposes opening of the MPT after exposure to DOX

Doxorubicin is known to decrease mitochondrial Ca^{2+} loading capacity[35] and β 1- and β 2-ARs regulate $[\text{Ca}^{2+}]_i$ through both parallel and opposing mechanisms. β 1-ARs couple to the stimulatory G protein, Gs, whereas β 2-ARs can couple to both Gs and Gi [36]. We have previously shown that resting cardiac contractile function is unaltered in β 2-/- mice [37], however, subtle changes in Ca^{2+} transients could be subthreshold to affect resting function, but critical in the setting of cardiac oxidative stress. In agreement with our hypothesis,

cardiomyocytes isolated from $\beta 2^{-/-}$ mice showed increases in the rate of Ca^{2+} release (75%) and in peak $[\text{Ca}^{2+}]_i$ (20%) compared to WT (Figure 5A-C). These changes were not associated with an increase in contractility in $\beta 2^{-/-}$ myocytes as measured by intrasarcomeric shortening (Figure 5D). Although we did not see statistically significant changes, there was a trend toward higher velocity of contraction and relaxation in the $\beta 2^{-/-}$ compared to WT (data not shown). Confirming the role of altered intracellular Ca^{2+} , blocking the L-type Ca^{2+} channel with verapamil (25 mg/kg) decreased mortality by 26% in $\beta 2^{-/-}$ mice treated with 10 mg/kg DOX (Figure 5E).

Finally, to determine if opening of the MPT was involved in this enhanced cardiotoxicity in the $\beta 2^{-/-}$, cyclosporine (10 mg/kg), a blocker of the MPT was administered 30 min. prior to 10 mg/kg DOX. Cyclosporine reduced DOX mortality from 85% to 50%. Since cyclosporine is also an inhibitor of the phosphatase calcineurin, we administered FK506, a blocker of calcineurin but not of the MPT, to determine whether this effect was related to calcineurin signaling. As expected if the effect was solely on the MPT, FK506 did not result in a reduction in DOX mortality (Figure 6). One traditional marker of MPT opening, cytochrome C release, was not detected in either WT mice or $\beta 2^{-/-}$ mice treated with DOX, however the rapid time to demise of these mice would likely be too soon for activation of the cytochrome C-intrinsic apoptotic pathway [38]. At baseline, $\beta 2^{-/-}$ mitochondria were not more sensitive to Ca^{2+} -mediated swelling than WT mitochondria (data not shown). Finally, to determine whether alterations in Akt and opening of the MPT were independent or dependent pathways, we administered prazosin and cyclosporine in combination 30 min before DOX. There was a synergistic marked decrease in DOX mortality from 85% to 12.5% (Figure 6).

4. Discussion

Our results suggest that $\beta 2$ -ARs mediate their cardioprotective effects in oxidative stress in part through crosstalk with mitochondrial cell death pathways. Anthracycline cardiotoxicity was used as model of oxidative stress to dissect the mechanisms by which a cell surface GPCR receptor such as the $\beta 2$ -AR can influence mitochondrial signaling. Although the exact mechanism of DOX cardiotoxicity is still incompletely defined, the mitochondria are a prime target for DOX action. Cardiomyocytes may be particularly susceptible to DOX toxicity due to their large number of mitochondria; redox cycling of DOX by complex I NADH dehydrogenase in mitochondria further increases the generation of free radicals and further increases cell damage [39].

We present evidence that $\beta 2$ -ARs mediate their cardioprotective effects in part through crosstalk with the pro-survival kinases ϵ PKC and Akt, which themselves regulated mitochondrial function. The cardioprotective role of ϵ PKC has previously been demonstrated in models of ischemia/reperfusion and preconditioning [40, 41]. Activation of ϵ PKC before ischemia protects mitochondrial function and diminishes apoptosis and oncosis; its cardioprotective mechanisms involve opening of mitochondrial ATP-sensitive K^+ channels, prevention of MPT opening and regulation of cytochrome c oxidase activity [42]. In contrast, δ PKC has been shown to mediate cardiotoxicity [8], δ PKC is required for DOX-induced apoptosis in Jurkat cells, mediated through sequential activation of caspase-2, δ PKC and JNK [43]. Here we demonstrate the role of ϵ PKC and δ PKC isozymes in $\beta 2$ -AR mediation of DOX cardiotoxicity. Although normal at baseline, ϵ PKC is markedly reduced in $\beta 2^{-/-}$ mice after DOX in both particulate and soluble fraction. The role of ϵ PKC in this enhanced toxicity was further demonstrated by administration of the ϵ PKC activator, $\psi\epsilon$ RACK, which partially rescues this acute toxicity. In contrast, although δ PKC was slightly increased at baseline in the $\beta 2^{-/-}$, its inhibition did not influence the cardiotoxicity. $\beta 2$ -AR signaling has been shown to crosstalk with ϵ PKC in regulating ERK activity,

although *in vitro* the effect of ϵ PKC appears to limit β 2-AR mediated cardioprotection [44]. The discrepancy between this *in vitro* study and the current *in vivo* study may be due to the different stresses used, the species, or in differences between isolated myocytes and intact myocardium.

Our results also provide evidence of β 2-AR crosstalk with Akt in mediating cardioprotection [3]. The role of Akt in DOX cardiotoxicity has been explored previously, with some studies suggesting that DOX induces the activation of both caspase-3 and Akt [45], whereas others show that DOX decreases Akt [13]. Akt activation may be enhanced by DOX-induced ROS generation [46], and may induce cardioprotection by attenuating cardiomyocyte apoptosis through phosphorylation of GSK-3 β [45]. If Akt is a protective mechanism induced in response to DOX, then in the absence of the β 2-AR, the marked dysregulation of Akt (where activity falls only in the β 2-/-) would participate in the enhanced toxicity. In support of this mechanism, the α 1-antagonist prazosin restores Akt activity to normal levels, and partially rescues the β 2-/- mice. The effect that we observed of α 1-AR blockade with prazosin on Akt activity is in agreement with findings by Ballou et al [47]. They have shown, using Rat-1 fibroblasts stably expressing the human α 1A-AR, that treatment of these cells with phenylephrine did not activate PI3-kinase or Akt. Furthermore, phenylephrine blocked the insulin-like growth factor-I-induced activation of PI3-kinase and the phosphorylation and activation of Akt-1. In their study, the effect of phenylephrine was not confined to signaling pathways that include insulin receptor substrate-1, as the α 1-AR agonist also inhibited the platelet-derived growth factor-induced activation of PI3-kinase and Akt-1. Thus, the α 1-AR negatively regulated the PI3-kinase/Akt pathway, resulting in enhanced cell death following apoptotic insult. Taken together with the current study, these results implicate altered regulation of Akt as one of the mechanisms for enhanced cardiotoxicity in the β 2-/-.

In addition to these alterations in pro-survival kinases, we hypothesized that altered Ca^{2+} transients would also provide a link between cell surface β 2-ARs and intracellular mitochondrial signaling. Altered Ca^{2+} uptake has previously been shown to increase mitochondrial ROS production [48] and DOX is known to decrease mitochondrial Ca^{2+} loading capacity. [35]. In the absence of β 2-AR signaling, peak $[\text{Ca}^{2+}]_i$ and the rate of Ca^{2+} release were increased, potentially due to the loss of inhibitory signaling through G_i . Devic et al. have shown increases in isoproterenol-stimulated contraction rate in β 1-/- neonatal cardiomyocytes treated with pertussis toxin [49]. Furthermore, Kuschel et al. have shown that inhibition of G_i with pertussis toxin permits a full phospholamban phosphorylation and a de novo relaxant effect following β 2-AR stimulation, converting the traditionally localized β 2-AR signaling to a global signaling mode similar to that of β 1-AR[50]. The current study would be the first demonstration of an alteration in $[\text{Ca}^{2+}]_i$ associated with the absence of β 2-AR signaling. We have previously shown that baseline cardiovascular function is not altered in the β 2-/- mouse [37], and here show that intrasarcomeric shortening is also not altered (Figure 5D), suggesting that these alterations in Ca^{2+} are subthreshold at rest. At baseline, β 2-/- mitochondria are not inherently more sensitive to Ca^{2+} -mediated swelling than WT mitochondria. However, *in vivo* when β 2-/- mice are stressed with DOX, the increase in $[\text{Ca}^{2+}]_i$, combined with alterations in ϵ PKC and Akt signaling, disrupt normal mitochondrial Ca^{2+} buffering capacity, predisposing to MPT opening and rapid demise. In support of this mechanism, both the L-type Ca^{2+} channel blocker verapamil and the MPT blocker cyclosporine rescue the enhanced toxicity. Opening of the MPT occurred without cytochrome c release in β 2-/- mice. Although cytochrome c release can be observed as early as one hour after ischemia-reperfusion injury [38], the more rapid demise of the β 2-/- after DOX may be too early for activation of the cytochrome c-intrinsic apoptotic pathway. In addition, MPT opening is not always a sufficient condition, in itself, to effect cytochrome c release [51]. We attempted to confirm MPT opening *in vitro* by treating myocytes isolated from WT and β 2-/- mice with DOX. Unfortunately, DOX autofluorescence interfered with

the ability to detect calcein release (a measure of MPT opening) or with the ability to measure mitochondrial membrane potential using TMRM.

The convergence of these pro-survival kinase pathways with intracellular Ca^{2+} regulation appears to be at the level of the MPT. The multi-component structure of the MPT integrates information on mitochondrial energy metabolism, oxidative stress and Ca^{2+} load to determine whether or not to activate cell death pathways, and to what extent [52]. Akt's cardioprotective actions are mediated in part through inhibition of GSK-3 β 's actions on the MPT complex [53]. We have shown that restoring Akt activity with prazosin combined with inhibition of the MPT with cyclosporine had a highly synergistic effect against DOX toxicity in the $\beta 2^{-/-}$ (Fig. 4C), nearly eliminating the enhanced toxicity. Further evidence that mitochondrial dysfunction plays a central role in DOX cardiotoxicity of the $\beta 2^{-/-}$ is evidenced by decreases in complex I and II activities and mitochondrial ultrastructural abnormalities seen only in the $\beta 2^{-/-}$ that survived more than 24 h after the highest dose of DOX combined with p38 inhibition and not in the WT [6]. Although the mitochondria in hearts from $\beta 2^{-/-}$ mice after DOX treatment are not typically swollen, MPT opening is not necessarily and readily followed by matrix swelling in intact cells, (Dr. Fabio Di Lisa, University of Padova, Italy, personal communication).

In conclusion, we have demonstrated a link between a cell surface GPCR receptor and mitochondrial cell death pathway signaling, mediated via pro-survival kinases including ϵ PKC and Akt, and by altered regulation of intracellular Ca^{2+} transients. When $\beta 2$ -AR signaling is ablated and this is coupled with the oxidative stress induced by DOX, these signaling alterations combine to dramatically increase cardiotoxicity by predisposing to opening of the MPT pore. A similar cardioprotective role for $\beta 2$ -AR signaling has been invoked in the suggestion that a $\beta 2$ -AR agonist, combined with a $\beta 1$ -AR antagonist, may be superior to a non-specific β -AR antagonist in the treatment of heart failure [54]. The implications of our data for the clinical use of subtype-specific or non-specific β -AR blockers remains to be determined, however, the extension of these previously observed subtype-specific effects to another model of cardiac stress (doxorubicin oxidative stress) is an important step.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- β 2-ARs protect against doxorubicin cardiotoxicity via modulation of PKC and Akt
- Doxorubicin decreases both ϵ PKC and Akt activity in β 2-/- mice but not in WT
- β 2-ARs attenuate mitochondrial dysfunction in doxorubicin cardiotoxicity
- Complex I and II activities decrease only in doxorubicin treated β 2-/- mice
- Calcium Blockade or MPT inhibition ameliorates doxorubicin toxicity in β 2-/- mice

Figure 1.

β2-ARs mediate cardioprotection through differential regulation of PKC isozymes. A) Baseline δPKC and εPKC levels in hearts from β2-/- and WT mice; Western blot image (top) and bar graph summarizing densitometry data (bottom); n=3, *p<0.05. B) δPKC is decreased and εPKC is increased in β2-/- vs. WT after 15 mg/kg DOX. Western blot image (top) and bar graph summarizing densitometry data (bottom); n=3, *p<0.01. C) The εPKC activator ψεRACK, but not the δPKC inhibitor δV1-1, reduces mortality in β2-/- mice receiving DOX at 8 mg/kg (LD₅₀) dose; DOX alone n=16, δV1-1 n=10, ψεRACK n=15.

Figure 2.

$\beta 2$ -ARs mediate cardioprotection through Akt signaling. A) Akt levels were similar in WT and $\beta 2^{-/-}$ mice before and after DOX; Western blot image (top) and bar graph summarizing densitometry data (bottom); $n=3$, $p=NS$. B) Phosphorylation of Akt Ser 473 was lower in the $\beta 2^{-/-}$ compared to WT at baseline. Only WT mice showed a decrease in phosphorylation of Akt after DOX, achieving levels similar to those in $\beta 2^{-/-}$ DOX-treated mice; Western blot image (top) and bar graph summarizing densitometry data (bottom); $n=3$, * $p<0.05$ compared to WT, # $p<0.05$ compared to non-treated. C) Baseline and DOX-treated Akt activity is shown. Akt activation is markedly decreased only in DOX treated $\beta 2^{-/-}$ mice. IP-kinase Akt activity assay (phospho-GSK3 β); Western blot image (top) and bar graph summarizing densitometry data (bottom); $n=4$, * $p<0.05$. D) Prazosin restores Akt activity in DOX treated $\beta 2^{-/-}$ mice; Western blot image (top) and bar graph summarizing densitometry data (bottom); $n=3$, * $p<0.05$. E) Prazosin reduces mortality in $\beta 2^{-/-}$ mice receiving 10 mg/kg DOX; DOX $n=20$, prazosin $n=11$.

Figure 3. β 2-ARs attenuate mitochondrial dysfunction in DOX cardiotoxicity. Electron micrographs of WT and β 2-/- mice treated with 15 mg/kg DOX for 24 h in the presence of a p38 inhibitor show cristae disruption only in the β 2-/-.

Figure 4.

Complex I and II activities are normal at baseline and decrease only in DOX treated $\beta 2^{-/-}$ mice; * $p < 0.05$ compared to WT.

Figure 5.

β2-ARs regulate Ca²⁺ signaling and enhance opening of the MPT. A) Representative Ca²⁺ transients from WT and β2^{-/-} mice. B) The rate of Ca²⁺ release is increased at baseline in β2^{-/-} vs. WT mice; n=14, *p<0.05. C) Baseline peak [Ca²⁺]_i is increased at baseline in β2^{-/-} vs. WT mice; n=14, *p<0.05 compared to WT. D) Intrasarcomeric shortening was similar between WT and β2^{-/-}; p=NS. E) The L-type calcium channel blocker verapamil reduces mortality in β2^{-/-} mice receiving 10 mg/kg DOX; DOX n=20, verapamil n=8.

Figure 6.

Cyclosporine (CsA, an inhibitor of the MPT and of calcineurin) partially rescues $\beta 2^{-/-}$ mice receiving 10 mg/kg DOX; FK506 (a calcineurin inhibitor which does not block the MPT) has no effect. The combination of Akt activation with prazosin (Pra) and inhibition of the MPT with cyclosporine (CsA) provides a synergistic cardioprotective effect against DOX cardiotoxicity, nearly eliminating the toxicity (mortality reduced to 12.5%). DOX n=20, CsA n=16, FK506 n=16, CsA + Pra n=8.