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Original article

# β2-adrenergic receptors mediate cardioprotection through crosstalk with mitochondrial cell death pathways

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ABSTRACT

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 $Ca^{2+}$-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. 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 $Ca^{2+}$ release by 75% and peak $[Ca^{2+}]_i$ by 20% respectively in isolated cardiomyocytes; the $Ca^{2+}$ 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%. 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 $Ca^{2+}$, thus predisposing the mitochondria to opening of the MPT.



## 1. Introduction

The two major cardiac β-adrenergic receptor (β-AR) subtypes, β1 and β2, 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 β1-ARs can induce apoptosis through PKA-dependent [1] and independent (e.g. CaMKII) pathways [2]. In contrast, stimulation of β2-ARs can be anti-apoptotic, mediated through Gi, Gβγ, phosphatidylinositol 3′-kinase and Akt [3]. In addition, β2-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 β1-ARs play a cardiotoxic and that β2-ARs play a cardioprotective role in oxidative stress, using as a model the anthracycline anti-cancer drug doxorubicin (DOX) [5,6]. β2-AR knockout (β2-/-) mice receiving a single therapeutic-level dose of DOX (which has no acute effect on WT or β1-/- 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 β2-/- and p38 inhibition partly rescued this phenotype. However, the exact mechanisms mediating the extremely rapid demise of the β2-/- mice and the additional pathways activated by β2-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 isozymes of PKC identified, opposing cardioprotective actions have been attributed to the novel subfamily

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members εPKC and δPKC. εPKC attenuates, whereas δPKC 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 β2-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 β2-/- 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 β2-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 β2-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 β2-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 β2-/- mice, both on a congenic FVB background, were injected with a single dose of DOX (NovaPlus, Bedford, OH) (200–300 μl) via the dorsal tail vein. Three different doses were administered in order to maximize the effect of the inhibitors tested: 8 mg/kg ($LD_{50}$ 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 h; 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 εPKC activator peptide ψεRACK (20 nmol in 150 μl) and the δPKC inhibitor peptide δV1-1 (20 nmol in 150 μl) were each given i.p. 10 min before DOX. The α1-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. εPKC and δPKC levels and translocation were determined by SDS-PAGE, followed by Western blot analysis with anti-PKC, anti-εPKC and anti-δPKC antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), GAPDH was used as loading control (Advance Immnunochemical, 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-3α/β (Ser21/9) antibody.

### 2.5. Electron microscopy

Hearts from WT and β2-/- 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 80 kV 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 β2-/- 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 β2-/- 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. $Ca^{2+}$ transient measurements

$Ca^{2+}$ 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