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Inhibition of PKCβ2 overexpression ameliorates myocardial ischaemia/reperfusion injury in diabetic rats via restoring caveolin-3/Akt signaling

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Abstract

Activation of PKCβ (protein kinase Cβ) plays a critical role in myocardial I/R (ischaemia/reperfusion) injury in non-diabetic rodents. In the myocardium of diabetes, PKCβ2 overexpression is associated with increased vulnerability to post-ischaemic I/R injury with concomitantly impaired cardiomyocyte Cav (caveolin)-3 and Akt signalling compared with non-diabetic rats. We hypothesized that myocardial PKCβ overexpression in diabetes exacerbates myocardial I/R injury through impairing Cav-3/Akt signalling. Streptozotocin-induced diabetic rats were treated with the selective PKCβ inhibitor ruboxistaurin (RBX, 1 mg/kg per day) for 4 weeks, starting from 1 week after diabetes induction, before inducing myocardial I/R achieved by occluding left descending coronary artery followed by reperfusion. Cardiac function was measured using a pressure–volume conductance system. In in vitro study, cardiac H9C2 cells were exposed to high glucose (30 mmol/l) and subjected to hypoxia followed by reoxygenation (H/R) in the presence or absence of the selective PKCβ2 inhibitor CGP41241 (1 µmol/l), siRNAs of PKCβ2 or Cav-3 or Akt. Cell apoptosis and mitochondrial membrane potential were assessed by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) and JC-1 staining respectively. RBX significantly decreased post-ischaemic myocardial infarct size (35 ± 5% compared with 49 ± 3% in control, P < 0.05) and attenuated cardiac dysfunction, and prevented the reduction in cardiac Cav-3 and enhanced phosphorylated/activated Akt (p-Akt) in diabetic rats (P < 0.05). H/R increased cardiomyocyte injury under high glucose conditions as was evident by increased TUNEL-positive and increased JC-1 monomeric cells (P < 0.05 compared with control), accompanied with increased phosphorylation/activation of Cav-3 expression. Either CGP41241 or PKCβ2 siRNA significantly attenuated all of these changes and enhanced p-Akt. Cav-3 gene knockdown significantly reduced p-Akt and increased post-hypoxic cellular and mitochondrial injury despite a concomitant reduction in PKCβ2 phosphorylation. PKCβ2 inhibition with RBX protects diabetic hearts from myocardial I/R injury through Cav-3-dependent activation of Akt.

Key words: caveolin-3, diabetes, myocardial ischaemia/reperfusion injury, protein kinase Cβ

Abbreviations: Δψm, mitochondrial membrane potential; AAR, area at risk; Cav, caveolin; CK-MB, creatine kinase-MB; CO, cardiac output; DMEM, Dulbecco’s modified Eagle’s medium; dP/dt max, peak rate of left ventricular pressure increase; dP/dt min, minimal rate of left ventricular pressure decrease; Ea, arterial elastance; 15-F2t-IsoP, 15-F2t-isoprostane; HR, heart rate; H/R, hypoxia/reoxygenation; HRP, horseradish peroxidase; I/R, ischaemia/reperfusion; IS, infarct size; LAD, left anterior descending; dP/dt, peak rate of left ventricular pressure change; EF, ejection fraction; LVP max, maximal left ventricular pressure; MTP, mitochondrial permeability transition pore; PKC, protein kinase C; PV, pressure-volume; RBX, ruboxistaurin; STZ, streptozotocin; SV, stroke volume; SW, stroke work; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling.

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INTRODUCTION

Diabetes is associated with significant acute myocardial infarction, a major cause of heart failure and high mortality rate [1–3]. Many therapeutic strategies that have been effective in the protection of non-diabetic hearts against I/R (ischaemia/reperfusion) injury lose effectiveness in diabetic states [4–6]. Hyperglycaemia-induced protein abnormalities, in particular activation of PKC (protein kinase C), a family of serine/threonine kinases that contribute to myocardial dysfunction [7,8], are potentially responsible for the exacerbation of acute myocardial infarction in diabetes [9,10].

PKC comprises a family of ten related serine/threonine kinases, which are grouped into one of three different subfamilies (classic, novel, and atypical PKC) on the basis of their requirements for Ca\(^{2+}\) for activation. Owing to the coexistence of multiple PKC isoenzymes in a tissue and lack of PKC isoenzyme-selective agonists or antagonists, the roles of PKC isoenzymes in myocardial I/R injury have been elusive. Recently, several studies have shown that a specific PKC\(\beta\) inhibitor ruboxistaurin (RBX) ameliorates I/R injury in various tissues including the heart, intestine and lung [11–13], and prevents myocardial hypertrophy and fibrosis of diabetes [14]. Despite the above important evidence, the role of PKC\(\beta\), which is preferably overexpressed in the myocardium of diabetic rodents, in myocardial I/R injury in diabetes has not been elucidated.

Our previous research suggests that PKC\(\beta\) requires Cav (caveolin)-3 for its activation, whereas its overactivation under diabetic or hyperglycaemic condition inhibits cardiac Cav-3 [8]. Cav-3 is a cardiac-specific caveolin and a membrane structural protein in caveolae of cardiomyocytes. Cav-3 is key to maintaining the normal structure of cardiomyocyte caveolae and retaining cardiomyocyte tolerance to I/R injury [15–17]. Our recent study shows that overexpressed PKC\(\beta\)2 in diabetic hearts negatively regulates both Cav-3 expression and the activation of Akt [8], a major molecule that promotes the survival of cardiomyocytes through the phosphorylation or inactivation of pro-apoptotic kinases [18,19], which is associated with impaired cardiac function and exacerbated post-ischaemic myocardial I/R injury in diabetes [9,20]. The present study aimed to explore whether or not hyperglycaemia-induced overexpression of PKC\(\beta\)2, via down-regulating cardiac Cav-3 and Akt, plays a critical role in exacerbating myocardial I/R injury in diabetes and to investigate the effects and mechanisms of PKC\(\beta\)2 inhibition in post-ischaemic myocardial reperfusion injury in diabetes.

MATERIALS AND METHODS

Induction of Type 1 diabetes and roboxistaurin treatment

Male Sprague–Dawley rats (250 ± 10 g, 6–8 weeks of age) were purchased from the Laboratory Animal Service Center at the University of Hong Kong. The animals were kept in a temperature-controlled room with a 12 h light/12 h dark cycle. Experiments were performed in accordance with the Animal Care Committee of the University of Hong Kong on the Use of Live Animals in Teaching and Research (CULATR). Diabetes was induced in rats by a single tail vein injection of streptozotocin (STZ) (65 mg/kg; Sigma–Aldrich) dissolved in citrate buffer (0.1 mol/l, pH 4.5) as described previously [14]. The glucose levels were measured 72 h after STZ injection, and rats were considered diabetic and used for the study only if they had blood glucose higher than 15 mmol/l. Diabetic rats were then randomized into three groups: sham-operated (Sham group), untreated control (Con group) and RBX-treated (RBX group). The rats remained in that particular group over the ensuing 5 weeks before being subjected to I/R or sham operation. At 1 week following diabetes induction, rats in the RBX group were treated with RBX through oral gavage at 1 mg/kg per day, a dose that was demonstrated to adequately inhibit PKC\(\beta\)2 activation in diabetic rat hearts [8,14], whereas rats in the Sham or Con group received same volume of saline as vehicle for 4 weeks. We previously found that plasma glucose levels remained stably high around 1 week after diabetes induction, whereas insulin levels were low [14]. STZ at a dose of 50–65 mg/kg induces hyperglycaemia at 20–30 mmol/l, but does not cause severe ketosis even without insulin supplement [21]. Therefore no insulin was given to the diabetic rats in the present study as we did previously [8,22]. We further found that administration of RBX (1 mg/kg per day) starting at 1 week after diabetes induction for 4 weeks was sufficient to attenuate oxidative stress and prevent the development of cardiomyopathy in this model [8]. Thus we used the same protocol as that of our previous study [8] to induce diabetes and administered RBX.

General characteristics such as water intakes were assessed on a daily basis, whereas food consumption, body weight and glucose levels were monitored every week.

Plasma level of free-15-F2t-isoprostane

15-F2t-IsoP (15-F2t-isoprostane), a specific biomarker of oxidative stress and lipid peroxidation in vivo [23], is produced by oxidation of tissue phospholipids, and was detected by using a commercial available competition enzyme immunoassay kit (Cayman Chemical) as described in [9,14]. Plasma samples were purified using Affinity Column and Affinity Sorbent (Cayman Chemical) following the manufacturer’s instructions. The assay relies on the competition between 15-F2t-IsoP in the plasma (free) and a 15-F2t-IsoP–acetylcholinesterase conjugate (15-F2t-IsoP tracer) for a limited number of 15-F2t-IsoP-specific rabbit antiserum-binding sites. The rabbit antiserum-free 15-F2t-IsoP complex can bind to the rabbit IgG mouse monoclonal antibody which was previously attached to the well to form a distinct yellow colour. The absorbance was assessed under the excitation of light at 412 nm. The values of free 15-F2t-IsoP were expressed as pg/ml in plasma samples.

In vivo myocardial ischaemia/reperfusion model

Myocardial I/R injury in vivo was induced by occluding the LAD (left anterior descending) coronary artery as described previously [9]. Our previous studies, which used that same rat model, have demonstrated that hearts from diabetic rats as early as 5 weeks after diabetes induction are more sensitive to myocardial I/R injury than hearts from non-diabetic subjects [9,24]. Briefly,
5 weeks after diabetes induction, diabetic rats were randomly divided into three groups ($n = 8$/group): Sham-operated (Sham), diabetic I/R untreated (Con) and I/R treated with RBX (RBX). Briefly, sodium pentobarbital-anaesthetized (65 mg/kg) control and RBX-treated diabetic rats were ventilated and supplemented with fresh air using a Harvard Apparatus Rodent Respirator at a rate of 60–70 breaths per min. A left thoracotomy was performed at the fourth intercostal space and the hearts were suspended in a pericardial cradle. The rats were subjected to 30 min of coronary occlusion followed by 2 h of reperfusion. Sham rats received all of the procedures without LAD ligation. At the end of the 2 h of reperfusion, 5% Evans Blue was intravenously administered. The stained region of the heart was identified as the area at risk (AAR) [24]. Then, heart tissues were quickly cut into six 2 mm cross-sectional slices and incubated in 1% TTC (2,3,5-triphenyltetrazolium chloride) buffer for 30 min at room temperature. The heart slices were then fixed with 10% formalin for 24 h. The area unstained by TTC was identified as the infarcted tissue. Myocardial IS (infarct size) was expressed as a percentage of the AAR.

In vivo haemodynamics measurements by PV (pressure–volume) loop

Left ventricular function in vivo was assessed by PV loops during ischaemia and reperfusion as described in [9,24]. Briefly, rats from Sham, Con and RBX groups were anaesthetized with sodium pentobarbital (65 mg/kg), and the right carotid artery and the left jugular vein in each heart were isolated. A Miller’s polyimide 1.9F PV conductance catheter (Scisense) connected to an Advantage PV control box (Scisense) was gently placed into the vessel and smoothly advanced into the left ventricle apex until the PV signal was displayed. Then the rats were stabilized for 15 min before baseline data were recorded. The chest was opened and the LAD coronary artery was ligated followed by reperfusion. Saline was given through a plastic tube cannulating the left jugular vein every 30 min for preventing dehydration. The haemodynamic cardiac functions recorded in diabetic hearts include HR (heart rate), LVP$_{max}$ (maximal left ventricular pressure), and load-independent contractility parameters including $dP/dt_{max}$ (peak rate of left ventricular pressure increase), $dP/dt_{min}$ (minimal rate of left ventricular pressure decrease), SV (stroke volume), SW (stroke work), CO (cardiac output) (CO = SV × HR) and Ea (arterial elastance) (Ea = ESP/SV). The data from the haemodynamic recordings were calculated using PVAN3.2 software.

In situ TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling)

In situ detection of apoptosis was assessed by TUNEL assay using an Apoptosis Detection Kit, POD (Roche) according to the manufacturer’s protocol as described in [22]. Paraffin-embedded left ventricular tissue blocks were sectioned at 5 mm and were dewaxed and rehydrated with serial changes of xylene and ethanol. Proteinase K (20 mg/l) was applied to the section for 15 min to produce optimal proteolysis. Hydrogen peroxide (3%) was used to inhibit the endogenous peroxidase. The terminal deoxynucleotidyltransferase reaction was carried out for 1 h at 37 °C, and HRP (horseradish peroxidase)-conjugated antidigoxigenin was applied at room temperature for 30 min followed by haematoxylin/eosin staining. The apoptosis in the left ventricle of heart tissue was assessed under light microscopy. Cell apoptosis was determined as the percentage of apoptotic cells in all cells.

Plasma CK-MB (creatine kinase-MB) and LDH (lactate dehydrogenase)

The levels of CK-MB in plasma were determined by rat ELISA assay (R&D Systems) according to the manufacturer’s instructions as described previously [9]. The cytoplasmic enzyme CK-MB reversibly catalyses the reaction of ATP and creatine to form phosphocreatine and ADP. Increased CK-MB concentration was used as an index of myocardial cell injury. Briefly, blood samples from diabetic hearts at the end of the experiments were collected and assayed in a 96-well plate pre-coated with antibody specific to CK-MB. After following the manufacturer’s guide, an increase in absorbance at a wavelength of 450 nm is an indication of increased CK-MB in plasma.

The release of the cytoplasmic enzyme LDH is an indicator of cell death and myocardial injury. LDH in plasma from diabetic hearts was assessed by using a commercially available Cytotoxicity Detection Kit (Roche) as described previously [9]. LDH catalyses the conversion of lactate into pyruvate with concomitant transfer of the electrons from NAD to NADH in the presence of lactate substrate. An increase in colorimetric signal at a wavelength of 450 nm is an indication of LDH release in plasma.

Genetic knockdown of PKCβ2 and induction of hypoxia/reoxygenation in vitro

Cardiac H9C2 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) with 5.5 mmol/l glucose (Gibco Life Technologies) containing 10% (v/v) FBS as described previously [22]. When cells reached 50% confluence, 1 μM compound CGP41274 (Sigma–Aldrich) was added or in alternative studies scrambled siRNA, PKCβ2 siRNA, Cav-3 siRNA and Akt siRNA (Santa Cruz Biotechnology) were respectively transfected into cells according to the manufacturer’s protocol.

At 2 before experiments, the medium was replaced with high-glucose (30 mmol/l) DMEM containing 10% (v/v) FBS and cells were cultured for 48 h. After 48 h, the cells were then exposed to 4 h of hypoxia (1% O$_2$, 5% CO$_2$, 94% N$_2$) in glucose- and serum-free DMEM followed by 4 h of reoxygenation (H/R) in high-glucose DMEM containing 10% (v/v) FBS.

Assessment of mitochondrial membrane potential ($\Delta \psi_m$) in vitro

Fluorescent images of JC-1 aggregated and monomeric cells were measured using a mitochondrial membrane potential assay kit (Cayman Chemical). In apoptotic cells, the loss of $\Delta \psi_m$ causes the formation of JC-1 monomeric mitochondria. In healthy cells with high $\Delta \psi_m$, JC-1 spontaneously forms complexes known as JC-1 aggregates. Briefly, H9C2 cells were incubated with JC-1 working solution for 30 min at 37 °C. Then the cells were...
Table 1  General characteristics of rats with STZ-induced diabetes
All values are expressed as means ± S.E.M. n = 8 per group. General parameters including food consumption, water intake, body weight, plasma 15-F2t-IsoP were measured before and after RBX treatment in diabetic rats (Sham or Con) before myocardial ischaemia. *P < 0.05 compared with before RBX treatment; #P < 0.05 compared with Sham and Con.

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<th>After RBX treatment</th>
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<td></td>
<td>Sham</td>
<td>Con</td>
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<tr>
<td>Food consumption (g/kg per day)</td>
<td>86 ± 6</td>
<td>87 ± 5</td>
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<tr>
<td>Water intake (ml/kg per day)</td>
<td>444 ± 25</td>
<td>442 ± 26</td>
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<tr>
<td>Body weight (g)</td>
<td>286 ± 6</td>
<td>270 ± 3</td>
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<td>Plasma glucose (mmol/l)</td>
<td>27 ± 1</td>
<td>25 ± 1</td>
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<td>Plasma 15-F2t-IsoP (pg/ml)</td>
<td>43 ± 4</td>
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Cardiomyocyte apoptotic cell death
In vitro apoptotic cell death was detected using a TUNEL Cytotoxicity Detection Kit as described in [25]. Briefly, H9C2 cells were fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. Then the fixed cells were incubated with 0.1% Triton X-100 for 10 min. After washing cells with PBS, 50 μl of TUNEL reaction mixture was added, and cells were incubated at 37°C for 1 h. Cells were then washed and labelled with DAPI to counterstain DNA. The green (TUNEL)/blue (DAPI) fluorescence ratios were calculated under fluorescence microscopy as ratios between JC-1 monomeric cells and JC-1 aggregates.

Immunoblotting
Left ventricular tissue was homogenized in protein lysis buffer (20 mM Tris/HCl, pH 7.5, 50 mM 2-mercaptoethanol, 5 mM EGTA, 2 mM EDTA, 10 mM NaF, 1 mM PMSF, 25 mg/ml leupeptin and 2 mg/ml aprotinin). H9C2 cells were lysed by cell lysis buffer (Cell Signaling Technology) with 1% protease-inhibitor cocktail (Sigma–Aldrich). The supernatant was collected and protein concentrations were determined by the Bradford protein concentration method. The samples were separated by SDS/PAGE (8% or 12% gel) and then transferred on to PVDF membranes (Bio-Rad Laboratories). The membranes were blocked at room temperature for 1 h with 5% blocking grade non-fat dry milk (Bio-Rad Laboratories) in TBS-T (TBS containing 0.1% Tween 20 solution). Then the membranes were incubated with antibodies against p-PKCβ2, cleaved caspase 3, p-Akt (Ser473), Akt, β-actin (Cell Signaling Technology), PKCβ2 (Abcam) and Cav-3 (Santa Cruz Biotechnology) overnight at 4°C. The membranes were incubated with HRP-conjugated secondary antibodies (Cell signaling) for 2 h at room temperature. The blots were visualized using ECL Western blotting detection reagents (GE Healthcare). ImageJ software (NIH) was used for densitometric analysis of immunoblots.

Statistical analysis
Data are presented as means ± S.E.M. One- and two-way ANOVA were used for statistical analysis (GraphPad Prism) of data obtained within the same group of rats and between groups of rats respectively, followed by Tukey’s test for multiple comparisons of group means. P < 0.05 was considered to indicate statistically significant differences.

RESULTS
RBX reduced plasma 15-F2t-IsoP in diabetic rats
As shown in Table 1, daily water and food intake were significantly increased in rats with 5-week STZ-induced diabetes compared with that in rats with 1-week diabetes (both P < 0.05).

Body weight gain in rats at 5-week diabetes was not significantly different from the body weight in rats with 1 week of STZ-induced diabetes. Plasma glucose levels reached as high as 27 ± 1 mmol/l in the first week and remained stable throughout the fifth week (28 ± 1 mmol/l). The levels of plasma-free 15-F2t-IsoP in rats with 5-week diabetes were significantly higher than that in rats with 1-week diabetes. RBX did not change water and food consumption or body weight, but significantly decreased plasma levels of 15-F2t-IsoP (P < 0.05) (Table 1).

RBX improved left ventricular function after I/R injury
Left ventricular function of rats was measured with a PV conductance catheter at baseline, 30 min after ischaemia, and 30, 60 and 120 min after reperfusion. As shown in Figure 1, there were no significant differences in all cardiac parameters measured among the three groups at baseline (P < 0.05). Myocardial ischaemia did not significantly decrease dP/dtmax, LVP, CO, SW, HR and Ea. However, reperfusion significantly decreased HR, dP/dtmax, dP/dtmin, LVPmax and Ea. All of these I/R injury-induced impairments in cardiac functions were significantly attenuated or reversed by RBX (P < 0.05).

RBX reduces post-ischaemic myocardial infarct size and apoptosis
Figure 2 shows the beneficial effects of RBX on reducing post-ischaemic myocardial infarct size. TUNEL-positive cells and the releases of LDH and CK-MB. There were no significant differences in AAR between groups. Coronary occlusion followed by reperfusion resulted in 49 ± 3% of IS/AAR in Con rats, which was significantly decreased to 35 ± 3% (P < 0.05) by RBX (Figure 2A). Apoptotic cardiomyocytes in the peri-infarct area were...
Inhibition of PKCβ2 ameliorates myocardial ischaemia/reperfusion injury in diabetic rats

Figure 1  Cardiac function measured by pressure–volume loop analysis

(A) HR; (B) dP/dt_{max}; (C) dP/dt_{min}; (D) LVP_{max}; (E) CO; (F) SV; (G) Ea. PV parameters were dynamically measured at baseline (B), 30 min after ischaemia (I30), and at 30 min (R30), 60 min (R60) and 120 min (R120) after reperfusion. Diabetic rats were treated with RBX or vehicle (Con) for 4 weeks. At the end of treatment, rats were subjected to 30 min of ischaemia followed by 120 min of reperfusion. Sham animals underwent all surgical procedures without ischaemia (Sham). Results are means ± S.E.M. (n = 5 rats per group). *P < 0.05 compared with Sham and RBX; #P < 0.05 compared with Sham.

evaluated by TUNEL staining. The number of TUNEL-positive cardiomyocytes in the Con group was significantly greater than in the Sham group (P < 0.05, Figure 2B). The I/R-induced increases in IS and apoptosis were significantly attenuated by RBX (P < 0.05). Both LDH and CK-MB are the necrotic cell markers. Myocardial I/R significantly enhanced LDH and CK-MB concentrations in the plasma of diabetic rats subjected to I/R compared with rats in the Sham group (P < 0.05, Figures 2C and
Figure 2. Effects of ruboxistaurin on in vivo myocardial I/R injury

(A) Myocardial infarct size expressed as a percentage of the AAR. The upper panels are the representative cross-sections of the hearts stained by TTC and Evans Blue from the rats of Sham, Con and RBX respectively. The white area is the infarct region, the black area is area not at risk, and the red area is the viable myocardium within the AAR. (n = 5 per group). (B) In situ TUNEL-positive cells expressed as the fold change relative to Sham. Upper panels are the representative left ventricle section of the hearts stained by TUNEL and haematoxylin/eosin staining from the rats of Sham, Con and RBX respectively. Apoptotic nuclei are stained dark brown and normal nuclei are stained blue (n = 5 rats per group, magnification ×200, scale bar, 25 μm). (C) LDH concentration in plasma (n = 5 rats per group). (D) CK-MB concentration in plasma (n = 5 rats per group). Diabetic rats were treated with RBX or vehicle (Con) for 4 weeks. At the end of treatment, rats were subjected to ischaemia followed by reperfusion. Sham animals underwent all surgical procedures without ischaemia (Sham). Results are means ± S.E.M. *P < 0.05 compared with Sham; #P < 0.05 compared with Con.

2D), whereas the pre-treatment with RBX significantly decreased the I/R-induced increases in LDH and CK-MB (P < 0.05).

Effect of RBX on post-ischaemic myocardial protein expressions of p-PKCβ2, Cav-3, p-Akt and cleaved caspase 3

Myocardial ischaemia followed by 2 h of reperfusion significantly increased cardiac p-PKCβ2 expression, but decreased Cav-3 and p-Akt expression in diabetic rats (all P < 0.05; Figures 3A, 3B and 3C). Myocardial I/R also increased the level of cleaved caspase 3, an active form of caspase 3 (P < 0.05, Figure 3D). The 4-week RBX pre-treatment could dramatically attenuate I/R-induced increases in p-PKCβ2 expression, restore Cav-3 expression and increase p-Akt expression further in diabetic rats (P < 0.05; Figures 3A, 3B and 3C).

Genetic knockdown or pharmacological inhibition of PKCβ attenuated H/R-induced increase in apoptotic cell death and reduction in ΔΨm

Cardiomyocyte apoptosis was evaluated by TUNEL (Figure 4, upper row images) and DAPI (Figure 4, lower row images) dual staining. As shown in Figure 4(A), the number of TUNEL-positive cells was significantly higher in H/R-stimulated cells (P < 0.05). This result was largely reduced by a specific PKCβ2 inhibitor, CGP41253, to an extent similar to that in normoxia group (P < 0.05; Figure 4A). Similarly, PKCβ2 siRNA significantly decreased the H/R-induced number of TUNEL-positive cells to an extent similar to that in normoxia group (P < 0.05; Figure 4B). Cav-3 or Akt siRNAs under normoxic conditions significantly increased the number of TUNEL-positive cells (P < 0.05; Figures 4C and 4D). Both Cav-3 siRNA and Akt siRNA exacerbated H/R-induced TUNEL-positive cells (P < 0.05; Figures 4C and 4D).
Inhibition of PKC/2 ameliorates myocardial ischaemia/reperfusion injury in diabetic rats

Figure 3 Effect of ruboxistaurin on myocardial protein expression in vivo

(A) Myocardial p-PKC/2 expression. Upper panels show representative Western blot bands of p-PKC/2 and β-actin from rat hearts of Sham, Con and RBX groups after 30 min of reperfusion. (B) Myocardial Cav-3 expression. Upper panels show representative Western blot bands of Cav-3 and β-actin from rat hearts of Sham, Con and RBX groups 30 min after reperfusion. (C) Myocardial p-Akt/Akt expression. Upper panels show representative Western blot bands of p-Akt, total Akt and β-actin from rat hearts of Sham, Con and RBX groups 30 min after reperfusion. (D) Myocardial cleaved caspase 3 expression. Upper panels show representative Western blot bands of cleaved caspase 3 and β-actin from rat hearts of Sham, Con and RBX groups 120 min after reperfusion. Diabetic rats were treated with RBX or vehicle (Con) and subsequently subjected to ischaemia (I) followed by reperfusion (R). Sham animals underwent all surgical procedures without ischaemia (Sham). Results are means ± S.E.M. (n = 3 rats per group). *P < 0.05 compared with Sham; #P < 0.05 compared with Con.

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Figure 4  Hypoxia/reoxygenation-induced apoptotic cells were reduced by either CGP41251 (CGP) or PKCβ2 siRNA, but increased by either Cav-3 siRNA or Akt siRNA.

(A) PKCβ2 inhibitor CGP41251 reduced H/R-induced apoptotic cells. Upper panels are representative fluorescent images of TUNEL and DAPI staining cells from Nor, CGP, H/R and H/R+CGP groups. Apoptotic cells are expressed as a percentage of TUNEL-positive nuclei of DAPI-positive nuclei. TUNEL-positive apoptotic nuclei show green fluorescence. DAPI-positive normal nuclei show blue fluorescence. 

(B) Knockdown of PKCβ2 reduces H/R-induced apoptotic cells. Upper panels show representative fluorescent images of TUNEL and DAPI staining cells from Nor, PKCβ2 siRNA, H/R and H/R+PKCβ2 siRNA groups. 

(C) Knockdown of Cav-3 increases H/R-induced apoptotic cells. Upper panels show representative fluorescent images of TUNEL and DAPI staining cells from Nor, Cav-3 siRNA, H/R and H/R+Cav-3 siRNA groups. 

(D) Knockdown of Akt increases H/R-induced apoptotic cells. Upper panels show representative fluorescent images of TUNEL and DAPI staining cells from Nor, Akt siRNA, H/R and H/R+Akt siRNA groups. H9C2 cells were exposed to high glucose for 48 h and subsequently treated with CGP or PKCβ2, Cav-3 or Akt siRNA for 24 h. Then the cells were exposed to 4 h of hypoxia followed by 4 h of reoxygenation (H/R). Normoxia cells underwent normal oxygen (Nor). Results are means ± S.E.M. (n = 5 per group, magnification ×100, scale bar, 12.5 μm). *P < 0.05 compared with Nor; #P < 0.05 compared with H/R.
The variations in apoptotic cell death assessed by DAPI staining mirrored those measured by TUNEL staining (results not shown).

Mitochondrial damage was measured using a mitochondrial membrane potential kit. The increase in the number of JC-1 monomeric cells reflected the loss of $\Delta \psi_m$. Compared with normoxia cells, the number of JC-1 monomeric cells was remarkably increased in H/R-stimulated cells ($P < 0.05$). The H/R-induced increase in monomeric form cells were reduced by CGP41254, to the extent of that in normoxia cells ($P < 0.05$; Figure 5A). Similarly, PKCβ2 siRNA significantly reduced the H/R-induced JC-1 monomeric cells to an extent similar to that in normoxia cells ($P < 0.05$; Figure 5B). Both Cav-3 siRNA and Akt siRNA under normoxic conditions significantly increased JC-1 monomeric cells ($P < 0.05$) to an extent similar to that under hypoxic conditions. Both Cav-3 siRNA and Akt siRNA exacerbated H/R-induced monomeric cells ($P < 0.05$; Figures 5C and 5D).

Protein expression of p-PKCβ2, Cav-3, p-Akt and cleaved caspase 3 following H/R and PKCβ2 or Cav-3 gene knockdown

Protein expression of p-PKCβ2, Cav-3, p-Akt and cleaved caspase 3 in H9C2 cells were measured by Western blotting. As shown in Figure 6(A), Knockdown of PKCβ2 with siRNA attenuated p-PKCβ2 expression by 23% in the normoxia group and by 29% in the hypoxia group ($P < 0.05$). Knockdown of Cav-3 reduced p-PKCβ2 expression by 40% in the normoxia group and by 42% in the hypoxia group ($P < 0.05$; Figure 6B). Cav-3 expression was lower in H/R-stimulated cells compared with normoxia cells. This reduction was restored by PKCβ2 siRNA in both the normoxia and the hypoxia group ($P < 0.05$; Figure 6C). Cav-3 siRNA suppressed Cav-3 expression by 68% under normoxic conditions, and by 40% under hypoxic conditions ($P < 0.05$; Figure 6D). p-Akt expression was significantly increased in H/R-stimulated cells. PKCβ2 siRNA remarkably increased, but Cav-3 siRNA decreased, p-Akt expression ($P < 0.05$; Figure 6E). Cleaved caspase 3 expression was decreased by PKCβ2 siRNA, but increased by Cav-3 siRNA ($P < 0.05$; Figure 6F).

DISCUSSION

In the present study, we demonstrated the protective effects of RBX, a selective PKCβ2 inhibitor at the dosage used [14], on acute myocardial I/R injury in rats with STZ-induced diabetes, and the role of the Cav-3/Akt pathway in PKCβ inhibition mediated protection against post-ischaemic and post-hypoxic cardiomyocyte injury. We found that (i) I/R induced significant cardiac dysfunction and myocardial infarction in diabetic rats, which was accompanied by increased protein expression of phosphorylated PKCβ2 and down-regulated Cav-3; (ii) RBX administration starting at the onset of diabetes for 4 weeks conferred cardioprotection through specific inhibition of PKCβ2, and restoration of Cav-3 and phosphorylated Akt; (iii) PKCβ2 gene knockdown attenuated the increase in H/R-induced apoptotic cardiomyocyte death and restored $\Delta \psi_m$ through up-regulation of Cav-3 and phosphorylated Akt in high-glucose-exposed cardiomyocytes; (iv) Cav-3 gene knockdown caused apoptotic cardiomyocyte death and exacerbated H/R-induced cardiomyocyte and mitochondrial injury by decreasing phosphorylated Akt. To our knowledge, the present study provides the first evidence that RBX ameliorates myocardial I/R injury via a PKCβ2-dependent mechanism, and the cardioprotection of RBX is achieved at least in part through Cav-3/Akt-mediated mitochondrial pathway.

Myocardial infarction and dysfunction are the major assessments of I/R injury in vivo [26–28]. RBX has previously been shown to have beneficial effects on microvascular dysfunction in diabetic hearts [29–32], and has a post-ischaemic infarction-limiting effect in non-diabetic hearts [33]. However, the cardiomyocyte-specific effect of RBX on I/R injury in diabetic rodents has not been studied. In the present study, chronic RBX administration in diabetic rat hearts sufficiently reduced infarct size by 29% (Figure 2A), and apoptosis by 22.2% (Figure 2B). In addition, RBX improved cardiac contractile function by preserving $dP/dt_{max}$ and $dP/dt_{min}$, two indices of contractility (Figure 1B and 1C) with concomitant reduction of plasma levels of CKMB and LDH, markers of cardiomyocyte cellular injury (Figure 2C and 2D). These results suggest that PKCβ activation plays detrimental roles in diabetes during I/R injury. However, we did not observe significantly beneficial effects of RBX on cardiac function at baseline or ischaemia. This may be due to the delayed administration of RBX (1 week after diabetes induction) that may have allowed some of the early adverse effects of diabetes to develop. We could not exclude the possibility that administration of RBX immediately at the onset of diabetes could largely improve cardiac function. The beneficial effect of RBX may due to its ability to prevent oxidative stress and delay the progression to myocardial dysfunction and remodelling. Our previous study provides evidence that RBX processes antioxidant properties, attenuates hyperglycaemia-induced oxidative stress, and preserves diastolic function of diabetic hearts [14]. Several studies have also supported the antioxidant capacity of RBX during I/R injury [11,34] and diabetes [8,14]. In line with our previous studies, we showed that RBX treatment significantly reduced plasma levels of 15-F2t-IsoP (Table 1), a specific index of oxidative stress. Reduction of the hyperglycaemia-mediated increase in oxidative stress should be a potential mechanism whereby RBX attenuates post-ischaemic myocardial infarction in diabetes, given that, in the same in vivo I/R model in type 1 diabetic rats, we were able to show that effective antioxidant treatments applied for 4 weeks before inducing I/R to reduce plasma 15-F2t-IsoP can significantly reduce post-ischaemic myocardial infarction [22,24], as seen in the present study. In the present study, we, for the first time, demonstrate that RBX has the potential to attenuate the early phase of post-ischaemic myocardial I/R injury in diabetes, although the longer-term cardioprotective effects in diabetes such as post-ischaemic myocardial remodelling have not yet been studied.

The mechanisms by which RBX protect hearts from I/R injury are not well understood. Although aberrant cell apoptosis has been shown to play crucial roles in the pathogenesis of myocardial infarction and dysfunction [35–37] in normal hearts, its role in diabetic hearts during I/R has not been studied. Distinct from necrosis, which is mainly associated with cell swelling
Hypoxia/reoxygenation-induced JC-1 monomeric mitochondria were reduced by either CGP41251 (CGP) or PKCβ2 siRNA, but increased by either Cav-3 siRNA or Akt siRNA.

(A) Inhibition of PKCβ2 with CGP reduced H/R-induced JC-1 monomeric mitochondria. Upper panels show representative fluorescent images of JC-1 monomeric mitochondria showing green fluorescence and JC-1 aggregated mitochondria showing intense red fluorescence from Nor, CGP, H/R and H/R+CGP groups. (B) PKCβ2 knockdown reduced H/R-induced JC-1 monomeric mitochondria. Upper panels show representative fluorescent images of JC-1 monomeric and aggregated mitochondria from Nor, PKCβ2 siRNA, H/R and H/R+PKCβ2 siRNA groups. (C) Cav-3 gene knockdown increased H/R-induced JC-1 monomeric mitochondria. Upper panels show representative fluorescent images of JC-1 monomeric and aggregated mitochondria from Nor, Cav-3 siRNA, H/R and H/R+Cav-3 siRNA groups. (D) Akt knockdown increased H/R-induced JC-1 monomeric mitochondria. Upper panels show representative fluorescent images of JC-1 monomeric and aggregated mitochondria from Nor, Akt siRNA, H/R and H/R+Akt siRNA groups. H9C2 cells were exposed to high glucose and subsequently treated with CGP or PKCβ2, Cav-3 or Akt siRNA. Then the cells were exposed to hypoxia followed by reoxygenation (H/R). Normoxia cells underwent normal oxygen (Nor). Results are means ± S.E.M. (n = 5 per group, magnification ×100, scale bar, 12.5 μm). *P < 0.05 compared with Nor; #P < 0.05 compared with H/R.
Inhibition of PKC/β2 ameliorates myocardial ischaemia/reperfusion injury in diabetic rats

Figure 6 Effect of the siRNA of PKC/β2, Cav-3 or Akt on cardiomyocyte protein expression in vitro

(A) Effect of PKC/β2 knockdown on p-PKCβ2 and PKCβ2 protein levels in high-glucose-exposed cardiac cells. (B) Effect of Cav-3 knockdown on p-PKCβ2 and PKCβ2 protein expression. (C) Effect of PKCβ2 knockdown on Cav-3 protein levels. (D) Effect of Cav-3 knockdown on Cav-3 protein expression. (E) Effect of PKCβ2 or Cav-3 knockdown on p-Akt/Akt expression. (F) Effect of PKCβ2 or Cav-3 knockdown on cleaved caspase 3 expression. H9C2 cells were exposed to high glucose and subsequently treated with PKCβ2, Cav-3 or Akt siRNA. Then the cells were exposed to hypoxia followed by reoxygenation (H/R). Normoxia cells underwent normal oxygen (Nor). Results are means ± S.E.M. (n = 3 per group). *P < 0.05 compared with Nor; #P < 0.05 compared with H/R.
and inflammation, apoptosis is a programmed cell death that involves DNA fragmentation and activation of caspase family, in particular caspase 3, which can in turn activate DNA degradation leading to cell death. In the present study, RBX significantly attenuated DNA fragmentation as was evident by increased TUNEL-positive cells (Figure 2B) and activated/cleaved caspase 3, markers of cell apoptosis (Figure 3D) in the myocardium of diabetic rats. Additionally, using high-glucose-stimulated cardiac cells, we demonstrated that inhibition of PKCβ2 with siRNA or CGP41254L attenuated the hyperglycaemia-induced increase in TUNEL-positive cells (Figure 4B) and activated/cleaved caspase 3 (Figure 6F). Our in vivo and in vitro data strongly suggest that increased PKCβ2 activation plays a critical role in exacerbating cardiomyocyte apoptosis, a major form of cardiomyocyte death following ischaemia/hypoxia under hyperglycaemic conditions.

Hyperglycaemia increased the interaction of Cav-3 with PKCβ2 on lipid rafts. Following PKCβ2 activation, PKCβ2 translocates from the cytosol to the cell membrane where it increases its association with Cav-3 in cardiomyocytes [8]. This process may increase the damage of cell membranes due to the decrease in Cav-3 levels in lipid rafts caused by hyperglycaemia [8]. Despite the findings regarding the alterations of Cav-3 in diabetic hearts, the role of Cav-3 in PKCβ2 activation and apoptosis in post-ischaemic myocardial injury has not been examined. We found that RBX down-regulated cardiac Cav-3 in diabetic myocardium (Figure 3B). In in vitro study, we confirmed that PKCβ2 gene knockdown and inhibition increased the post-ischaemic Cav-3 expression (Figure 6C). In contrast with PKCβ2 knockdown of Cav-3 enhanced cell apoptosis (Figures 4C and 6F), but concomitantly reduced PKCβ2 phosphorylation (Figure 6B), which indicated that Cav-3 is a salvage molecule for cardiomyocyte survival and the basal level of Cav-3 is required for PKCβ2 activation. Akt is a well-established cardioprotective molecule for cell survival during I/R injury [24,38]. In the present study, RBX enhanced phosphorylated Akt, although Akt phosphorylation was not significantly altered following I/R in diabetic myocardium (Figure 3C). In high-glucose-exposed cardiac cells, we observed the decreased phosphorylated Akt following H/R, whereas PKCβ2 siRNA enhanced p-Akt levels (Figure 6E). The difference of this result may be partially due to the experimental complicity in in vivo and in vitro study including the duration of diabetes, degree of ischaemia and reperfusion that may differentially contribute to p-Akt expression in the heart.

The consequence of abnormal signalling transduction induced by I/R is the mPTP (mitochondrial permeability transition pore) opening and depolarization of the Δψ_m to increase cell death. The mPTP opening results in loss of Δψ_m, uncoupling of respiratory chain, Ca^{2+} accumulation, release of cytochrome c and free radicals, and mitochondrial swelling [39–42]. As shown in Figures 5(A) and 5(B), the Δψ_m was regulated by PKCβ2 as was evident as a lower number of JC-1 monomeric cells after pharmacological inhibition or genetic knockdown of PKCβ2. Cav-3 and Akt are both required for the PKCβ2-mediated protection against mitochondrial dysfunction, as both Cav-3 and Akt siRNA significantly increased JC-1 monomeric cells in the H/R group (Figures 5C and 5D).

In summary, the present study demonstrates that RBX ameliorates myocardial I/R injury by attenuating post-ischaemic myocardial infarction and dysfunction. The plausible mechanism underlying the cardioprotection of RBX is via Cav-3/Akt-mediated attenuation of post-hypoxic mitochondrial injury.

**CLINICAL PERSPECTIVES**

- This study may have high translational potential given that ruboxistaurin has now been approved by the U.S. FDA (Food and Drug Administration) for clinical trials to treat diabetic peripheral neuropathy.
- Our findings that ruboxistaurin, as an oral PKCβ inhibitor with antioxidant properties that can attenuate post-ischaemic myocardial infarction and dysfunction in functionally impaired hearts of diabetic rodents, strongly suggest that ruboxistaurin may have great potential to prevent the development of ischaemic heart diseases in patients with diabetes, especially when antioxidants or other medications are not clinically effective at clinical settings nowadays.
- Meanwhile, the mechanisms identified as being involved in ruboxistaurin-induced cardioprotection may help in the development of strategies to improve the prognoses of acute myocardial infarction after surgery such as revascularization in patients with diabetes.

**AUTHOR CONTRIBUTION**

Zhengyuan Xia and Yanan Liu designed the research. Yanan Liu, Jiqin Jin, Shigang Qiao, Shaqing Lei, Songyan Liao, Haobo Li and Gordon Wong performed the research. Yanan Liu, Jiqin Jin, Zhi-Dong Ge, Michael Irwin and Zhengyuan Xia analysed the data. Yanan Liu, Michael Irwin and Zhengyuan Xia wrote the paper.

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