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Inhibition of PKC α / β with ruboxistaurin antagonizes heart failure in pigs after myocardial infarction injury

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Abstract

Rationale—Protein kinase C α (PKC α) activity and protein level are induced during cardiac disease where it controls myocardial contractility and propensity to heart failure in mice and rats. For example, mice lacking the gene for PKC α have enhanced cardiac contractility and reduced susceptibility to heart failure following long-term pressure overload or after myocardial infarction injury. Pharmacologic inhibition of PKC α / β with Ro-32-0432, Ro-31-8220 or ruboxistaurin (LY333531) similarly enhances cardiac function and antagonizes heart failure in multiple models of disease in both mice and rats.

Objective—Large and small mammals differ in several key indexes of heart function and biochemistry, lending uncertainty as to how PKC α / β inhibition might affect or protect a large animal model of heart failure.

Methods and Results—Here we demonstrate that ruboxistaurin administration to a pig model of myocardial infarction-induced heart failure was protective. Twenty-kilogram pigs underwent left anterior descending artery (LAD) occlusion resulting in myocardial infarctions and were then divided into vehicle or ruboxistaurin feed groups, after which they were monitored monthly for the next 3 months. Ruboxistaurin administered pigs showed significantly better recovery of myocardial contractility 3 months after infarction injury, greater ejection fraction, and greater cardiac output compared with vehicle treated pigs.

Conclusions—These results provide additional evidence in a large animal model of disease that PKC α / β inhibition (with ruboxistaurin) represents a tenable and novel therapeutic approach for treating human heart failure.

Keywords

Heart failure; contractility; PKC; signaling; cardiomyopathy

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Disclosures:

The ruboxistaurin compound was obtained from Eli Lilly under an MTA.

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Introduction

The protein kinase C family of Ca^{2+} and/or lipid-activated serine-threonine protein kinases are critical mediators of signal transduction in the heart where they regulate disease responsiveness.^{1,2} The PKC family is broadly classified by activation characteristics, such that the conventional PKC isozymes ($\text{PKC}\alpha$, $\beta\text{I/II}$, and γ) are Ca^{2+} - and lipid-activated, while the novel isozymes (ϵ , θ , η , and δ) and atypical isozymes (ζ , and λ) are Ca^{2+} independent but activated by distinct lipids.^{1,2} $\text{PKC}\alpha$ is the predominant conventional PKC isoform expressed in the mouse, rabbit, and human heart,³⁻⁵ and many different disease causing stimuli result in its activation, including heart failure.⁶⁻¹³

Transgenic mice with greater $\text{PKC}\alpha$ activity showed decreased cardiac contractility, ventricular dilation, and secondary hypertrophy, suggesting that increased $\text{PKC}\alpha$ signaling is detrimental to the heart.^{14,15} Indeed, $\text{PKC}\alpha^{-/-}$ mice are protected from insults or genetic mutations that would otherwise induce heart failure.^{14,16} By comparison, $\text{PKC}\beta/\gamma^{-/-}$ mice showed more severe heart failure when stressed,¹⁷ suggesting that $\text{PKC}\alpha$ is the primary disease affecting isoform in the heart and the best candidate for inhibition. Transgenic mice with inducible expression of a dominant negative $\text{PKC}\alpha$ mutant in cardiomyocytes of the heart also showed reduced failure progression after myocardial infarction (MI) injury.¹⁶

Results in genetically modified animal models and in isolated adult myocytes clearly showed a cardioprotective effect with $\text{PKC}\alpha$ inhibition. Thus, we and others carefully examined the effects of cPKC inhibitors of the bisindolylmaleimide class, such as ruboxistaurin (LY333531), Ro-32-0432 or Ro-31-8220, in different rodent heart failure models. Short-term or long-term treatment with Ro-31-8220 in the *Csrp3* null mouse model of heart failure augmented cardiac contractility and restored pump function.⁵ PKC inhibition with Ro-31-8220 or Ro-32-0432 also reduced mortality and cardiac contractile abnormalities in a mouse model of myotonic dystrophy type 1.¹⁸ Another $\text{PKC}\alpha/\beta$ inhibitor, ruboxistaurin, prevented death in wildtype mice throughout 10 weeks of pressure-overload stimulation, reduced ventricular dilation, enhanced ventricular performance, reduced fibrosis, and reduced pulmonary edema comparable to or better than metoprolol treatment.¹⁷ Ruboxistaurin was also administered to $\text{PKC}\beta/\gamma$ null mice subjected to pressure overload, resulting in less death and heart failure, strongly suggesting $\text{PKC}\alpha$ as the primary target of this drug in mitigating heart disease.¹⁷ In addition, Boyle et al. showed that ruboxistaurin reduced ventricular fibrosis and dysfunction following MI in rats.¹⁹ Ruboxistaurin treatment also significantly decreased infarct size and enhanced recovery of left ventricular function and reduced markers of cellular necrosis in mice subjected to 30 min of ischemia followed by 48 h of reperfusion.²⁰ Connelly et al. demonstrated that ruboxistaurin attenuated diastolic dysfunction, myocyte hypertrophy, collagen deposition, and preserved cardiac contractility in a rat diabetic heart failure model.²¹ These results in rodents overwhelmingly support the contention that $\text{PKC}\alpha/\beta$ inhibition with ruboxistaurin, or related compounds, protects the heart from failure after injury. Hence, if ruboxistaurin is similarly protective in a large animal model of heart failure, there should be little resistance remaining towards initiating clinical trials in patients with heart failure, especially given the apparent safety of this compound in other human trials.²²

Methods

Animal studies and MI model

This study, using female Yorkshire pigs (~ 20 kg body weight) was approved by the Institutional Animal Care and Use Committee. All procedures were performed under propofol (2-10 mg/kg/h) anaesthesia. For MI generation, we introduced an 8F sheet into the femoral artery and cannulated the left anterior descending (LAD) coronary artery with an 8F

hockey stick guiding catheter (Cordis Infiniti, Johnson & Johnson). After injecting 100 mg nitroglycerin and obtaining a baseline coronary angiogram, we placed a 5F balloon catheter (Cordis Infiniti, Johnson & Johnson) into the LAD after the first diagonal branch, thus occluding 2/3rds of the LAD tributary for 90 minutes. The resulting infarct size was approximately 15% of the left ventricle, determined by TTC staining. The 48-hr survival rate was 86% (n = 15). Thirteen animals were randomised to receive either control pig chow or pig chow enriched with Ruboxistaurin (Eli Lilly).

Oral treatment with ruboxistaurin

Pigs were given 10 mg/kg/day ruboxistaurin in separate doses twice a day starting immediately after MI until 12 weeks. Ruboxistaurin was administered mixed with the regular animal diet. Four days of oral ruboxistaurin treatment (2 days at 5 mg/kg/day and 2 days at 10 mg/kg/day) produced plasma levels of 93 +/- 31 ng/ml of ruboxistaurin and 817 +/- 179 ng/ml of the primary metabolite.

Assessment of myocardial function and structure

We assessed myocardial function and structure at baseline (i.e. before MI generation), 48 hours, 1 month, 2 months, and 3 months after MI. We performed echocardiography with an iE33 ultrasound machine (Philips Medical Systems) equipped with an X3-1 and S8-3 transducer during end-expiratory breath-hold in an R-wave-triggered mode. Images were obtained in the standard LV apical and short axis views with a high frame rate (> 60 frames/s). QLab software (Philips) was used for analysis of strain rate. Two stable and well-defined consecutive cardiac cycles were acquired digitally for each measurement.

For hemodynamic catheterization, we accessed the femoral artery and vein with 7F sheaths and placed a 6F Millar Micro-Tip catheter (Millar Instruments Inc.) into the aorta, the left ventricle, and the right ventricle. We determined the following parameters: systolic pressure, end-diastolic pressure, peak LV pressure rate of rise $(dP/dt)_{max}$ and Tau value (time constant of isovolumic relaxation); $(dP/dt)_{max}/P$ was calculated as $(dP/dt)_{max}/(\text{systolic} - \text{end-diastolic pressure})$. The mean of at least 3 consecutive cardiac cycles was calculated for each measurement.

We performed coronary angiography on day 2, after one, and three months using an Integris H5000 single-plane fluoroscopy system (Philips Medical Systems). All images were acquired and analysed by an investigator blinded to the study arm. We euthanized pigs by i.v. injection of EuthasolR (pentobarbital, phenytoin, 1 ml/4.5 kg), removed the hearts, resected the right ventricle, and cut the left ventricle into 6 slices of the same thickness. We visualised viable myocardium by staining 5 of these slices with TTC and quantified scar volume.

Western blot assessment of PKC target proteins

Hearts were collected at the end of the study for protein extraction and western blotting as previously described.⁵ Primary antibodies included PKC α , PKC β I, PKC β II, PKC γ , Cav1.2, RyR (Santa Cruz Biotechnology), GAPDH (Research Diagnostics), Phospho-PLN, Phospho-Cav1.2, Phospho-RyR2 (Badrilla) PLN (Pierce), Phospho-PKC, GRK2, TnI, Phospho-TnI (Cell Signaling), Phospho-PKC (Upstate), Phospho-TnI, TnT (Abcam), SERCA2 (Affinity Bioreagents), MyBP-C, and Phospho-MyBP-C (gift from Jeffrey Robbins, Cincinnati Children's Hospital, Cincinnati, OH). For analysis of TnT, GRK2 and SERCA2 phosphorylation, the Phos-Tag reagent (Wako Chemicals) was used at 30 μ M. Chemifluorescent detection was performed with Vistra ECF reagent (Amersham Pharmacia Biotech) and scanned with a Gel-Doc XR (Bio-Rad).

Statistical analysis

All data analysis was performed in a blinded manner. Data were presented as mean \pm standard error of mean. Statistical analysis was performed with SPSS software (SPSS Inc.) using non-parametric Wilcoxon test.

Results and Discussion

Analysis of pig heart function after MI with or without ruboxistaurin treatment

While ruboxistaurin was shown to dramatically attenuate heart failure in select mouse and rat models of disease, its applicability to large mammals with heart failure remains uncertain. Here we utilized 20 kg Yorkshire pigs to evaluate heart failure over a 3-month period after MI injury. The study was limited to 3 months to curtail costs and suffering, to conserve limited quantities of ruboxistaurin, to reduce the effect of rapid weight gain that typically occurs in juvenile pigs that would otherwise skew data interpretation, and because 3 months is sufficient time to uncover heart failure in control animals. Two days after LAD occlusion (MI injury) heart rate was significantly increased in both vehicle and ruboxistaurin treated pigs, which gradually dropped back to pre-MI levels by 2-3 months (Figure 1A). Vehicle and ruboxistaurin treated pigs showed a loss of cardiac contractility, ejection fraction, and cardiac output 2 days after MI injury (Figure 1B,C,D). This loss of cardiac performance remained depressed in vehicle treated pigs over the 3 month period, although ruboxistaurin treated pigs showed a significant recovery of cardiac contractility, ejection fraction, and cardiac output 3 months after MI injury (Figure 1B,C,D). There was no difference in infarct size normalized to the left ventricular area between the control and ruboxistaurin treated groups assessed by TTC staining at the end of the study (Figure 1E,F). No overt differences in cardiac histopathology were observed between the treated and control pigs (data not shown). Taken together, these results showed that ruboxistaurin treatment benefitted the heart and augmented myocardial recover following MI injury, suggesting a novel therapeutic approach for heart failure post MI in humans.

The mechanism whereby ruboxistaurin treatment protected the pig heart post MI is uncertain. Ventricular dilation post MI injury over the 3 months of the study remained the same between vehicle and ruboxistaurin treated groups (data not shown) and infarct and scar size was not different (Figure 1D,E), suggesting that ventricular remodeling was not altered by PKC α/β inhibition, or that not enough time had passed to permit accurate assessment of beneficial changes in ventricular geometry with ruboxistaurin treatment. We also failed to observe a change in ventricular remodeling in transgenic mice expressing a dominant negative PKC α protein in the heart after MI injury, which might suggest that inhibition of PKC α protects the heart through a unique mechanism of action that is independent of structural remodeling.²¹ Indeed, we have previously proposed that the overwhelming beneficial effect associated with PKC α inhibition/deletion on the heart was due to an increase in cardiac contractility or the efficiency of myofilament function.²³ However, attempts to identify a direct PKC α/β phosphorylation target that might underlie an alteration in cardiac contractility in ruboxistaurin treated pig hearts were unsuccessful (Figure 2). Quantitation of these blots showed no significant changes when anterior and inferior regions were combined (Table 1). Similarly, we also failed to identify a change in phosphorylation of these same nodal control proteins in the hearts of mice treated with the PKC α/β inhibitors Ro-31-8220 or Ro-32-0432.⁵ Thus, while we previously demonstrated that PKC $\alpha^{-/-}$ mice have increased sarcoplasmic reticulum calcium levels due to changes in inhibitor-1 phosphorylation and the subsequent phosphorylation of phospholamban, PKC α/β inhibitory drugs may not be “potent” enough at the physiologic doses employed to show the same effect. However, acute administration of Ro-31-8220, Ro-32-0432 or ruboxistaurin to mice or rats *in vivo*, or in an isolated work performing heart preparation, did significantly

enhanced cardiac contractility (within minutes), suggesting that the same regulatory mechanisms are in place and likely being affected. Despite these observations, the employed PKC α/β antagonists might only partially inhibit phosphorylation of downstream PKC α targets over an integrated period of time, and at any single time-point the effect is too subtle to detect. Regardless of this issue, our results strongly support the contention that inhibition of PKC α/β activity with ruboxistaurin has a significant beneficial effect on the pig heart under conditions that would otherwise induce failure. Taken together with similar beneficial effects observed in mice and rats in heart failure, a unified front emerges with overwhelming data that support the contention that PKC α inhibitory drugs should be translated to the heart failure clinic in appropriate patients, especially since drugs like ruboxistaurin are apparently safe and have already been used in large late phase clinical trials.²²

The dosage of ruboxistaurin used here was 10 mg/kg/day, which achieved a blood level of 93 ng/ml with an active metabolite level of 817 ng/ml. These concentrations are similar to that achieved by us previously with mice receiving 120 mg/kg/day,¹⁷ and similar to a lower end of what has been achieved in human patients receiving a 32 mg dosage.²⁴ However, considering the half life of ruboxistaurin in humans of 6-12 hours, a dosage of approximately 30-60 mg bid should be considered for future application in heart failure patients. Another issue is that ruboxistaurin appears to have a vascular protective effect, hence its prior use in human clinical trials for diabetic retinopathy.²² These results suggest that ruboxistaurin might also protect the heart by preserving endothelial cell function and microvascular integrity, in addition to an effect on contractility or diminution of reactive signaling in myocytes and possibly fibroblasts.

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Non-Standard Abbreviations

LAD	left anterior descending
MI	myocardial infarction
PKC	protein kinase C

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Novelty and Significance

What is known

- Genetic or pharmacologic inhibition of PKC α in mouse protects from heart failure
- Pharmacologic inhibition of PKC α in rats protects from heart failure
- PKC α activity is increased in heart failure

What new information does this article contribute

- We provide the first proof that pharmacologic inhibition of PKC α/β reduces heart failure in a large animal model
- Ruboxistaurin promotes recovery of myocardial function in a pig model of MI-induced heart failure

This study was designed to examine if inhibition of PKC α/β with ruboxistaurin would be efficacious in a large animal model of heart failure following myocardial infarction injury. The significance is that if this drug is effective in the pig in reducing heart failure, it will suggest a clinical therapy to try in humans with heart failure. Ruboxistaurin also appears to be relatively safe in previous clinical trials, so it could be rapidly evaluated in heart failure patients.

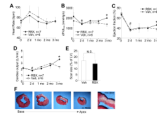
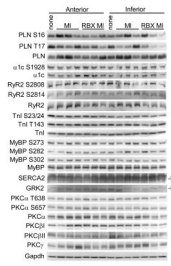


Figure 1.

Ruboxistaurin attenuates heart failure in pigs post MI. A, Millar catheter-based analysis of heart rate and B, contractility in vehicle or ruboxistaurin treated (10 mg/kg/day) pigs subjected to MI injury at the indicated times after injury (days or months). # $P < 0.05$ versus 0 time point. * $P < 0.05$ versus vehicle treated pigs at 3 months. C, left ventricular ejection fraction measured by ventriculography in vehicle or ruboxistaurin treated pigs after MI for the indicated periods of time (days or months). * $P < 0.05$ versus vehicle at 3 months. D, Cardiac output measured with a Swan Ganz catheter in vehicle or ruboxistaurin treated pigs after MI for the indicated periods of time. * $P < 0.05$ versus vehicle at 3 months. E, Quantitation of scar size after TTC staining to show area of injury between the 2 groups. N.S.=not significantly different. F, Pig heart slices after MI injury stained with TTC (white area is not stained by TTC and represents the area of infarction).

**Figure 2.**

Western blot analysis of calcium handling proteins, myofilament proteins, and PKC $\alpha/\beta/\gamma$ for the native protein or for the indicated specific phosphorylation site from pig hearts after 3 months of ruboxistaurin treatment or no treatment after myocardial infarction injury. One non-infarcted control is shown. The indicated proteins or phosphoproteins were analyzed from pieces of anterior or inferior portions of the left ventricle. The “-P” designation represents specialized gel electrophoresis conditions that separate proteins with differential phosphorylation.

Table 1

Quantitation of western blotting from Figure 2.*

	Control MI		RBX MI		N
	1	±SE	6	±SE	
P-PLN S16	1	0.1484	6	0.6989	6
P-PLN T17	1	0.1686	6	0.6179	6
P-CaV1.2 S1928	1	0.06157	6	0.9453	6
P-RyR2 S2808	1	0.07453	6	1.007	6
P-RyR2 S2814	1	0.09668	6	1.174	6
P-Tnl S23/24	1	0.07356	6	1.02	6
P-Tnl T143	1	0.03434	6	0.9662	6
P-MyBP-C S273	1	0.06311	6	1.012	6
P-MyBP-C S282	1	0.1388	6	1.185	6
P-MyBP-C S302	1	0.07872	6	0.9289	6
P-PKC $\alpha/\beta/\gamma$ T638	1	0.02391	6	0.9056	6
P-PKC α S657	1	0.06466	6	1.056	6
PKC α	1	0.04457	6	0.8684	6
PKC β I	1	0.09436	6	0.949	6
PKC β II	1	0.07333	6	0.8084	6
PKC γ	1	0.08483	6	0.7911	6

* None of the values were significantly different between treated and untreated controls. N=6 ventricular samples from 3 different animals (2 samples each).