Inhibition of PKCα/β With Ruboxistaurin Antagonizes Heart Failure in Pigs After Myocardial Infarction Injury

Dennis Ladage, Lisa Tilemann, Kiyotake Ishikawa, Robert N. Correll, Yoshiaki Kawase, Steven R. Houser, Jeffery D. Molkentin, Roger J. Hajjar

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

Methods and Results: 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 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 PKCα/β inhibition (with ruboxistaurin) represents a tenable and novel therapeutic approach for treating human heart failure. (Circ Res. 2011;109:1396-1400.)

Key Words: heart failure • contractility • PKC • signaling • cardiomyopathy

The protein kinase C (PKC) family of Ca2+ 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 (PKCα, βI/II, and γ) are Ca2+- and lipid-activated, whereas the novel isozymes (ε, θ, η, and δ) and atypical isozymes (ζ, and λ) are Ca2+-independent but activated by distinct lipids.1–2 PKCα 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 PKCα activity showed decreased cardiac contractility, ventricular dilation, and secondary hypertrophy, suggesting that increased PKCα signaling is detrimental to the heart.14,15 Indeed, PKCα−/− mice are protected from insults or genetic mutations that would otherwise induce heart failure.14,16 By comparison, PKCβ/γ−/− mice showed more severe heart failure when stressed,17 suggesting that PKCα is the primary disease affecting isoform in the heart and the best candidate for inhibition. Transgenic mice with inducible expression of a dominant negative PKCα 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 PKCα inhibition. Thus, we and others carefully examined the effects of C PKC 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...
null mouse model of heart failure augmented cardiac contractility and restored pump function. 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. Another PKCα/β inhibitor, ruboxistaurin, prevented death in wild-type 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. Ruboxistaurin was also administered to PKCB/γ null mice subjected to pressure overload, resulting in less death and heart failure, strongly suggesting PKCα as the primary target of this drug in mitigating heart disease. In addition, Boyle et al showed that ruboxistaurin reduced ventricular fibrosis and dysfunction after MI in rats. 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 minutes of ischemia followed by 48 hours of reperfusion. Connelly et al demonstrated that ruboxistaurin attenuated diastolic dysfunction, myocyte hypertrophy, collagen deposition, and preserved cardiac contractility in a rat diabetic heart failure model. These results in rodents overwhelmingly support the contention that PKCα/β 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 toward initiating clinical trials in patients with heart failure, especially given the apparent safety of this compound in other human trials.

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 per hour) anesthesia. For MI generation, we introduced an 8F sheath 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 two-thirds of the LAD tributary for 90 minutes. The resulting infarct size was approximately 15% of the left ventricle, determined by TTC staining. The 48-hour survival rate was 86% (n=15). Thirteen animals were randomized to receive either control pig chow or pig chow enriched with ruboxistaurin (Eli Lilly).

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

Assessment of MI and Structure
We assessed myocardial function and structure at baseline (ie, 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)\text{max} and Tau value (time constant of isovolumic relaxation); (dP/dt)\text{max}/P was calculated as (dP/dt)\text{max}/(systolic–end-diastolic pressure). The mean of at least 3 consecutive cardiac cycles was calculated for each measurement.

In 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. Primary antibodies included PKCe, PKCβ, PKCβ1, PKCy, Ca2+/CaM, Ryr2 (Alomone Labs), GAPDH (Research Diagnostics), Phospho-PLN, Phospho-Ca2+, Phospho-RyR2 (Badrilla) PLN (Pierce), Phospho-PKC, GRK2, TnI, Phospho-TnI (Cell Signaling), Phospho-PKC (Upstate), Phospho-TnI, TnI (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 μmol/L. Chemiluminescent 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±SEM. Statistical analysis was performed with SPSS software (SPSS Inc), using nonparametric Wilcoxon test.

Results
Analysis of Pig Heart Function After MI With or Without Ruboxistaurin Treatment
Whereas 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. We used 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 to 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, 1C, and 1D). 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, 1C, and 1D). 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 and 1F). 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 recovery after MI injury, suggesting a novel therapeutic approach for heart failure after MI in humans.

The mechanism whereby ruboxistaurin protected the pig heart after MI is uncertain. Ventricular dilation after 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 and 1E). There was no difference in cardiac contractility or the efficiency of myofilament function. However, attempts to identify a direct PKCα/β phosphorylation target that might underlie an alteration in cardiac contractility in ruboxistaurin treated pig hearts were unsuccessful (Figure 2). Quantification of these blots showed no significant changes when anterior and inferior regions were combined (Table). Similarly, we also failed to identify a change in phosphorylation of these same nodal control proteins in the hearts of mice treated with the PKCα/β inhibitors Ro-31-8220 or Ro-32-0432. Whereas we previously demonstrated that PKCα−/− mice have increased sarcoplasmic reticulum calcium levels due to changes in inhibitor-1 phosphorylation and the subsequent phosphorylation of phospholamban, PKCα/β inhibitory drugs may not be “potent” enough at the physiological doses used 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 working heart preparation, did significantly enhance cardiac contractility (within minutes), suggesting that the same regulatory mechanisms are in place and probably being affected. Despite these observations, the PKCα/β antagonists that were used might only partially inhibit phosphorylation of downstream PKCα 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 PKCα/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 PKCα inhibitory drugs should be translated to the heart failure clinic in appropriate patients, especially because drugs such as ruboxistaurin are apparently safe and have already been used in large, late-phase clinical trials.

The dosage of ruboxistaurin used here was 10 mg/kg per 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 per 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 remodeling after myocardial infarction injury. One noninfarcted 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.

Source Funding
This work was supported by the National Institutes of Health (J.D.M., R.J.H.), a grant from the Fondation Leducq (J.D.M., R.J.H.), and the Howard Hughes Medical Institute (J.D.M.). R.N.C. (J.D.M., R.J.H.), a grant from the Fondation Leducq (J.D.M., R.J.H.), and the Howard Hughes Medical Institute (J.D.M.). R.N.C.

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Ruboxistaurin promotes recovery of myocardial function in a pig model of myocardial infarction–induced heart failure.

What New Information Does This Article Contribute?

- We provide the first proof that pharmacological inhibition of PKCα/β reduces heart failure in a large animal model.
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Circ Res. 2011;109:1396-1400; originally published online October 13, 2011;
doi: 10.1161/CIRCRESAHA.111.255687
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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