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

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Rationale: Protein kinase C (PKC) 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 PKCα have enhanced cardiac contractility and reduced susceptibility to heart failure after long-term pressure overload or after myocardial infarction injury. Pharmacological inhibition of PKCα/β with Ro-32 to 0432, Ro-31 to 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 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;109:00-00.)

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

The protein kinase C (PKC) family of Ca2+/lipid-activated serine-threonine protein kinases are critical mediators of signal transduction in the heart, where they regulate disease responsiveness. The PKC family is broadly classified by activation characteristics, such that the conventional PKC isozymes (PKCα, β/II, and γ) are Ca2+/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. PKCα is the predominant conventional PKC isoform expressed in the mouse, rabbit, and human heart, and many different disease-causing stimuli result in its activation, including heart failure.

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. Indeed, PKCα−/− mice are protected from insults or genetic mutations that would otherwise induce heart failure. By comparison, PKCβ/γ−/− mice showed more severe heart failure when stressed, 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.

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 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. 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 PKCβ/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 by an investigator blinded to the study arm. We euthanized pigs by intravenous 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 visualized viable myocardium by staining 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. Primary antibodies included PKCo, PKCβI, PKCβII, PKCγ, CaMKII, RyR (Santa Cruz Biotechnology), GAPDH (Research Diagnostics), Phospho-PLN, Phospho-CaMKII, Phospho-RyR2 (Badrilla) PLN (Pierce), Phospho-PKC, GRK2, Tnl, Phospho-Tnl (Cell Signaling), Phospho-PKC (Upstate), Phospho-Tnl, 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 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 treatment 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), suggesting that ventricular remodeling was not altered by PKCα/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 PKCα protein in the heart after MI injury, which might suggest that inhibition of PKCα 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 PKCα 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 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

![Figure 1. Ruboxistaurin attenuates heart failure in pigs after MI. A, Millar catheter-based analysis of heart rate, and B, contractility in vehicle- or ruboxistaurin-treated (10 mg/kg per 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, Quantification of scar size after TTC staining to show area of injury between the 2 groups. N.S. indicates 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).](http://circres.ahajournals.org/)

![Figure 2. Western blot analysis of calcium handling proteins, myofilament proteins, and PKCα/β 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 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.](http://circres.ahajournals.org/)
hearts of mice treated with the PKCa/β inhibitors Ro-31–8220 or Ro-32–0432.\textsuperscript{5} Whereas we previously demonstrated that PKCa\textsuperscript{-/-} mice have increased sarcoplasmic reticulum calcium levels due to changes in inhibitor-1 phosphorylation and the subsequent phosphorylation of phospholamban, PKCa/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 work performing heart preparation, did significantly enhanced cardiac contractility (within minutes), suggesting that the same regulatory mechanisms are in place and probably being affected. Despite these observations, the ePKCa/β antagonists that we used might only partially inhibit phosphorylation of downstream PKCa 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 PKCa/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 PKCa 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.\textsuperscript{22}

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Disclosures

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