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

**Rationale:** Protein kinase Cα (PKCα) 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
PKC in rodents overwhelmingly support the contention that overload, resulting in less death and heart failure, strongly 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 hockey stick guiding catheter (Cordis Infiniti, Johnson & Johnson). After injecting 100 mg nitroglycerin (Cincinnati Children’s Hospital, Cincinnati, OH). For analysis of PKCα/βII, PKCα/βII, PKCα/βII, and PKCα/βII, 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 nonparametric Wilcoxon test.

Results

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 performed 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/(systolic–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 1 and 3 months, using an Integris HS900 single-plane fluoroscopy system (Philips Medical Systems). All images were acquired and analyzed by an investigator blinded to the study arm. We euthanized pigs by intravenous injection of Euthasol (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 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. Primary antibodies included PKCα, PKCβII, PKCδ, CaV1.2, RyR (Santa Cruz Biotechnology), GAPDH (Research Diagnostics), Phospho-PLN, Phospho-CaV1.2, Phospho-RyR2 (Badrilla) PLN (Pierce), Phospho-PKC, GRK2, TnI, TnT, 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 (Wakoh 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).

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 administered 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.

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. 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. 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.\textsuperscript{5} Whereas we previously demonstrated that PKC\textsuperscript{α/β}–/– 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 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 ePKCα/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 PKC\textsuperscript{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 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.\textsuperscript{22}

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

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**Novelty and Significance**

**What Is Known?**
- Genetic or pharmacological inhibition of Protein kinase C (PKCalpha) in mouse protects from heart failure.
- Pharmacological inhibition of PKCalpha in rats protects from heart failure.
- PKCalpha activity is increased in heart failure.

**What New Information Does This Article Contribute?**
- We provide the first proof that pharmacological inhibition of PKCalpha reduces heart failure in a large animal model.
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