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 PKC overload, resulting in less death and heart failure, strongly better than metoprolol treatment.17 Ruboxistaurin was also fibrosis, and reduced pulmonary edema comparable to or ular dilation, enhanced ventricular performance, reduced 10 weeks of pressure-overload stimulation, reduced ventric-

ruboxistaurin, prevented death in wild-type mice throughout /H11006
179 ng/mL of the primary metabolite.

of ruboxistaurin and 817 /H11006
31 ng/mL /H11006

2 days at 10 mg/kg per day) produced plasma levels of 93 /H11006
months of oral ruboxistaurin treatment (2 days at 5 mg/kg per day and /H11006

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.

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)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 H5000 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 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 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.3 Primary antibodies included PKCa, PKCB, PKCγ, Ca2+, RyR (Santa Cruz Biotechnology), GAPDH (Research Diagnostics), Phospho-PLN, Phospho-Ca2+, 1.2, Phospho-RyR2 (Badrilla) PLN (Pierce), Phospho- PKC, GRK2, TnI, Phospho-TnI, TnT (Abcam), SERCA2 (Affinity Biore-
agents), 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. 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

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).

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.
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, and similar to a lower end of what has been achieved in human patients receiving a 32 mg dosage. 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. 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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Disclosures

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

References


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Table. Quantification of Western Blotting From Figure 2

<table>
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<th></th>
<th>Control MI ± SEM</th>
<th>n</th>
<th>RBX MI ± SEM</th>
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<td>0.6989 ± 0.1233</td>
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<tr>
<td>P-PLN T17</td>
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<td>P-Ca, 1.2</td>
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<td>P-MyBP-C S282</td>
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None of the values were significantly different between treated and untreated controls; n = 6 heart samples each.


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