A Rho-kinase inhibitor improves cardiac function after 24-hour heart preservation

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Objective: The Rho-kinase signaling pathway is associated with coronary vasculopathy and myocardial dysfunction after cardiac transplantation. This study evaluated whether using a Rho-kinase inhibitor during allograft storage could limit early endothelial dysfunction and improve myocardial performance after reperfusion.

Methods: This experiment was performed with an isolated working rabbit heart model and a support rabbit. Donor hearts (control group, n = 8) were arrested with an extracellular type of cardioplegia, preserved with University of Wisconsin solution, and then immersed in University of Wisconsin solution for 24 hours (1°C). The Rho-kinase inhibitor (Rho-kinase inhibitor group, n = 8) was administrated in the cardioplegic solution, the preservation University of Wisconsin solution, and the storage University of Wisconsin solution. Left ventricular performance was evaluated from the modified Frank–Starling curve in the working mode. Coronary blood flow and donor heart rate were measured in Langendorff mode. Effective evaluation of the Rho-kinase inhibitor was inferred from phosphorylated myosin light chain. The expression of endothelial nitric oxide synthase mRNA was analyzed to assess endothelial function.

Results: The Frank–Starling curve showed a significant left and upward shift in the Rho-kinase inhibitor group compared with the control group (P < .05). The coronary blood flow and heart rate in the Rho-kinase inhibitor group at 120 minutes was significantly higher than in the control group (P < .05). Phosphorylated myosin light chain was significantly suppressed in the Rho-kinase inhibitor group (P < .05). Endothelial nitric oxide synthase mRNA levels in the Rho-kinase inhibitor group increased 4-fold relative to those seen in the control group.

Abbreviations and Acronyms
CBF = coronary blood flow
CKMB = creatine kinase–MB
Ct = cycle threshold
eNOS = endothelial nitric oxide synthase
LAP = left atrial pressure
LV = left ventricular
MLC = myosin light chain
NO = nitric oxide
p-MLC = phosphorylated myosin light chain
RKI = Rho-kinase inhibitor
TUNEL = terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling
UW = University of Wisconsin

under the “Guidelines for animal experiments” of the Faculty of Medicine, Kyushu University, Legal Ruling (no. 105) and Notification (no. 6) of the Japanese Government.

Animal Preparation
Forty male Japanese white rabbits (16 donor rabbits, 16 support rabbits, and 8 blood donors) weighing 3.0 to 3.3 kg were used. General anesthesia was induced with sodium thiopental (20 mg/kg) through the dorsal marginal ear vein, and then a tracheal tube was inserted through the mouth and connected to a mechanical ventilator with 100% oxygen. Additional vecuronium bromide (1 mg/kg) and fentanyl citrate (30 μg/kg) were intravenously administered. The femoral arterial pressure of both donor and support rabbits was monitored continuously. This study was performed with an isolated working rabbit heart model using support rabbit blood, as described in detail by Kajihara and colleagues.11,13

Donor Heart Management
The donor rabbit heart and aortic arch were exposed through a median sternotomy. After heparinization (300 U/kg), the innominate artery was cannulated to administer the cardioplegic solution. The inferior vena cava was transected to decompress the heart, and then the aortic arch was cross-clamped. In the control group (n = 8) cardiac arrest was induced with an infusion of 10 mL/kg extracellular-type Kyushu University cardioplegic solution,13,14 and then 20 mL/kg University of Wisconsin (UW) preservation solution was infused to wash out the coronary vascular cardioplegia. The excised heart was immersed in UW solution (55 mL) for 24 hours. The temperature and administration velocity of both cardioplegic and UW solutions were maintained at 4°C and 12 mL/min, respectively. In the RKI group 0.1 mg/kg RKI was administered with the cardioplegia and UW solutions, and 0.5 mg/kg RKI was added to the immersion UW solution. All excised hearts were preserved at 1°C by using heat exchange for 24 hours, followed by 120 minutes of reperfusion.

Support Rabbit and Cross-circulation System
After induction of general anesthesia and heparinization (300 U/kg), the common carotid arteries and external jugular veins of support rabbits were exposed and cannulated. Fentanyl citrate (100 μg/h), vecuronium bromide (1 mg/h), and heparin (1000 U/h) were used to maintain anesthesia and prevent coagulation. Oxygenated blood from the common carotid artery of the support rabbit was introduced to a cannula connected to the ascending aorta of the donor heart with a microtube pump. Blood draining from the system was returned to the jugular vein through another microtube pump. The pulmonary vein and vena cava of the donor heart were closed, and a double-lumen cannula was inserted into the left atrium. One lumen was connected to a pressure transducer to measure left atrial pressure (LAP). The other lumen was connected to an atrial reservoir (preload). During systemic circu-

The remaining block was fixed in 10% formaldehyde.

Modified Frank–Starling Curve
The donor heart was perfused and stabilized with constant coronary perfusion at 60 mm Hg in Langendorff mode for 60 minutes, and then the working mode was started. In working mode the heart was paced atrially at 250 beats/min, and the aortic afterload pressure was fixed at 60 mm Hg. LAP was gradually increased by raising the atrial reservoir, and aortic flow rates were measured at each level of LAP. Based on these data, we constructed a modified Frank–Starling curve. After measurement of left ventricular (LV) function, the working mode was reversed to Langendorff mode, and at 120 minutes after reperfusion, LV function was evaluated in working mode again.

Coronary Blood Flow and Heart Rate
The maximum coronary blood flow (CBF) and heart rate of the donor heart were measured in Langendorff mode at 10, 60, and 120 minutes after reperfusion.

Measurement of Creatine Kinase–MB
Serum creatine kinase–MB (CKMB) levels were measured with a chemiluminescent immunoassay (SRL, Inc, Tokyo, Japan). The change of CKMB level between 60 and 120 minutes is presented, with the level at 60 minutes representing 100%.

Quantitative Real-time Reverse Transcriptase–Polymerase Chain Reaction
The gene expression of eNOS in myocardium was measured by means of quantitative real-time reverse transcriptase–polymerase chain reaction performed according to the recommendations of the Premix Ex Taq-Perfect Real-Time protocol (Takara Bio, Inc, Shiga, Japan). Total RNA was isolated from homogenized myocardium with ISOGEN (Nippon Gen Co Ltd, Toyama, Japan). The PrimeScript first strand cDNA (Takara Bio, Inc) was made with random primers to reverse transcribe RNA to cDNA. The eNOS probe and primers were designed based on the published sequence of rabbit eNOS (GenBank accession no. AY964103)13 and were produced by Applied Biosystems Japan Ltd. The amount of target cDNA was quantified on a Sequence Detection System ABI 7000 (Applied Biosystems Ltd, Foster City, Calif) by using rabbit eNOS-specific primers and probe. Relative gene expression was calculated by using the 66 cycle threshold (Ct) method. Relative eNOS levels were determined by normalizing the Ct value to the rabbit glyceraldehyde-3-phosphate dehydrogenase signal measured from the same sample. The sample with lowest eNOS expression was arbitrarily selected as a calibrator value, and the other quantities are expressed as an n-fold difference relative to this calibrator.

Western Blot Analysis for Phosphorylated Myosin Light Chain
The extent of phosphorylation of the 20-kd myosin light chain (MLC) in myocardium was quantified by means of Western blot analysis, as previously described, to evaluate Rho-kinase activity.10 Protein samples were separated by means of SDS-PAGE with 15% Tris–HCL gels (Bio-Rad
Laboratories, Inc, Tokyo, Japan). Twenty microliters of each sample containing 50 μg of protein was applied to the gel and run for 90 minutes at 100 V. Separated proteins were transferred to a membrane at 100 V for 60 minutes. The membrane was incubated with an anti-mouse monoclonal phosphorylated-MLC (p-MLC) antibody (Cell Signaling Technology, Inc, Danvers, Mass), followed by incubation with an anti-mouse IgG horseradish peroxidase–labeled secondary antibody. The region containing MLC was detected with a monoclonal anti-mouse MLC primary antibody (Sigma–Aldrich, Inc, St Louis, Mo) and an anti-mouse IgM horseradish peroxidase–labeled secondary antibody. After detection was performed with a luminoimage analyzer, the intensity of both the unphosphorylated and phosphorylated MLC bands was quantified with National Institutes of Health Image J software. The percentage of the phosphorylated form was measured to express the extent of the MLC phosphorylation. HEK293 cells were used as a positive control for both specific primary antibodies.

Histology in the Myocardium
Cardiac tissue was fixed in 10% formaldehyde, dehydrated, and embedded in paraffin. Each block was cut into 5-μm-thick slices and stained with hematoxylin and eosin to evaluate infiltrating cells.

Apoptotic myocytes were detected with a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay by using a Mebstain Apoptosis Kit II (Medical and Biological Laboratories Co, Ltd, Nagoya, Japan), according to the manufacturer’s instructions. Myer’s hematoxylin was used as the counterstain. The ratio of TUNEL-positive nuclei per 1000 total nuclei was measured.

Statistical Analysis
SPSS statistical software for Windows (version 15.0; SPSS, Inc, Chicago, Ill) was used for data analysis. Data are expressed as the mean value ± standard error of the mean. Statistical comparisons were performed by using unpaired Student t tests. The relationship between LAP and aortic flow was analyzed by using a multiple regression model with a dummy variable technique to investigate intergroup differences.

RESULTS
Modified Frank–Starling Curve
Modified Frank–Starling curves were obtained from each group at both 60 and 120 minutes after reperfusion, and the equations obtained from multiple regression analyses are shown in Figure 1. The values of the x-intercepts and the slopes at the x-intercepts obtained from regression curves in each group are shown in Table 1. The value of the x-intercept in the RKI group was significantly higher than that in the control group, indicating better function in the control group than in the RKI group. The value of the slope at the x-intercept obtained from individual regressions in the control group was significantly lower than in the RKI group, indicating better function in the control group than the RKI group.

There was no difference between 60 and 120 minutes in the RKI group at both 60 and 120 minutes after reperfusion, and the x-intercept obtained from individual regressions in the control group at both 60 and 120 minutes after reperfusion, and the x-intercept obtained from individual regressions in the control group was significantly higher than that in the RKI group. The value of the slope at the x-intercept obtained from individual regressions in the control group was significantly lower than in the RKI group, indicating better function in the control group than the RKI group.

There was no difference between 60 and 120 minutes in the RKI group at 10 and 120 minutes after reperfusion in each group is shown in Figure 2, A. The CBF in the RKI group at 10 and 120 minutes was significantly higher than in the control group (P < .05). The heart rate of the donor after reperfusion was more similar to the normal rabbit rate in the RKI group than in the control group (Figure 2, B).

Serum Levels of CKMB
Serum CKMB levels in the coronary effluent at 10 and 120 minutes were not significantly different between the control and RKI groups. However, the CKMB change from 60 to 120 minutes was lower in the RKI group than in the control group (P < .05; Figure 3, A).

eNOS Synthesis
The relative amount of eNOS mRNA was quantified by means of linear extrapolation of the Ct values, and eNOS mRNA levels in the RKI group were 4-fold greater than those in the control group (Figure 3, B).

Suppression of MLC Phosphorylation by RKI
RKI treatment significantly blocked the extent of MLC phosphorylation (the phosphorylated form as a percentage of total MLC) at 2 hours after reperfusion (P < .05; Figure 3, C).

Pathologic Findings
There was no obvious change in myocardial or vascular structure in the control and RKI groups, and infiltrating cells were not observed. TUNEL-positive nuclei were detected in 0.3% per 1000 nuclei in the control group and 0.1% per 1000 nuclei in the RKI group (P = .4).

DISCUSSION
We found that inhibition of Rho-kinase during allograft harvest and storage markedly improved recovery of myocardial function. The improved myocardial function seemed to result from heightened preservation of endothelium and vascular smooth muscle. This study clearly showed the cardioprotective effect of RKI during cardiac allograft storage in the isolated working rabbit heart model with a support rabbit.

This experimental model offers major advantages, in particular by demonstrating pharmacologic effects at the organ level. Use of a working mode and a support rabbit provided a closer approach to in vivo conditions. Perfusate containing erythrocytes, leukocytes, platelets, and inflammatory cytokines is more commonly compared with crystalloid perfusion fluid, which not only affords oxygen-carrying capacity and coronary flow autoregulation within the physiologic range but also provides biochemical functions, such as buffering of oxygen radicals and catabolism of nitric oxide (NO).

RKI
RKIs are beneficial in organ transplantation. Shiotani and colleagues demonstrated that RKI reduced acute hepato-cellular necrosis and apoptosis induced by cold ischemia–reperfusion injury in a rat model of liver transplantation.
Hattori and associates\(^9\) described that long-term treatment with RKI suppressed cardiac allograft vasculopathy in mice. The influence of Rho-kinase during cardiac allograft storage is not clear.

The report by Yada and coworkers\(^10\) showed that RKI supplanted directly to the coronary artery dose-dependently caused vasodilatation and increased CBF in a canine model. The most effective dose of RKI was 0.1 mg/kg. Thus we used that dose of RKI in the supplanted solution directly. Our preliminary experiment of 1 mg/kg RKI supplanted into the cardioplegic solution did not improve cardiac recovery. Moreover, RKI added to the support rabbit during reperfusion produced profound systemic vasodilation and instability. Therefore we assessed RKI during harvest and allograft storage to minimize changes in the systemic pressure of the support rabbit.

Inokuchi and colleagues\(^18\) reported that fasudil (1.5 mg/kg) administered into intractable spastic arteries was effective in a clinical setting and showed few systemic adverse effects. Species differences could account for these different dosing needs.

Fasudil hydrochloride and Y27632 are available RKIs. We chose the former because it is already available in clinical settings.\(^4,5\) The latter is used only in animal studies because of its metabolic byproducts.

Enhanced Cardiac Function Recovery

Allograft contractile performance was evaluated with modified Frank–Starling curves. RKI enhanced the recovery of LV function after reperfusion. The curve at 60 minutes in the RKI group was close to that of a normal heart, as was shown in our previous study.\(^3\) After ischemia and reperfusion, intracellular Rho-kinase is induced by angiotensin II, thrombin, and endothelin I. Antagonists or inhibitors for those agonists usually attenuate ischemia–reperfusion injury, with efficacy similar to that of RKI, indicating that RKI influences multiple signaling pathways.

In the RKI group increased CBF might result from preserved endothelial function and coronary vasodilatation. Improved endothelial function could result in enhanced contractile function by improving myocardial perfusion and a washout of toxic metabolites that accumulate during storage. RKI treatment slightly increased CKMB levels at 120 minutes but did not change necrosis or myocardial structure under hematoxylin and eosin staining and did not affect

![Modified Frank–Starling curves obtained by means of multiple regression analysis with dummy variables in each group. The black broken lines show the individual curves in each group. The black solid lines represent the regression equations for each curve. Representative equations are as follows: (A) y = −2.01 x^2 + 36.75 x −19.85 at 60 minutes in the control group; (B) y = −5.91 x^2 + 62.08 x +18.13 at 60 minutes in the Rho-kinase inhibitor group; (C) y = −0.16 x^2 + 19.05 x + 27.24 at 120 minutes in the control group; (D) y = −5.49 x^2 + 63.44 x −3.74 at 120 minutes in the Rho-kinase inhibitor group. The values of the x-intercept and the slope at the x-intercept obtained from individual curves in each group are shown in Table 1.

**TABLE 1. Values of the x-intercept and the slope at the x-intercept in each group**

<table>
<thead>
<tr>
<th>Group</th>
<th>60 min</th>
<th>120 min</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x-Intercept</td>
<td>Slope</td>
<td>x-Intercept</td>
<td>Slope</td>
</tr>
<tr>
<td>Control</td>
<td>0.59 ± 0.77</td>
<td>34.39 ± 3.09</td>
<td>−1.40 ± 1.26</td>
<td>42.39 ± 5.06</td>
</tr>
<tr>
<td>RKI</td>
<td>−0.28 ± 0.30</td>
<td>65.38 ± 3.49</td>
<td>0.08 ± 0.50</td>
<td>61.15 ± 5.87</td>
</tr>
</tbody>
</table>

\(P\) value: \(.017\) < .01, \(.013\) < .01

\(\text{RKI, Rho-kinase inhibitor.}\)
apoptosis. Such changes would presumably appear later because the allograft was appropriately preserved and incidental apoptosis in ischemia–reperfusion injury develops during late reperfusion.19

**Preserved Endothelial Function**

Postischemic endothelial dysfunction occurs in several models of cardiac injury.20 The primary consequence of impaired endothelial function is the reduction of NO.
production released by endothelial cells. NO is synthesized from L-arginine by NO synthase and modulates vascular tone, attenuates platelet aggregation and neutrophil function, and reduces free radical damage. NO synthesis inhibitors and eNOS-deficient mice increase infarct size after ischemia and reperfusion. Therefore restored NO synthesis is mandatory for appropriate cardiac performance. RKI exerts cardioprotective effects on coronary ischemia–reperfusion injury in vivo, possibly by preserving eNOS expression. The overexpression of a dominant-negative mutant of Rho A in cultured endothelial cells upregulates the expression of eNOS mRNA, and the activation of Rho A downregulates eNOS mRNA. Inhibition of Rho-kinase signaling helps preserve eNOS mRNA levels.

NO production can be evaluated by measuring the amount of NO in serum samples, Western blotting for eNOS, and detection of eNOS mRNA by means of polymerase chain reaction, but we prefer real-time reverse transcriptase–polymerase chain reaction. RKI treatment increased eNOS mRNA 4-fold compared with that seen in the control group. RKI could improve cardiac function through NO-dependent endothelial protection after ischemia and reperfusion.

Analysis of RKI Activity

We added RKI to all solutions, including the cardioplegic solution, UW solution, and storage solution. However, it is unclear whether the chemical can penetrate cells at a low temperature. To evaluate the bioactivity of Rho A and Rho-kinase, phosphorylation of the α-adducin, ezrin, radixin, and moesin family and the myosin-binding subunit of myosin phosphatase, as well as expression of Rho A are usually measured. We used an anti-p-MLC antibody that is specific to MLC in the endothelium and vascular smooth muscle but not cardiomyocytes. RKI reduced p-MLC levels in the allograft for at least 2 hours after reperfusion. RKI is a vasorelaxant in the endothelium and vascular smooth muscle in vivo and in vitro.

LIMITATIONS

Our data are limited to the acute setting after reperfusion. Although a combination of early endothelial and myocardial protection will presumably provide long-term benefits, further chronic RKI treatment is required to test this. Rho and Rho-kinase activation is difficult to measure in rabbits because of limited antibody selection.

Although the transition of the donor heart rate in each group after reperfusion was in direct proportion to CBF, the relationships were inversely proportional. At 60 minutes, heart rate in the RKI group decreased but was increased in the control group. We are unable to explain the reason for this difference.

Coronary vasculopathy after cardiac transplantation is an important cause of poor outcomes after cardiac transplantation, including reduced survival. Improvement of both endothelial and myocardial protection during allograft storage might improve early and long-term outcomes. These results might have clinical relevance for the prevention of low coronary flow phenomenon and postoperative low output syndrome induced by prolonged cardiac ischemia.

CONCLUSIONS

With a working model using cross-circulated blood perfusion in the rabbit heart, we showed that RKI during allograft harvest and storage enhanced CBF and ventricular recovery through NO-dependent endothelial protection after reperfusion. Rho and Rho-kinase are implicated in endothelial dysfunction induced by means of ischemia and reperfusion. These results indicate that RKI could be a potential therapy for preventing early myocardial dysfunction after transplantation.

We thank Tomomi Yamada, MS, for statistical analysis; Miho Miyakawa and Eiko Iwata for pathologic technical advice and assistance; and Kensuke Egashira, MD, PhD, Department of Cardiovascular Medicine, for pathologic experimental support.

References


