Administration of the Rho-kinase inhibitor fasudil before ischemia or just after reperfusion, but not 30 min after reperfusion, protects the stunned myocardium in swine.

Author(s)
Shibata, Itsuko; Yoshitomi, Osamu; Use, Tadasuke; Ureshino, Hiroyuki; Cho, Sungsam; Maekawa, Takuji; Hara, Tetsuya; Sumikawa, Koji

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Title

Administration of the Rho-kinase inhibitor Fasudil before ischemia or just after reperfusion, but not 30 min after reperfusion, protects the stunned myocardium in swine.

Authors and Affiliation

Itsuko Shibata, MD*, Osamu Yoshitomi, MD*, Tadasuke Use, MD*, Hiroyuki Ureshino†, Sungsam Cho, MD*, Takuji Maekawa, MD*, Tetsuya Hara, MD*, and Koji Sumikawa, MD, PhD‡.

* Staff Anesthesiologist, † Research assistant, ‡ Professor and Chair, Department of Anesthesiology, Nagasaki University School of Medicine, Nagasaki, Japan

Name and address for correspondence and reprint requests

Sungsam Cho, MD
Department of Anesthesiology, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, JAPAN.
Abbreviated title: fasudil protects stunned myocardium
Abstract

We assessed the effect of administration time for fasudil treatment of the stunned myocardium in 40 anesthetized open chest swine. All swine were subjected to 12 min ischemia followed by reperfusion to generate stunned myocardium. Group A (n = 11) received saline in place of fasudil both before ischemia and after reperfusion. Group B (n = 10) received 30 min intravenous fasudil at a rate of 13 µg/kg/min starting 45 min before ischemia and received saline after reperfusion. Groups C (n = 10) and D (n = 9) received saline before ischemia, and received fasudil at a rate of 13 µg/kg/min starting just before reperfusion in group C and 30 min after reperfusion in group D. In both groups, treatment lasted 30 min. Myocardial contractility was assessed by percent segment shortening (%SS). Three swine in group A, 2 swine in each of groups B and C, and one swine in group D had ventricular fibrillation or tachycardia after reperfusion and were excluded from further analysis. The changes of %SS from baseline at 90 min after reperfusion in groups B and C were 68 ± 8% and 75 ± 8%, respectively, which were significantly higher than in group A or D (47 ± 10% or 43 ± 8%). We conclude that fasudil administered before ischemia or
just after reperfusion, but not 30 min after reperfusion, protects the stunned myocardium. (228 words)

Key words

Rho-kinase, fasudil, ischemia, reperfusion, swine, stunned myocardium.
Introduction

Fasudil, a Rho-kinase inhibitor, has been clinically used as an antispasm drug for treatment of cerebral vasospasm after subarachnoid hemorrhage (SAH) in humans [1]. Recently, several studies have shown that fasudil may be useful for the treatment of a wide range of other cardiovascular diseases including angina pectoris, hypertension, pulmonary hypertension, stroke, and heart failure [2]. Intracoronary fasudil prevents myocardial ischemia in patients with coronary microvascular spasm [3] and treats intractable severe coronary spasm after coronary artery bypass grafting (CABG) [4].

Cardioprotective effects of Rho-kinase inhibitors for myocardial ischemia-reperfusion injuries have been reported in some studies. Preischemic administration of the Rho-kinase inhibitors fasudil and Y-27632 decreased the incidence of arrhythmias and myocardial infarct size in rats subjected to transient LAD occlusion [5, 6]. Recently, administration of the Rho-kinase inhibitor Y-27632 at early reperfusion was reported to have a cardioprotective effect against myocardial infarction [7, 8].
Myocardial stunning is defined as prolonged reversible postischemic contractile dysfunction following a brief ischemic episode that does not result in necrosis. It occurs in patients with coronary artery diseases such as unstable angina, exercise-induced ischemia, percutaneous transluminal coronary angioplasty (PTCA), and open heart surgery [9]. In large mammals, dogs, and pigs, myocardial stunning can be induced by a single completely reversible episode of regional ischemia lasting less than 20 min, or multiple completely reversible episodes of ischemia [10-12]. Adenosine triphosphate-sensitive potassium (K$_{ATP}$) channel openers, adenosine, and angiotensin-converting enzyme (ACE) inhibitors administered before ischemia contribute to cardioprotection against not only myocardial infarction but also myocardial stunning [13]. However, the mechanisms involved in pathophysiology could be different between myocardial infarction and stunning, and it is not known whether Rho-kinase inhibitors provide protection against myocardial stunning.

The present study was carried out to clarify whether fasudil administered before ischemia could protect against myocardial stunning, and moreover to clarify the
effect of fasudil administration time during reperfusion, *i.e.* administration during early and late after reperfusion.
**Materials and methods**

**Surgical procedure**

All experimental procedures used in this investigation were reviewed and approved by the Institutional Animal Care Committee of Nagasaki University. Forty swine of either sex weighing 19-33 kg were sedated with 20 mg/kg intramuscular ketamine hydrochloride. Surgical preparation was performed as described previously [14]. Swine were anesthetized with 100 mg/kg intravenous \( \alpha \)-chloralose and 10 \( \mu \)g/kg fentanyl, followed by continuous infusion of 10 mg/kg/h \( \alpha \)-chloralose and 5 \( \mu \)g/kg/h fentanyl throughout the study period. Through a midline cervical incision, the trachea was intubated for connection to a Harvard respiratory pump (Harvard Apparatus Co., South Natick, MA, USA). Mechanical ventilation was facilitated by intermittent infusion of 0.2 mg/kg vecuronium, and adjusted to maintain the arterial carbon dioxide tension at \((\text{PaCO}_2)\) 35-40 mmHg and the arterial oxygen tension \((\text{PaO}_2)\) at 100-300 mmHg. The esophageal temperature was maintained at 36-37°C throughout the study period using a warmer blanket and a heating lamp. A catheter was inserted into the right carotid vein to administer fluid and drugs. Lactated
Ringer’s solution was infused at a rate of 5 ml/kg/h. Systemic anticoagulation was achieved with intravenous 750 U/kg sodium heparin followed by a continuous infusion of sodium heparin at 250 U/kg/hour. Sodium bicarbonate was administered to maintain the base deficit within 5 mEq/l. Arterial blood glucose concentration was measured before and during ischemia and maintained at the baseline value. A standard peripheral lead electrocardiogram was monitored continuously. Medial sternotomy was performed and the pericardium was opened to expose the heart. The LAD distal to the first diagonal branch was cannulated with a stainless steel cannula and perfused with blood from the left carotid artery through an extracorporeal circuit. Coronary blood flow (CBF) of the perfused area of LAD was measured with an ultrasonic flow probe (ADP17; Crystal Biotech, Hopkinton, MA, USA) attached at the extracorporeal circuit. Pressure transducer-tipped catheters (PC500; Millar Instruments, Huston, TX, USA) were connected to the left ventricular (LV) chamber cannula through an incision in the apex and right internal carotid artery cannula for continuous recording of left ventricular pressure (LVP) and arterial blood pressure. The peak rate of increase in LVP (LVdP/dt max) was determined by electric
differentiation of the LV pressure waveform. Two ultrasonic segment length transducers were implanted 10-15 mm apart in the subendocardium of the perfused area of the extracorporeal circuit and aligned such that the intercrystal axis was parallel to the direction of myocardial fiber shortening. The regional contractile function was accessed by changes in percent segment shortening (%SS). Segment length was monitored by ultrasonic amplifiers (VF-1; Crystal Biotech, Hopkinton, MA, USA). The end-systolic segment length (ESL) was determined 10 ms before maximum negative LVdp/dt, and the end-diastolic segment length (EDL) was determined 10 ms before the LVdP/dt first exceeded 140 mmHg/s (immediately before the onset of LV isovolemic contraction). %SS was calculated using the formula: %SS = (EDL – ESL) × 100 × 1/EDL. All hemodynamic data were continuously monitored on a polygraph and digitized through a computer interfaced with an analog-to-digital converter (HEM; Physio-Tech, Tokyo, Japan).

**Experimental Protocols**

Figure 1 shows the experimental time course. Baseline systemic and coronary hemodynamics and %SS were recorded 30 min after instrumentation was
completed. Forty swine were randomly assigned to one of four groups. Group A (n = 11) received saline in place of fasudil both before and after ischemia. Group B (n = 10) received intravenous fasudil at a rate of 13 µg/kg/min for 30 min until 15 min before coronary occlusion, and received saline after reperfusion. Groups C (n = 10) and D (n = 9) received saline in place of fasudil before ischemia, and received fasudil at a rate of 13 µg/kg/min starting just before reperfusion in group C and 30 min after reperfusion in group D. In both groups, the treatment lasted 30 min. The dose and duration of fasudil treatment (13 µg/kg/min for 30 min) used in groups B, C, and D correspond to those clinically applied for prevention of cerebral vasospasm after SAH.

All swine were subjected to 12 min ischemia with complete occlusion of the extracorporeal circuit followed by a 90 min reperfusion. Hemodynamics and contractile function were monitored continuously throughout the experiment and recorded at the time points illustrated in figure 1 (P₀: baseline, P₃₀: 30 min after administration of fasudil, P₄₅: 15 min after discontinuation of fasudil and just before ischemia, R₀: just before reperfusion, R₅, R₃₀, R₆₀, and R₉₀: 5, 30, 60, and 90 min after reperfusion, respectively).
All swine received 2 mg/kg intravenous lidocaine at 1 min before reperfusion. If five or more premature ventricular contractions per minute or multifocal premature ventricular contractions were observed after reperfusion, 1 mg/kg intravenous lidocaine was administered and repeatedly given if necessary. Swine with continuous ventricular fibrillation (VF) or ventricular tachycardia (VT) after reperfusion were excluded from the study. The effects on reperfusion-induced arrhythmias were evaluated with regard to the incidence of VF or VT and the total amount of lidocaine used for 10 min after reperfusion.

Statistics

All data are expressed as mean ± SD. One-way analysis of variance (ANOVA) for non-repeated measures followed by the Student-Newman-Keuls (SNK) post hoc test was used to test for differences in baseline hemodynamics, %SS, and total dose of lidocaine administered after reperfusion among groups. Data within groups were analyzed with one-way ANOVA for repeated measures, and data between groups were analyzed with two-way repeated measures ANOVA followed by the SNK post
hoc test. The incidence of VF or VT was analyzed by the $\chi^2$ test. P values < 0.05 were considered statistically significant.
Results

There were no significant differences in weight or sex among the groups. Arterial blood gas values and blood glucose were maintained within physiological range in all swine throughout the study period (data not shown).

Table 1 shows the systemic and coronary hemodynamics from baseline throughout the time course of the study. There were no significant differences in any measured systemic or coronary hemodynamics at baseline among groups. There were no significant differences among groups at any measured point in heart rate (HR), mean arterial pressure (MAP), or LV end-diastolic pressure (LVEDP). LVdp/dt max was significantly decreased in group A at R$_5$, in group B at R$_0$ and R$_5$, and in group D at R$_5$ and R$_{90}$ from the baseline value. CBF increased significantly from the baseline in all groups and returned thereafter, and there were no significant differences at any measured point when compared to group A.

Table 2 summarizes the assessment of reperfusion-induced arrhythmias. Three swine in group A, two swine in each of groups B and C, and one swine in group D each had VF or VT after reperfusion and were excluded from further analysis. The
incidence of VF or VT and the total amount of lidocaine used were not significantly different among groups.

Baseline values of %SS were 24.2 ± 6.1 in group A, 26.3 ± 2.9 in group B, 25.6 ± 5.0 in group B, and 23.0 ± 2.6 in group B, respectively, and there were no significant differences among groups. Figure 2 shows the percent changes of %SS from baseline throughout the time course of the study. All swine showed negative %SS values at the end of the ischemic period (R0), which indicates bulging. At R60 and R90, the values of %SS in group B (57 ± 11% and 68 ± 8% of baseline) were significantly higher than those in group A. At R30, R60, and R90, the values of %SS in group C (57 ± 10%, 66 ± 5%, and 75 ± 8% of baseline, respectively) was significantly higher than those in group A (42 ± 7%, 44± 6%, and 47 ± 10% of baseline, respectively). However, there were no significant differences in the values of %SS between groups A and D at any measured point.
Discussion

Our findings demonstrate that fasudil administered before ischemia can protect the stunned myocardium. Fasudil is cleared quickly from the blood with a half-life of less than 15 min, whereas its hydroxylated metabolite hydroxyfasudil, which preferentially inhibits Rho-kinase, remains in the blood for as long as 8 hr after infusion [15, 16]. In the present study, hydroxyfasudil might have been retained during the reperfusion phase and produced Rho-kinase inhibition. To clarify the effects of hydroxyfasudil remaining in the blood during the reperfusion phase, we carried out an additional study. Intracoronary infusion of fasudil for 30 min until 15 min before coronary occlusion through an extracorporeal circuit also improved myocardial contractility: the values of %SS at R_{60} and R_{90} (61 ± 8% and 67 ± 9% of baseline) were significantly higher than those in group A. The concentration used for intracoronary fasudil administration correspond to that in the blood used in group B, and the total dose was about 1/100 of that in group B. Thus it is possible that cardioprotection by fasudil administered before ischemia could occur through a mechanism other than maintenance of hydroxyfasudil in the blood during reperfusion.
Wolfrum et al. reported that acute inhibition of Rho-kinase led to cardiovascular protection mediated by the rapid activation of the phosphatidylinositol 3-kinase/Akt/endothelial nitric oxide synthase (eNOS) pathway [5]. Hannan et al. showed that deletion of eNOS in knockout mice resulted in increased myocardial dysfunction following ischemia–reperfusion [17]. It is therefore possible that fasudil protects the stunned myocardium via eNOS activation.

The majority of the injury responsible for myocardial stunning and infarction develops during the early phase of reperfusion [18]. In the present study, we showed that fasudil administered just after reperfusion but not 30 min after reperfusion protected the stunned myocardium. Hamid et al. reported that Rho-kinase activity increased 10 min after reperfusion but not during ischemia, and that Y27632 administered during early reperfusion significantly reduced the infarct size [8]. Thus fasudil administration during early reperfusion may protect against myocardial stunning as well as myocardial infarction.

In this study, there were no significant differences in HR, MAP or LVEDP among groups at any measured point. Yada et al. [19] reported that hydroxyfasudil
caused significant coronary vasodilation of both small arteries and arterioles in a
dose-dependent manner. In the present study, the CBF did not increase after
administration of fasudil at a clinically relevant dose. A previous study in dogs showed
that the infarct-limiting effects of Rho-kinase inhibition could be independent of
changes in systemic hemodynamics or recruitment of collateral blood flow [7].
Taking these findings into consideration, it is unlikely that systemic and coronary
hemodynamic changes played any role in the present results.

It was reported that preischemic administration of low-dose fasudil (0.3
mg/kg) had proarrhythmic potential in an anesthetized rat model of myocardial
infarction [20]. Stevens et al. [21] reported that VF often occurred at reperfusion in
an in vivo swine model of myocardial stunning. Therefore, we administered lidocaine
to all swine prior to reperfusion, and added it when premature ventricular contraction
occurred. Consequently, there were no significant differences in the incidence of VF
or VT, or in the total amount of lidocaine among groups.

In summary, administration of the Rho-kinase inhibitor fasudil before
ischemia or just after reperfusion, but not 30 min after reperfusion, protects the
stunned myocardium in anesthetized open-chest swine. This cardioprotective effect of fasudil against myocardial stunning was probably not related to systemic or coronary hemodynamic changes. Rho-kinase inhibition during early reperfusion could therefore protect against myocardial stunning as well as myocardial infarction.
References


11. Kim SJ, Ghaleh B, Kudej RK, Huang CH, Hintze TH, Vatner SF. Delayed enhanced nitric oxide-mediated coronary vasodilation following brief ischemia and


## Table 1. Hemodynamic data

<table>
<thead>
<tr>
<th></th>
<th>Paradox in Group B</th>
<th>Ischemia</th>
<th>Paradox in Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HR</strong></td>
<td>84 ± 17</td>
<td>61 ± 17</td>
<td>82 ± 21</td>
<td>85 ± 23</td>
</tr>
<tr>
<td>Group B</td>
<td>92 ± 14</td>
<td>91 ± 15</td>
<td>94 ± 16</td>
<td>93 ± 18</td>
</tr>
<tr>
<td>Group C</td>
<td>84 ± 12</td>
<td>83 ± 11</td>
<td>86 ± 13</td>
<td>85 ± 19</td>
</tr>
<tr>
<td>Group D</td>
<td>95 ± 12</td>
<td>96 ± 13</td>
<td>97 ± 12</td>
<td>96 ± 10</td>
</tr>
<tr>
<td><strong>MAP</strong></td>
<td>123 ± 14</td>
<td>112 ± 15</td>
<td>113 ± 16</td>
<td>107 ± 17</td>
</tr>
<tr>
<td>Group B</td>
<td>99 ± 15</td>
<td>91 ± 17</td>
<td>95 ± 15</td>
<td>92 ± 16</td>
</tr>
<tr>
<td>Group C</td>
<td>115 ± 17</td>
<td>114 ± 18</td>
<td>115 ± 18</td>
<td>112 ± 20</td>
</tr>
<tr>
<td>Group D</td>
<td>95 ± 17</td>
<td>95 ± 17</td>
<td>96 ± 16</td>
<td>95 ± 21</td>
</tr>
<tr>
<td><strong>LVEDP</strong></td>
<td>123 ± 2.5</td>
<td>12.2 ± 2.2</td>
<td>12.1 ± 2.0</td>
<td>11.8 ± 4.3</td>
</tr>
<tr>
<td>Group B</td>
<td>11.3 ± 3.4</td>
<td>10.6 ± 3.9</td>
<td>10.9 ± 2.8</td>
<td>10.6 ± 2.8</td>
</tr>
<tr>
<td>Group C</td>
<td>9.5 ± 2.2</td>
<td>9.8 ± 2.5</td>
<td>9.5 ± 2.4</td>
<td>9.5 ± 2.3</td>
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<tr>
<td>Group D</td>
<td>10.3 ± 1.8</td>
<td>10.3 ± 1.5</td>
<td>10.3 ± 2.2</td>
<td>10.6 ± 1.6</td>
</tr>
<tr>
<td><strong>LVd’/dt max</strong></td>
<td>2322 ± 399</td>
<td>2553 ± 445</td>
<td>2516 ± 461</td>
<td>2320 ± 432</td>
</tr>
<tr>
<td>Group B</td>
<td>2064 ± 377</td>
<td>2523 ± 325</td>
<td>2045 ± 303</td>
<td>2104 ± 332</td>
</tr>
<tr>
<td>Group C</td>
<td>2387 ± 1227</td>
<td>2694 ± 1197</td>
<td>2703 ± 1191</td>
<td>2369 ± 1083</td>
</tr>
<tr>
<td>Group D</td>
<td>2427 ± 506</td>
<td>2456 ± 515</td>
<td>2466 ± 480</td>
<td>2195 ± 367</td>
</tr>
<tr>
<td><strong>CBF</strong></td>
<td>24.0 ± 10.1</td>
<td>25.8 ± 10.8</td>
<td>25.9 ± 10.8</td>
<td>24.2 ± 10.1</td>
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<tr>
<td>Group B</td>
<td>29.3 ± 9.4</td>
<td>27.9 ± 9.2</td>
<td>29.6 ± 10.3</td>
<td>27.5 ± 9.5</td>
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<td>Group C</td>
<td>24.2 ± 7.0</td>
<td>24.4 ± 6.4</td>
<td>24.0 ± 6.5</td>
<td>24.9 ± 7.0</td>
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<tr>
<td>Group D</td>
<td>26.2 ± 7.3</td>
<td>26.3 ± 7.4</td>
<td>26.4 ± 7.5</td>
<td>34.0 ± 8.3</td>
</tr>
<tr>
<td><strong>%SS</strong></td>
<td>24.2 ± 6.1</td>
<td>24.3 ± 6.0</td>
<td>24.1 ± 5.9</td>
<td>-3.0 ± 2.4*</td>
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<tr>
<td>Group B</td>
<td>26.3 ± 2.9</td>
<td>26.8 ± 2.83</td>
<td>27.2 ± 2.9</td>
<td>-2.5 ± 1.7*</td>
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<tr>
<td>Group C</td>
<td>25.5 ± 5.0</td>
<td>25.4 ± 4.6</td>
<td>24.9 ± 5.0</td>
<td>-1.4 ± 1.3*</td>
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<tr>
<td>Group D</td>
<td>23.1 ± 2.6</td>
<td>22.1 ± 2.5</td>
<td>22.0 ± 2.7</td>
<td>-3.0 ± 1.4*</td>
</tr>
</tbody>
</table>

Values are Mean ± SD. *p < 0.05 vs Group B. **p < 0.05 vs Group A.
HR: Heart rate (beats/min), MAP: Mean Arterial Pressure (mmHg), LVEDP: Left Ventricular End-diastolic Pressure (mmHg), LVd’/dt max: maximal rate of increase of left ventricular pressure (mmHg/sec), CBF: Coronary Blood Flow (ml/min), %SS: Segment Shortening
Table 2. Incidence of ventricular fibrillation (VF) or ventricular tachycardia (VT) and total amount of lidocaine used during the first 10 min after reperfusion

<table>
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<tr>
<th></th>
<th>Group A</th>
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<tr>
<td>Total Number</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Number of VF or VT</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
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<tr>
<td>Lidocaine (mg/kg)</td>
<td>2.4 ± 0.8</td>
<td>2.3 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>2.2 ± 0.9</td>
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Values are mean ± SD.
**Figure 1:** Experimental time course.

<table>
<thead>
<tr>
<th>Group</th>
<th>Saline</th>
<th>Ischemia</th>
<th>Saline</th>
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<th>Saline</th>
<th>Fasudil</th>
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<tr>
<td>Group A</td>
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<td></td>
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<td></td>
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<tr>
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<td>fasudil</td>
<td></td>
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<tr>
<td>Group D</td>
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<td>fasudil</td>
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- $P_0$, $P_{30}$, $P_{45}$, $R_{0}R_{3}$, $R_{30}$, $R_{60}$, $R_{90}$
Figure 2: Recovery of percent segment shortening (%SS).
Figure legends

Figure 1: Experimental time course.

All swine were subjected to 12 min ischemia followed by 90 min reperfusion. Group A received saline in place of fasudil both before and after ischemia. Group B received fasudil (13 µg/kg/min for 30 min) before ischemia, and Groups C and D received fasudil (13 µg/kg/min for 30 min) just before reperfusion or 30 min after reperfusion. Hemodynamic and percent segment shortening (%SS) measurements were performed at the times indicated by triangles (▲) in the figure. P0: baseline, P30: 30 min after administration of fasudil, P45: 15 min after discontinuation of fasudil and just before ischemia, R0: just before reperfusion, R5, R30, R60, and R90: 5, 30, 60, and 90 min after reperfusion, respectively.

Figure 2: Recovery of percent segment shortening (%SS).

Values are expressed as mean ± SD. †; p < 0.05 vs group A.

■: group A, ▲: group B, ▼: group C, and ♦: group D. P0: baseline, P30: 30 min after administration of fasudil, P45: 15 min after discontinuation of fasudil and just before
ischemia, R₀: just before reperfusion, R₅, R₃₀, R₆₀, and R₉₀: 5, 30, 60, and 90 min after reperfusion, respectively.