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# Fasudil Administered during Early Reperfusion Protects against Myocardial Infarction through Activation of PI3K/Akt/NOS Pathway in Rats

Takuji Maekawa*1, Sungsam Cho*1, Taiga Ichinomiya*1, Shinya Tosaka*3, Shuhei Matsumoto*1, Itsuko Shibata*1, Tetsuya Hara*1, Koji Sumikawa*2

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**Abstract**

**Purpose:** The authors examined whether fasudil, a Rho-kinase inhibitor, administered during reperfusion could protect the heart against myocardial infarction and, if so, whether phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK1/2), and nitric oxide synthase (NOS) pathways would be involved in the mechanism.

**Methods:** All rats underwent 30 min of coronary artery occlusion followed by 2 h of reperfusion. Rats received fasudil at the beginning of reperfusion, or at 30 min after reperfusion. In other groups, rats received fasudil after administration of wortmannin, a PI3K inhibitor, PD98059, an ERK1/2 inhibitor, or N($\omega$)-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor.

**Results:** Fasudil at the beginning of reperfusion (22±9%), but not at 30 min (42±12%) after reperfusion reduced infarct size as compared to the control group (42±7%). The beneficial effect of fasudil was blocked by wortmannin (36±9%) or L-NAME (47±6%), but not PD98059 (26±8%).

**Conclusions:** Fasudil administered early reperfusion protects the heart against myocardial infarction in anesthetized rats, and that this beneficial effect is mediated through PI3K and NOS, but not ERK1/2 activation.

**Key words;** Rho-kinase inhibitor, fasudil, ischemia reperfusion injury, myocardial infarction

## Introduction

Ischemic postconditioning is the phenomenon that the application of repetitive ischemia during early reperfusion results in reduced myocardial injury, which has led to renewed interest in the development of protective maneuvers against lethal reperfusion injury[1]. Pharmacological postconditioning is produced by agents in stead of ischemia and would be a more practical solution. In this respect a variety of diverse pharmacological postconditioning agents including inhalational anesthetics[2], G-protein coupled receptor ligands such as opioids[3] and adenosine[4], growth factors such as insulin[5] and erythropoietin[6], and natriuretic peptides[7] have been linked to the activation of reperfusion injury salvage kinase (RISK) pathway.

*1 Staff Anesthesiologist, Department of Anesthesiology, Nagasaki University School of Medicine, Nagasaki, Japan
*2 Professor and Chair, Department of Anesthesiology, Nagasaki University School of Medicine, Nagasaki, Japan
*3 Department of Anesthesiology, National Hospital Organization Nagasaki Medical Center, Nagasaki, Japan

An intravenous formulation of fasudil, a selective Rho-kinase inhibitor, is approved in Japan to prevent cerebral vasospasm after subarachnoid hemorrhage (SAH) and recently, has been considered useful for the treatment of a wide range of cardiovascular diseases including angina pectoris[8], hypertension[9], pulmonary hypertension[10], and heart failure[11]. The Rho and Rho-kinase pathway is involved in the pathogenesis of cardiac dysfunction, and plays a critical role in the failing heart[11]. Cardioprotective effects of Rho-kinase inhibitors against ischemia-reperfusion injury was studied in some animal models, and fasudil, hydroxyfasudil, a metabolite of fasudil, and Y-27632, a structurally unrelated Rho-kinase inhibitor were shown to have preconditioning effects against myocardial infarction in rats[12-14], mice[15] and dogs[16]. Recently, we have demonstrated that fasudil administered not only before but also just after reperfusion protects stunned myocardium in swine[17]. It was reported that pre-ischemic administration of Rho-kinase inhibitor activates phosphatidylinositol 3-kinase (PI3K)/Akt and endothelial nitric oxide synthase (eNOS), which would mediate reduction of myocardial infarct size[12]. Based on several lines of evidence, we hypothesized that PI3K/Akt pathway would be activated specifically after reperfusion rather than during ischemia, and that Rho-kinase inhibition during early reperfusion period could limit infarct size through augmentation of Akt activation and NO production in the myocardium.

The objective of this study, therefore, was to determine whether fasudil administered during reperfusion could protect against myocardial infarction in anesthetized rat, and if so, whether PI3K, ERK1/2, and NOS pathways might be involved in the mechanism.

## Methods and Materials

### A. Animal Care

This study was performed in accordance with the guideline of the Institutional Animal Care and Experimentation Committee.

### B. Surgical procedure

Male Sprague-Dawley rats (320-570g) were anesthetized with sodium pentobarbital (a 50 mg/kg intraperitoneal bolus injection followed by a continuous intravenous infusion of 10-20mg/kg/h). The rats received tracheotomy and lungs were ventilated with pure oxygen by means of a small animal ventilator (SAR-830 CWE, PA, USA) at a rate of 45-60 breaths/min. Body temperature was measured via a rectal probe and maintained at 37±1℃ with a heating lamp. The right carotid artery was cannulated with a polyethylene catheter and connected to a pressure transducer (Blood pressure monitor link sck-9082; Becton Dickinson, Tokyo, Japan) to monitor arterial blood pressure and heart rates. Systemic hemodynamics were continuously monitored and recorded throughout the experiment using an AP-641G blood pressure amplifer (Nihon-Kohden, Tokyo, Japan) and shown on a polygraph system (Nihon-Kohden). The right jugular vein was cannulated for the administration of drugs. The chest was opened by a left thoracotomy between the fourth and the fifth ribs, the pericardium incised, and the heart gently exteriorized. A 6-0 prolene ligature was placed around the left anterior descending coronary artery (LAD), close to its origin. The heart was immediately replaced in the chest cavity with the ligature ends exteriorized. Both ends of the ligature were then passed through a small rubber tube to form a snare for a reversible LAD occlusion. Any rat, in which this procedure itself produced dysrhythmias or a sustained fall in systolic arterial pressure to less than 70 mm Hg, was excluded from the study at this point. Following a stabilization period of 15 min, the snare around the LAD was tightened and held in place using a small clip to induce transient regional myocardial ischemia for 30 min. Successful occlusion was confirmed by an appearance of epicardial cyanosis. Reperfusion was initiated by releasing the ligature and removing the tube.

### C. Experimental protocol

The experimental design is illustrated in **Fig. 1**. The rats were randomly assigned to one of 9 groups. Group C (n = 9) received no intervention during ischemia or reperfusion period. Group F0 (n = 9),

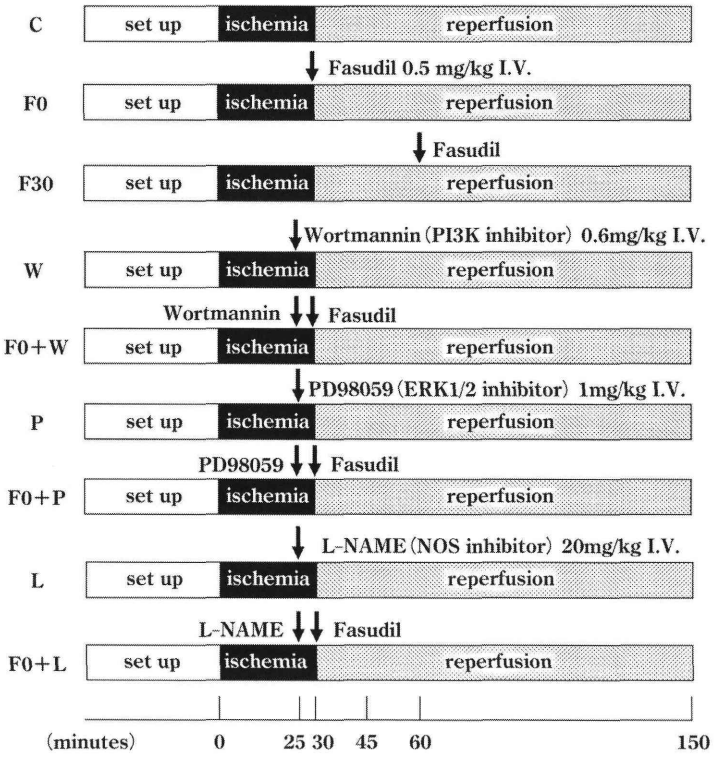


**Figure 1 A schematic illustration of experimental protocols**
C=Control; F0=administration of fasudil at just reperfusion; F30=administration of fasudil at 30 min after reperfusion; W=Wortmannin; P=PD98059; L=L-NAME.

and F30 (n=9) received 0.5 mg/kg of fasudil intravenously at the beginning of reperfusion, at 30 min after reperfusion, respectively. Group W (n=8) received 0.6 mg/kg of wortmannin (dimethylsulfoxide 1% aqueous solution), a PI3K inhibitor, intravenously, 5 min before reperfusion. Group F0+W (n=7) received the same procedures as the combination of groups F0 and W. Group P (n=9) received 1 mg/kg of PD98059 (dimethylsulfoxide 1% aqueous solution), an ERK1/2 inhibitor, intravenously, 5 min before reperfusion. Group F0+P (n=9) received the same procedures as the combination of groups F0 and P. Group L (n=9) received 20 mg/kg of N ($\omega$)-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, intravenously, 5 min before reperfusion. Group F0+L (n=9) received the same procedures as the combination of groups F0 and L. The doses of wortmannin[18], PD98059[19] and L-NAME[20] were set on the basis of previous studies. The dose of fasudil in this study corresponds to that clinically applied for prevention of cerebral vasospasm after SAH. Dimethylsulfoxide 1% aqueous solution, vehicle of wortmannin or PD98059 had no effect on the myocardial infarct size in our previous study[21], therefore we did not evaluate the effect of the vehicle on myocardial infarct size in the current study.

### D. Determination of myocardial infarct size

Myocardial infarct size was measured as previously described[22]. At the end of reperfusion, the LAD was reoccluded, and then patent blue dye was injected into the left ventricle (LV) to identify ischemic (area at risk [AAR], unstained) and non-ischemic myocardium (area not at risk, stained blue). Then the hearts were harvested and rinsed in normal saline. The atria, right ventricle and great vessels were removed. The LV was cut into slices of 3-4-mm thickness, and the AAR from the apex to the base was separated from the non-ischemic area. The AAR was incubated at 37℃ for 15 min in 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC, in 20 mM

phosphate buffer, pH 7.4). The tissues were fixed overnight in 10% formaldehyde. The AAR and the blue-stained non-ischemic area were weighed for determination of AAR/LV. The myocardial infarct size was expressed as the percentage of AAR.

**E. Statistical analyses**

All values were expressed as mean±SD. Statistical analysis of hemodynamic data within and between groups was performed with analysis of variance for repeated measures followed by Dunnett test. Inter-group differences in body weight, age, LV weight, the ratio of AAR to LV, and the ratio of infarct size to AAR were analyzed using one-way analysis of variance followed by Student-Newman-Keuls test. A p value less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 15.0 software (SPSS Japan, Tokyo, Japan) or GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

## Results

There were no significant differences in body weight or age among the groups. Ninety-four rats were used to obtain 78 successful myocardial infarct size experiments. Four rats were excluded as a result of technical difficulties with the experimental preparation. Malignant ventricular arrhythmias developed in 5 rats, and fall in arterial blood pressure continued in 7 rats before completion of the experiment, and these rats were excluded further analysis.

**A. Hemodynamics**

There were no significant differences in heart rate (HR) or mean arterial pressure (MAP) at baseline among the groups. In any group, neither HR nor MAP had a significant change from the baseline value throughout the experimental period (**Table 1, 2**).

**Table 1 Systemic hemodynamics (Heart rates: beats/min)**

| Group | Baseline | Occlusion | Reperfusion | | |
|---|---|---|---|---|---|
| | | 20min | 10min | 1h | 2h |
| C | 430±23 | 437±21 | 431±31 | 420±25 | 415±27 |
| F0 | 410±35 | 404±40 | 400±33 | 404±41 | 392±55 |
| F30 | 373±52 | 353±58 | 377±63 | 365±55 | 396±61 |
| W | 390±43 | 376±50 | 376±50 | 365±34 | 409±58 |
| F0+W | 407±65 | 404±69 | 383±64 | 376±64 | 380±76 |
| P | 380±60 | 359±64 | 355±64 | 363±69 | 365±64 |
| F0+P | 383±51 | 375±47 | 376±48 | 353±61 | 359±57 |
| L | 404±36 | 400±42 | 392±36 | 389±40 | 380±41 |
| F0+L | 400±46 | 393±54 | 389±56 | 392±56 | 387±55 |

Values are expressed as mean±SD. C=Control; F0=administration of fasudil at just reperfusion; F30=administration of fasudil at 30 min after reperfusion; W=Wortmannin; P=PD98059; L=L-NAME.

**Table 2 Systemic hemodynamics (Mean arterial pressure: mmHg)**

| Group | Baseline | Occlusion | Reperfusion | | |
|---|---|---|---|---|---|
| | | 20min | 10min | 1h | 2h |
| C | 115±12 | 123±11 | 123±9 | 112±11 | 103±13 |
| F0 | 104±15 | 106±20 | 101±18 | 112±13 | 118±16 |
| F30 | 98±11 | 93±16 | 92±19 | 102±23 | 107±24 |
| W | 102±14 | 100±17 | 99±17 | 116±26 | 111±25 |
| F0+W | 100±29 | 108±24 | 115±25 | 108±24 | 89±30 |
| P | 90±24 | 91±24 | 91±24 | 97±21 | 97±22 |
| F0+P | 89±15 | 82±20 | 83±14 | 96±26 | 93±39 |
| L | 103±9 | 95±9 | 101±12 | 104±16 | 107±19 |
| F0+L | 108±17 | 98±16 | 108±15 | 107±19 | 98±17 |

Values are expressed as mean±SD. C=Control; F0=administration of fasudil at just reperfusion; F30=administration of fasudil at 30 min after reperfusion; W=Wortmannin; P=PD98059; L=L-NAME.

**Table 3 Left ventricular area at risk**

| Group | Number | Area at risk/ Left ventricle (%) |
|---|---|---|
| C | 9 | 53±8 |
| F0 | 9 | 52±9 |
| F30 | 9 | 48±10 |
| W | 8 | 49±10 |
| F0+W | 7 | 49±9 |
| P | 9 | 52±13 |
| F0+P | 9 | 48±8 |
| L | 9 | 44±10 |
| F0+L | 9 | 52±9 |

Values are expressed as mean±SD. C=Control; F0=administration of fasudil at just reperfusion; F30=administration of fasudil at 30 min after reperfusion; W=Wortmannin; P=PD98059; L=L-NAME.

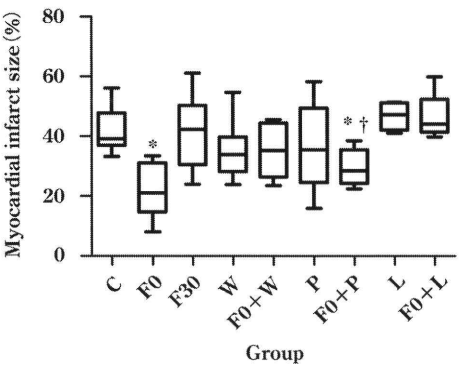


**Figure 2 Myocardial infarct size expressed as a percentage of the left ventricular area at risk**
C=Control; F0=administration of fasudil at just reperfusion; F30=administration of fasudil at 30 min after reperfusion; W=Wortmannin; P=PD98059; L=L-NAME. Values are expressed as mean±SD.
*Significantly ($p<0.05$) different from C. †Significantly ($p<0.05$) different between P and F0+P.

### B. Infarct size

LV weight, AAR weight, and the ratio of AAR to total LV mass were similar among the groups (**Table 3**). The data of the infarct size of each group are shown in **Fig. 2**. Group F0 (22±9%), but not Group F30 (42±12%) showed significantly smaller infarct sizes as compared to Group C (42±7%). The protective effect of fasudil was blocked by wortmannin (Group F0+W; 36±9%) or L-NAME (Group F0+L; 47±6%), but not by PD98059 (Group F0+P; 26±8%). Wortmannin (Group W; 35±10%), PD98059 (Group P; 37±15%) or L-NAME (Group L; 47±4%) alone had no effect on the myocardial infarct size.

## Discussion and Conclusion

The results show that fasudil administered at the beginning of, but not 30 min after reperfusion could limit myocardial infarct size in anesthetized rats. Some previous reports demonstrated that Rho-kinase inhibitors exert preconditioning effect against myocardial infarction in various mammalian species, i.e., hydroxyfasudil in rats[12] and dogs[16], fasudil in rats[13] and Y-27632 in rats[14] and mice[15]. Recently, it was reported that Y-27632 administered at beginning of reperfusion reduced infarct size in perfused rat hearts[23]. To the best of our knowledge, this is the first report that demonstrates the protective effect of fasudil at a clinical dose administered during reperfusion against myocardial ischemia/reperfusion injury *in vivo* model. This new appreciation of Rho-kinase as a mediator of ischemia/reperfusion injury has an important implication for potent therapeutic manipula-

tion of survival pathways in patients undergoing reperfusion for acute myocardial infarction. In the current results, fasudil administered at the beginning of, but not at 30 min after reperfusion reduced infarct size. Pharmacological postconditioning is likely to need a protective strategy during a few minutes of reperfusion. Jonassen reported that insulin administered at first 15 min of reperfusion reduced myocardial infarct size in isolated perfused rat hearts[5]. They also demonstrated that insulin mediated activation of PI3K/Akt pathway during the first 15 min of reperfusion, which might support our results.

The dose of fasudil adopted in the current study corresponds to that clinically applied for prevention of cerebral vasospasm after SAH. Fasudil is clinically used in doses of 30 mg/30 min intravenous infusion three times a day for cerebral vasospasm after subarachnoid hemorrhage[24], and 60 mg/60 min intravenous infusion twice daily for acute ischemic stroke[25]. Recently, our group reported that both 0.5mg/kg and 0.15mg/kg of fasudil produced reductions of myocardial infarct size under normoglycemia in rats[26]. Thus, relatively small dose of fasudil as compared to clinical dose could induce postconditioning. They also demonstrated that hyperglycemia completely abolished the protective effect of 0.15mg/kg, but not 0.5mg/kg of fasudil. Thus, fasudil is likely to induce postconditioning in a dose dependent manner under hyperglycemia. Fasudil would cause hypotension, as a side effect, in a dose dependent manner[27], and it was reported that intravenous injection of 3mg/kg or more of fasudil causes a significant decrease in blood pressure in rats[28]. In the present study, there were no significant changes in MAP after administration of 0.5mg/kg of fasudil in any of fasudil groups (**Table 2**). Demiryürek, et al. reported that intravenous administration of 1 or 10mg/kg of fasudil caused significant decreases in blood pressure. They also reported that pre-ischemic administration of 10mg/kg but not 0.3 or 1mg/kg of fasudil reduced myocardial infarct size. In the current results, post-ischemic administration of 0.5mg/kg of fasudil reduced myocardial infarct size, showing a discrepancy with Demiryürek's results. This discrepancy could be explained as follows. Firstly, the deleterious action of Rho-kinase in ischemia-reperfusion injury is mediated almost exclusively during reperfusion, rather than during ischemia[23]. Therefore, it is likely that the effect of fasudil during early reperfusion would be important for myocardial protection against ischemia-reperfusion injury. Secondly, fasudil has a short elimination half-life, i.e., 16 min after intravenous bolus injection of 0.4mg/kg in humans[29]. Therefore, it is possible that pre-ischemic administration of 0.3mg/kg or 1mg/kg of fasudil did not exert the protective effect because of low blood concentrations during the early reperfusion period in Demiryürek's study.

In the current study, fasudil administered at the beginning of reperfusion exerted cardioprotective effects through activation of PI3K/Akt/NOS but not ERK1/2. Wolfrum reported that pre-ischemic administration of hydroxyfasudil led to the activation of the PI3K/Akt/NOS pathway and cardiovascular protection[12]. Hamid demonstrated that Rho-kinase activation occurred specifically after reperfusion and was sustained for 120 min after reperfusion in the infarct region[23]. Moreover, they also demonstrated that inhibition of Rho-kinase at reperfusion limited myocardial infarct size through an Akt/NOS-dependent mechanism. These previous reports and the current results suggest that Rho-kinase negatively regulates PI3K, upstream of Akt/NOS. On the other hands, the current results show that ERK1/2 dose not relate to fasudil-induced myocardial protection. There have not been reports on the role of ERK1/2 in Rho-kinase inhibitor-induced postconditioning. Interestingly, Zhang reported that ERK-MAPK signaling is required in ischemic preconditioning to oppose Rho-kinase activity in cadiomyocyte apoptosis, indicating that ERK-MAPK signaling leads to upregulation of Rho-kinase activity[30], which does not contradict our results.

We used three enzyme inhibitors to investigate the mechanism of fasudil-induced cardioprotection, i.e. wortmannin, a PI3K inhibitor, PD98059, an ERK1/2 inhibitor, and L-NAME, an NOS inhibitor. It was

reported that wortmannin, given at 15 min prior to prolonged ischemia, failed to block ischemic postconditioning *in vivo* rat model[18]. In contrast, they also reported that administration of wortmannin 5 min prior to reperfusion, as we did in the current study, could successfully abolish the ischemic postconditioning. In the current study, wortmannin had no effect on myocardial infarct size, in accordance with other post-conditioning studies using wortmannin[2,18]. PD98059 was also reported to have no effect on myocardial infarct size in desflurane preconditioning, in accordance with the current result[19]. We used L-NAME to investigate the effect of NOS on the fasudil-induced myocardial protection. The previous study targeting Rho-kinase inhibitor-induced preconditioning used L-NAME for investigating the role of NOS on the mechanisms involved, as we did[12]. Moreover, the current results support the finding of the other study implicating a role of NOS in postconditioning, which demonstrated that PI3K-mediated phosphorylation of NOS was central to this process[31].

In conclusion, fasudil in a clinical dose administered at the beginning of, but not at 30 min after reperfusion limits myocardial infarct size *in vivo* rat myocardial ischemia-reperfusion model. The protective effect of fasudil at beginning of reperfusion could be mediated through activation of PI3K/Akt/NOS but not ERK1/2 pathway.

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