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Effects of fasudil, a Rho-kinase inhibitor, on myocardial preconditioning in anesthetized rats

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Abstract

The aim of this study was to examine the effects of fasudil, a Rho-kinase inhibitor, on ischemic preconditioning and carbachol preconditioning in anesthetized rats. The total number of ventricular ectopic beats was markedly augmented with fasudil at 0.3 mg/kg and depressed with fasudil at 10 mg/kg. Fasudil at 10 mg/kg also markedly decreased the ventricular tachycardia incidence. Ischemic preconditioning, induced by 5 min coronary artery occlusion and 5 min reperfusion, decreased the incidence of ventricular tachycardia and abolished the occurrence of ventricular fibrillation. The incidences of ventricular tachycardia and ventricular fibrillation in the fasudil (10 mg/kg)+ischemic preconditioning group were found to be similar to the ischemic preconditioning group. However, low doses of fasudil (0.3 and 1 mg/kg) appeared to prevent the antiarrhythmic effects of ischemic preconditioning. Carbachol (4 μg/kg/min for 5 min) induced marked reductions in mean arterial blood pressure, heart rate and abolished ventricular tachycardia. Marked reductions in ventricular ectopic beats and ventricular tachycardia were noted in the fasudil (10 mg/kg) +carbachol preconditioning group. Lactate levels were markedly reduced in the ischemic preconditioning group and this reduction was prominently inhibited with fasudil at 1 mg/kg. Ischemic preconditioning caused a marked decrease in plasma malondialdehyde levels. Fasudil (10 mg/kg), ischemic preconditioning and carbachol preconditioning each generated marked reductions in ischemic myocardial malondialdehyde levels. Decreases in infarct size were observed with fasudil (10 mg/kg) treatment, ischemic preconditioning and carbachol preconditioning when compared to control. These results suggest that low doses of fasudil (0.3 and 1 mg/kg) appeared to prevents the effects of ischemic preconditioning and carbachol preconditioning, but a high dose of fasudil (10 mg/kg) was able to produce cardioprotective effects on myocardium against arrhythmias, infarct size or biochemical parameters and mimic the effects of ischemic preconditioning in anesthetized rats.

Keywords: Arrhythmia; Fasudil; Infarct size; Preconditioning; Rho-kinase

1. Introduction

Rho-kinase has been known as an effector of the small GTPase Rho, which plays an important role in various cellular functions, including smooth muscle contraction, actin cytoskeleton organization, and cardiovascular remodeling (Hall, 1998; Shimokawa, 2002). The Rho and Rho-kinase pathway is involved in the pathogenesis of cardiac dysfunction, and inhibition of Rho-kinase plays a critical role in the failing heart (Kobayashi et al., 2002). Fasudil, a Rho-kinase inhibitor, has been previously shown to act as a vasodilator in vivo when administered in animals (Asano et al., 1989). Fasudil significantly dilated spastic arteries in a swine model of coronary artery spasm induced by chronic treatment with interleukin (IL)-1â (Katsumata et al., 1997) and prevented endothelin-1-induced myocardial injury in rabbits (Yamamoto et al., 2000). Recent studies suggest that inhibition of Rho-kinase with fasudil or hydroxyfasudil, the main active metabolite of fasudil, protect the myocardium in experimental models of vasospastic or effort angina (Utsunomiya et al., 2001; Satoh et al., 2001a). There is also evidence that fasudil is effective in suppressing coronary artery spasm in patients with vasospastic angina

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(Masumoto et al., 2002). Fasudil may be useful for treatment of intractable severe coronary spasm resistant to intensive conventional vasodilator therapy after coronary artery bypass grafting (Inokuchi et al., 2004).

Murry et al. (1986) described for the first time that short periods of ischemia with intermittent reperfusion protect myocardium from a subsequent prolonged ischemic insult, defined as ischemic preconditioning. We tested the hypothesis that whether fasudil can pharmacologically mimic cardiac preconditioning. We have recently showed that Rho-kinase inhibition with Y-27632 is able to induce cardioprotective effects in rats (Demiryurek et al., 2005). Although it has been reported that ischemia/reperfusion upregulated expression of RhoA and subsequently activated Rho-kinase in ischemic myocardium from the mice (Bao et al., 2004), and treatment with fasudil decreased myocardial infarct size in rats subjected to transient coronary artery occlusion (Wolfrum et al., 2004), the effects of fasudil, with chemical structure different from Y-27632, on myocardial preconditioning are not known. Additionally, the effects of fasudil on ischemia-induced arrhythmias have not been studied. Therefore, the aim of this study was to investigate the effects of Rho-kinase inhibitor fasudil on cardiac effects of ischemic preconditioning and carbachol preconditioning in anesthetized rats.

2. Materials and methods

2.1. Animals and surgical preparation

Male Wistar rats, weighing 250–320 g, were used in this study. Animals were kept in colony rooms with 12-h-light/dark cycles at a room temperature of 21±1 °C and supplied with standard laboratory diet and tap water ad libitum. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study was approved by the Local Ethics Committee.

Rats were anesthetized with thiopental sodium (120 mg/kg, i.p., Pental Sodyum, I.E. Ulagay, Istanbul, Turkey) and anesthesia was maintained by supplementary injections (~10 mg/kg, i.v.) of thiopental sodium as required (Demiryurek et al., 2005). The rats were intubated and ventilated with room air by means of a small animal ventilator (SAR-830, IITC Life Science, California, USA) with a rate of 70 strokes/min. A standard limb lead II electrocardiogram (ECG) was continuously monitored and recorded on a computer throughout the experiment by using a computerized data acquisition system (MP30, BIOPAC Systems, Inc., California, USA). Body temperature was measured via a rectal probe and maintained at 37±1 °C with a lamp. The left carotid artery was cannulated with a polyethylene PE-50 catheter and connected to a pressure transducer to monitor mean arterial blood pressure. Body temperature and arterial blood pressure were also continuously monitored and recorded throughout the experiment by the same data acquisition system. The left jugular vein was cannulated for the administration of drugs. An infusion pump (74900 series, Cole-Parmer, Illinois, USA) was used for i.v. drug infusion. Rats were then given heparin i.v. (200 IU/kg), and then the chest was opened by a left thoracotomy performed between the fourth and the fifth ribs approximately 3 mm from the sternum, the pericardium incised, and the heart gently exteriorized by pressure on the abdomen. A loose ligature, 6/0 braided silk suture attached to a 10-mm micro-point reverse-cutting needle, was placed around the left anterior descending coronary artery, 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 short piece of polyethylene tube (1 mm i.d. and 15 mm long) to form a snare. Any animal, in which this procedure itself produced dysrhythmias or a sustained fall in mean arterial pressure to less than 60 mm Hg, was discarded from the study at this point. Following a stabilization period of 15 min, the snare around the left anterior descending coronary artery was tightened and held in place with a small clip to induce transient regional myocardial ischemia for 30 min. Reperfusion was initiated by releasing the ligature and removing the tube. Successful occlusion was confirmed by a 20–30% reduction in the arterial blood pressure compared to the pre-ischemic values.

2.2. Measured parameters

For all the groups, heart rate was measured from the recordings of electrocardiogram and the incidences of arrhythmias were registered, in accordance with the Lambeth Conventions (Walker et al., 1988), as ventricular tachycardia, ventricular fibrillation, and ventricular ectopic beat. A ventricular ectopic beat is defined as a discrete and identifiable premature QRS complex. Ventricular tachycardia was diagnosed as four or more consecutive ventricular ectopic beats. Ventricular fibrillation was diagnosed when the ECG recording showed chaotic activity with amplitude less than that of the normal ECG. Complex forms (e.g., bigeminy) were included in the count of ventricular ectopic beats and were not analyzed separately. Ventricular fibrillation may be sustained or may revert spontaneously to a normal sinus rhythm in the rat. Irreversible ventricular fibrillation was defined as ventricular fibrillation, which did not reverse within 5 min of onset. The onset and duration of arrhythmias were also measured. The arrhythmia score for these experiments was calculated by using the previously published scale (Demiryurek et al., 2002). The following values were given:

0. 0–50 ventricular ectopic beats with no ventricular tachycardia or ventricular fibrillation over the 30 min ischemia period,
1. 50–500 ventricular ectopic beats only,
2. More than 500 ventricular ectopic beats, or one episode of spontaneously reversible ventricular tachycardia or ventricular fibrillation,
3. Spontaneously reversible ventricular tachycardia and/or ventricular fibrillation for 2–30 episodes,
4. Spontaneously reversible ventricular tachycardia and/or ventricular fibrillation for more than 30 episodes,
5. Occurrence of irreversible ventricular fibrillation.

2.3. Cardiac area at risk and infarct size determination

At the end of experiments, the left anterior descending coronary artery was occluded again at the same site as previously, and 3 ml of a 2% solution of Evans blue dye was infused into the jugular vein catheter to distinguish between perfused and non-perfused (area at risk) sections of the heart. Following the staining of the perfused myocardium, the heart was excised and the entire ventricle was cut from the apex to the base into slices of 3–4 mm, the right ventricular wall was removed, and the area at risk (pink) was separated from the non-ischemic (blue) area. The area at risk was cut into small pieces and incubated with 1% solution of 2,3,5-triphenyltetrazolium chloride (in 20 mM phosphate buffer, pH 7.4) stain for a period of 30 min at 37 °C to visualize the infarct area. Pieces were separated according to staining and weighed to determine the infarct size as a percentage of the weight of the area at risk. Area at risk was expressed as a percentage of the left ventricle as described previously (Murry et al., 1986; Demiryurek et al., 2005).

2.4. Experimental protocols

After completing surgical procedures, all hearts were allowed to stabilize for 15 min prior to the experimental protocol. These protocols are diagrammatically represented in Fig. 1. In the first group of experiments (protocol 1, control, $n=25$), hearts were subjected to 30 min left anterior descending coronary artery occlusion and 30 min reperfusion. In the second group of experiments (protocol 2, fasudil, $n=8$–18), rats were injected with fasudil at 0.3, 1 or 10 mg/kg (i.v. bolus) (Yamamoto et al., 2000; Utsunomiya et al., 2001; Satoh et al., 2001b; Wolfrum et al., 2004) and 20 min later were subjected to 30 min occlusion and 30 min reperfusion. In the third group of experiments (protocol 3, ischemic preconditioning, $n=25$), hearts were preconditioned by 5 min occlusion and 5 min reperfusion before giving 30 min ischemia and reperfusion. In protocol 4 (fasudil+ischemic preconditioning, $n=7$–18), animals received i.v. bolus administration of fasudil followed after 10 min by preconditioning with 5 min ischemia, than as in protocol 3. In the fifth group of experiments (protocol 5, carbachol preconditioning, $n=10$), animals received a 5-min carbachol infusion (4 μg/kg/min, i.v.) followed after 5 min by 30 min ischemia and reperfusion. This dose of carbachol has been shown to induce pharmacological preconditioning in rat heart (Yamaguchi et al., 1997). For the last series of experiments (protocol 6, fasudil +carbachol preconditioning, $n=5$–8), hearts were preconditioned with 5 min carbachol infusion as in protocol 5, but received i.v. bolus administration of fasudil 10 min prior to carbachol infusion.

2.5. Biochemical analysis

Blood samples were collected at the end of the experiment. Then samples were promptly centrifuged at 2500×*g*, 4 °C, for 15 min, the plasma removed, and stored at −40 °C until assayed. Plasma lactate and cardiac troponin T levels, creatine kinase and creatine kinase-MB activities were quantified as

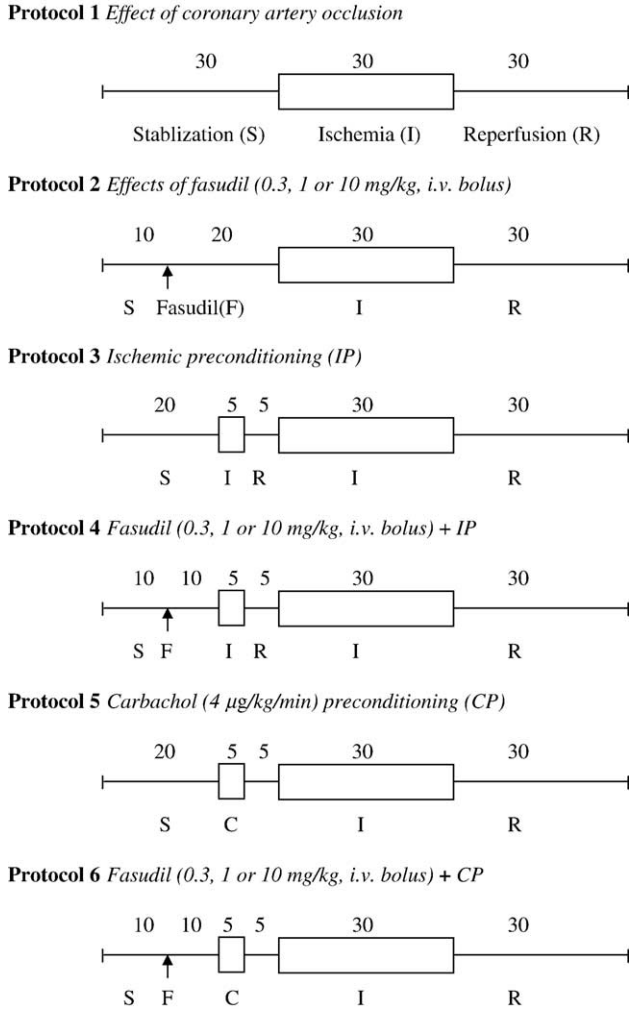

Fig. 1. Experimental protocol for the study. Numerical values represent the duration in minutes.

reported previously (Demiryurek et al., 2005). The protein content of homogenates was determined according to the procedure of Lowry et al. (1951).

2.6. Malondialdehyde measurements

As an index for lipid peroxidation and free radical generation, malondialdehyde was measured from plasma or tissue homogenates by using thiobarbituric acid reactivity method as previously described (Ohkawa et al., 1979; Draper and Hadley, 1990). Ischemic myocardium was separated with Evans blue staining as described above and the unstained region (area at risk) was used for the malondialdehyde measurement. Malondialdehyde generation was evaluated by the assay of thiobarbituric acid-reacting substances. Myocardial tissues were homogenized in a four volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.4) using a homogenizer (Branson sonifier 150, Danbury, CT, USA) after cutting of the tissue into small pieces with a scissors. The homogenate was then centrifuged at 2500×*g* for 5 min and clear supernatant was used for malondialdehyde assay. Briefly, 100 μl of sample was added to a mixture

Table 1
Mean arterial blood pressure values (mm Hg) during coronary occlusion and reperfusion in anesthetized rats

	n	Baseline	i.v. bolus fasudil		Preconditioning or drug infusion		Reperfusion or no infusion		Occlusion		Reperfusion	
			1 min	20 min	1 min	5 min	1 min	5 min	1 min	30 min	1 min	30 min
Control	25	142±5	–	–	–	–	–	–	117±4*	100±5*	111±5*	98±7*
Fasudil (0.3 mg/kg)	9	153±10	149±9	143±10	–	–	–	–	122±8	112±10	129±9	128±14
Fasudil (1 mg/kg)	18	133±4	118±4*	117±4*	–	–	–	–	105±4*	95±5*	105±3*	100±3*
Fasudil (10 mg/kg)	8	139±7	117±7*	110±6*	–	–	–	–	101±7*	94±7*	102±6*	95±5*
IP	25	130±4	–	–	108±3*	101±4*	114±4*	120±4	104±4*	103±3*	111±4*	108±5*
Fasudil (0.3 mg/kg)+IP	7	145±9	141±10	138±8[a]	127±10	123±10	125±12	131±9	112±8	110±9	108±11	104±10
Fasudil (1 mg/kg)+IP	18	135±5	121±3	123±6[a]	109±3*	106±6*	117±5	124±5	100±4*	95±5*	102±4*	97±5*
Fasudil (10 mg/kg)+IP	8	136±5	115±3*	109±3[a,]*	103±5*	99±4*	97±4*	104±2*	96±3*	87±3*	101±2*	92±2*
CP	10	140±7	–	–	109±8*	101±7*	107±8*	111±5*	97±5*	89±5*	99±5*	99±3*
Fasudil (0.3 mg/kg)+CP	5	146±7	141±7	137±10[a]	137±9	125±8	132±10	127±10	119±10	96±2	120±8	124±8
Fasudil (1 mg/kg)+CP	7	135±6	119±7	116±6[a]	112±7	104±10	104±9	106±10	99±10	87±8*	103±9	92±6*
Fasudil (10 mg/kg)+CP	8	137±6	102±3*	100±2[a,]*	99±3*	92±3*	87±3*	92±2*	88±2*	78±5*	82±4*	80±2*

IP, ischemic preconditioning; CP, carbachol preconditioning.
[a] 10 min after i.v. bolus fasudil.
* $P<0.05$ compared to baseline values.

containing 400 μl PBS, 13 μl butylated hydroxytoluene and 250 μl trichloroacetic acid. After mixing, samples were kept at 4 °C for 2 h and then centrifuged at 2000×*g* for 15 min. The supernatant (500 μl) was added to a mixture of 38 μl disodium EDTA and 126 μl thiobarbituric acid and then incubated for 15 min at 95 °C. After cooling, the absorbance of the mixture was read at 532 nm in a spectrophotometer (Shimatzu UV-1601, Kyoto, Japan) and the results expressed as nmol/ml and nmol/mg protein for plasma and ischemic myocardium, respectively. The amount of thiobarbituric acid-reacting substances was calculated as malondialdehyde equivalents using 1,1,3,3-tetramethoxypropane as standard.

2.7. Drugs

Fasudil hydrochloride was purchased from Tocris Cookson Ltd. (Bristol, UK). Evans blue, carbachol, and 2,3,5-triphenyltetrazolium chloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other materials used were in analytical grade and all stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Eczacibasi-Baxter, Istanbul, Turkey) immediately before use.

2.8. Statistical analysis

All data are expressed as mean±S.E.M. or the percentage incidence. Statistical comparison of more than two groups was performed by a one-way analysis of variance followed by Student–Newman–Keuls multiple comparisons test. A Fisher's exact test was used to detect significant differences in the incidence of ventricular tachycardia, ventricular fibrillation and irreversible ventricular fibrillation between groups. The Mann–Whitney *U*-test was used to detect significant differences between arrhythmia scores. In all tests, *P*

Table 2
Heart rate values (beats/min) during coronary occlusion and reperfusion in anesthetized rats

	n	Baseline	i.v. bolus fasudil		Preconditioning or drug infusion		Reperfusion or no infusion		Occlusion		Reperfusion	
			1 min	20 min	1 min	5 min	1 min	5 min	1 min	30 min	1 min	30 min
Control	25	390±9	–	–	–	–	–	–	392±7	371±14	399±13	365±13
Fasudil (0.3 mg/kg)	9	387±11	386±12	374±10	–	–	–	–	370±12	387±15	376±12	378±17
Fasudil (1 mg/kg)	18	380±8	376±9	398±9	–	–	–	–	393±9	379±9	385±11	370±9
Fasudil (10 mg/kg)	8	402±13	377±12	387±12	–	–	–	–	383±16	384±12	361±7	362±8
IP	25	381±8	–	–	369±7	378±8	377±9	380±7	387±8	382±8	391±11	396±9
Fasudil (0.3 mg/kg)+IP	7	388±12	385±12	403±12[a]	406±8	400±14	403±13	397±13	400±9	381±9	403±13	397±13
Fasudil (1 mg/kg)+IP	18	368±7	360±9	372±8[a]	381±10	376±11	379±8	375±7	375±7	373±7	376±7	367±9
Fasudil (10 mg/kg)+IP	8	379±7	369±12	390±8[a]	378±12	377±10	390±8	382±8	379±7	377±14	381±12	380±13
CP	10	396±9	–	–	369±10	215±17*	214±20*	306±12*	296±14*	336±23*	311±26*	320±20*
Fasudil (0.3 mg/kg)+CP	5	362±15	342±11	364±12[a]	322±32	189±35*	181±39*	241±37*	234±35*	209±45*	274±26*	279±15*
Fasudil (1 mg/kg)+CP	7	393±6	377±8	363±14[a]	362±13	258±23*	233±17*	301±11	301±9	289±29*	310±27	316±29
Fasudil (10 mg/kg)+CP	8	391±9	379±12	374±8[a]	284±21*	246±26*	217±21*	293±17*	304±21*	294±12*	316±12*	307±13*

IP, ischemic preconditioning; CP, carbachol preconditioning.
[a] 10 min after i.v. bolus fasudil.
* $P<0.05$ compared to baseline values.

Table 3
Effects of ischemic preconditioning, carbachol preconditioning, and fasudil on the severity of arrhythmias induced by 30 min of coronary artery occlusion in anesthetized rats

	n	Total ventricular ectopic beats	% ventricular tachycardia	% Total ventricular fibrillation	% Irreversible ventricular fibrillation
Control	25	664±131 (25)	100 (25)	44.0 (11)	12 (3)
Fasudil (0.3 mg/kg)	9	1340±432 *	100 (9)	55.6 (5)	0 (0)
Fasudil (1 mg/kg)	18	700±126 (18)	88.9 (16)	16.7 (3)	0 (0)
Fasudil (10 mg/kg)	8	57±20 * (8)	50 * (4)	0 * (0)	0 (0)
IP	25	35±10 * (19)	24 * (6)	0 * (0)	0 (0)
Fasudil (0.3 mg/kg)+IP	7	446±109 (7)	71.4 *,** (5)	0 (0)	0 (0)
Fasudil (1 mg/kg)+IP	18	364±114 (18)	66.7 *,** (12)	0 * (0)	0 (0)
Fasudil (10 mg/kg)+IP	8	44±16 * (8)	25 * (2)	0 * (0)	0 (0)
CP	10	285±144 (10)	80 (8)	0 * (0)	0 (0)
Fasudil (0.3 mg/kg)+CP	5	314±62 (5)	60 (3)	0 (0)	0 (0)
Fasudil (1 mg/kg)+CP	7	418±72 (7)	100 (7)	0 (0)	0 (0)
Fasudil (10 mg/kg)+CP	8	144±50 * (8)	62.5 * (5)	0 * (0)	0 (0)

Numbers in parentheses are the number of hearts that exhibited that particular type of arrhythmia. IP, ischemic preconditioning; CP, carbachol preconditioning.
* $P<0.05$ compared to control group.
** $P<0.05$ compared to IP group.

values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Hemodynamics

Table 1 summarizes mean arterial blood pressure in all groups. Occlusion of the left anterior descending coronary artery produced a marked decrease in blood pressure in the control group. Fasudil at 1 and 10 mg/kg doses caused significant decreases in mean arterial blood pressure. Blood pressure changes with these doses of fasudil were similar to control group during occlusion and reperfusion periods. However, no significant change in mean arterial blood pressure was observed with fasudil at 0.3 mg/kg dose. Ischemic preconditioning and carbachol preconditioning produced marked decreases in blood pressure, which were slightly further reduced with 10 mg/kg fasudil administration. However, there were no marked changes in blood pressure with fasudil at 0.3 mg/kg dose in fasudil+ischemic preconditioning and fasudil+carbachol preconditioning groups. Table 2 summarizes heart rate in all groups. No significant differences were observed in heart rate between groups except in carbachol-treated groups.

3.2. Effects on ischemia-induced arrhythmias

The total number of ventricular ectopic beats was markedly increased with fasudil at 0.3 mg/kg (from 664±131, $n=25$, to 1340±432, $n=9$) and decreased with fasudil at 10 mg/kg (to 57±20, $n=8$) as shown in Table 3. There were marked reductions in the total number of ventricular ectopic beats in ischemic preconditioning and fasudil (10 mg/kg)+ischemic preconditioning groups, but no significant decreases were observed in

Table 4
Effects of ischemic preconditioning, carbachol preconditioning, and fasudil on the time of onset of first arrhythmias, durations of ventricular tachycardia and ventricular fibrillation, and on arrhythmia scores in anesthetized rats

	n	Time of onset of first arrhythmias (s)	Duration of ventricular tachycardia (s)	Duration of ventricular fibrillation (s)	Arrhythmia scores
Control	25	38.8±13.2	78.5±20.0	18.6±4.3	3.3±0.1
Fasudil (0.3 mg/kg)	9	99.4±45.5	173.2±70.4 *	98.8±81.1	3.3±0.2
Fasudil (1 mg/kg)	18	86.8±26.8	79.3±16.1	166.3±126.8	2.9±0.2
Fasudil (10 mg/kg)	8	220.6±49.8 *	9.5±4.1	0	1.4±0.5 *
IP	25	135.6±36.8	7.8±4.3	0	0.6±0.2 *
Fasudil (0.3 mg/kg)+IP	7	75.2±27.0	47.0±4.7	0	2.6±0.3 **
Fasudil (1 mg/kg)+IP	18	149.9±38.1	57.1±17.3	0	2.2±0.3 *,**
Fasudil (10 mg/kg)+IP	8	169.3±28.1	6.5±1.5	0	0.8±0.4 *
CP	10	86.3±54.7	37.8±17.4	0	2.2±0.4 *
Fasudil (0.3 mg/kg)+CP	5	280.4±60.0 *	15.7±8.2	0	2.2±0.5
Fasudil (1 mg/kg)+CP	7	244.4±73.7 *	41.0±12.1	0	2.9±0.1
Fasudil (10 mg/kg)+CP	8	328.3±30.4 *,***	21.2±12.3	0	1.8±0.5 *

IP, ischemic preconditioning; CP, carbachol preconditioning.
* $P<0.05$ compared to control group.
** $P<0.05$ compared to IP group.
*** $P<0.05$ compared to carbachol preconditioning.

0.3 and 1 mg/kg fasudil+ischemic preconditioning groups. A marked reduction in the total ventricular ectopic beats number was also observed in 10 mg/kg fasudil+carbachol preconditioning group. All the rats produced ventricular tachycardia in the control group. Fasudil at 10 mg/kg, but not at 0.3 or 1 mg/kg, markedly decreased the ventricular tachycardia incidence (from 100%, $n=25$, to 50%, $n=8$, $P<0.05$). The most marked reduction in ventricular tachycardia incidence was noted in the ischemic preconditioning group (to 24%, $n=25$). This reduction was partially reversed in the presence of 0.3 and 1 mg/kg fasudil. However, fasudil at 10 mg/kg produced similar ventricular tachycardia incidence to the ischemic preconditioning group. Marked reduction in ventricular tachycardia incidence was also observed in the 10 mg/kg fasudil+carbachol preconditioning group. The ventricular fibrillation incidence was 44% ($n=25$) in the control group. Although 55.6% and 16.7% ventricular fibrillation incidences were found in 0.3 and 1 mg/kg doses fasudil, respectively, no ventricular fibrillation was recorded in other groups. Irreversible ventricular fibrillation was observed only in the control group (Table 3).

Effects of ischemic preconditioning, carbachol preconditioning and fasudil on the time of onset of the first arrhythmias, durations of ventricular tachycardia and ventricular fibrillation and on arrhythmia scores are shown in Table 4. The time of onset of first arrhythmias was markedly delayed in fasudil at 10 mg/kg and in fasudil+carbachol preconditioning groups. There was a marked increase in the duration of ventricular tachycardia with 0.3 mg/kg dose of fasudil. There were no significant differences in this parameter in other groups. No marked changes were observed in the durations of ventricular fibrillation between the groups.

Significant reductions in arrhythmia scores were observed with fasudil at 10 mg/kg, but not 0.3 or 1 mg/kg, dose. The most marked reduction in the arrhythmia scores was observed in the ischemic preconditioning group (from 3.3 ± 0.1, $n=25$, to 0.6 ± 0.2, $n=25$, $P<0.05$). This reduction was markedly inhibited in the presence of 0.3 and 1 mg/kg fasudil. The effect of 10 mg/kg fasudil+ischemic preconditioning on arrhythmia score was similar to the ischemic preconditioning group. Carbachol preconditioning generated significant reduction in the arrhythmia score (to 2.2 ± 0.4, $n=10$). Marked attenuation in the arrhythmia score was also observed with the 10 mg/kg fasudil+carbachol preconditioning group (Table 4).

3.3. Biochemical analysis

Lactate levels were markedly reduced in the ischemic preconditioning group (Fig. 2A). This reduction was markedly inhibited with fasudil 1 mg/kg. The changes in troponin T levels did not reach a significance level (Fig. 2B). Neither

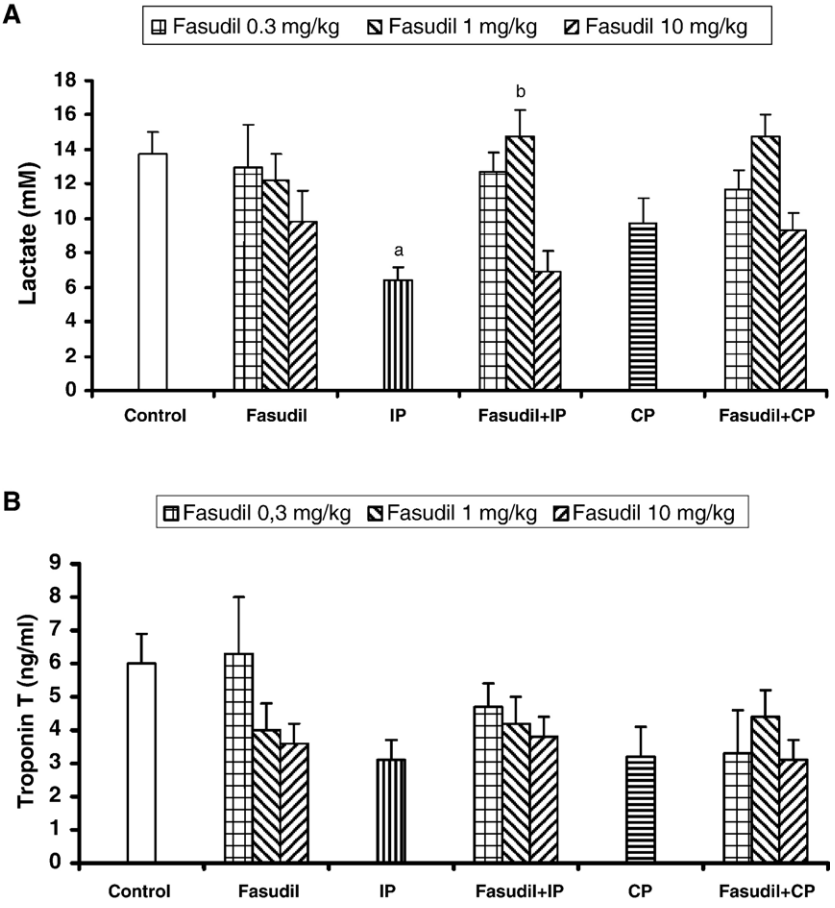

Fig. 2. Effects of fasudil on lactate (A) and cardiac troponin T levels (B) in plasma of the anesthetized rats. All values are the mean±S.E.M., $n=5$–25. [a]$P<0.05$ vs. control group, [b]$P<0.05$ compared to IP group. IP, ischemic preconditioning; CP, carbachol preconditioning.

preconditioning nor fasudil-treated groups had significant effects on creatine kinase and creatine kinase-MB levels (Fig. 3). Plasma malondialdehyde levels were markedly diminished only in the ischemic preconditioning group (Fig. 4A). Decreases in plasma malondialdehyde levels were also noted in other treatment groups, but these attenuations were not significant. However, fasudil 10 mg/kg, ischemic preconditioning and carbachol preconditioning generated marked reduction in ischemic myocardial malondialdehyde levels (Fig. 4B). Myocardial malondialdehyde levels were significantly higher in fasudil 1 mg/kg+carbachol preconditioning group when compared to carbachol preconditioning (Fig. 4B).

3.4. Area at risk and infarct size measurements

No significant differences were recorded in the left ventricular area at risk between the groups (Fig. 5A). However, the necrotic area was markedly reduced in fasudil 10 mg/kg, ischemic preconditioning and carbachol preconditioning groups when compared to control group (Fig. 5B). Marked reductions in the necrotic area in ischemic preconditioning and carbachol preconditioning groups were not found in rats treated with fasudil 0.3 or 1 mg/kg.

4. Discussion

4.1. Dose-dependent effects of fasudil

The results of this study provided the first experimental evidence that fasudil is able to produce a dual effect on the ischemia–reperfusion, and ischemic and pharmacological preconditioning in anesthetized rats. Low doses of fasudil (0.3 and 1 mg/kg) appeared to prevent the cardioprotective effects of ischemic preconditioning and carbachol preconditioning, whereas a high dose of fasudil (10 mg/kg) generated cardioprotective effects and did not inhibit the cardioprotection induced by ischemic preconditioning or carbachol preconditioning. To our knowledge, this is the first experimental study to demonstrate that fasudil has a dose-dependent cardiac effect in an in vivo setup. These results are different in our previous study showing that Y-27632, another selective Rho-kinase inhibitor, was cardioprotective at the dose of 0.1 mg/kg (Demiryurek et al., 2005). The underlying mechanisms for the prevention of cardioprotective effects with the low doses of fasudil in the present study are not known. Since fasudil has recently been shown to prevent pinacidil-induced cardioprotection in rat isolated hearts, it is likely

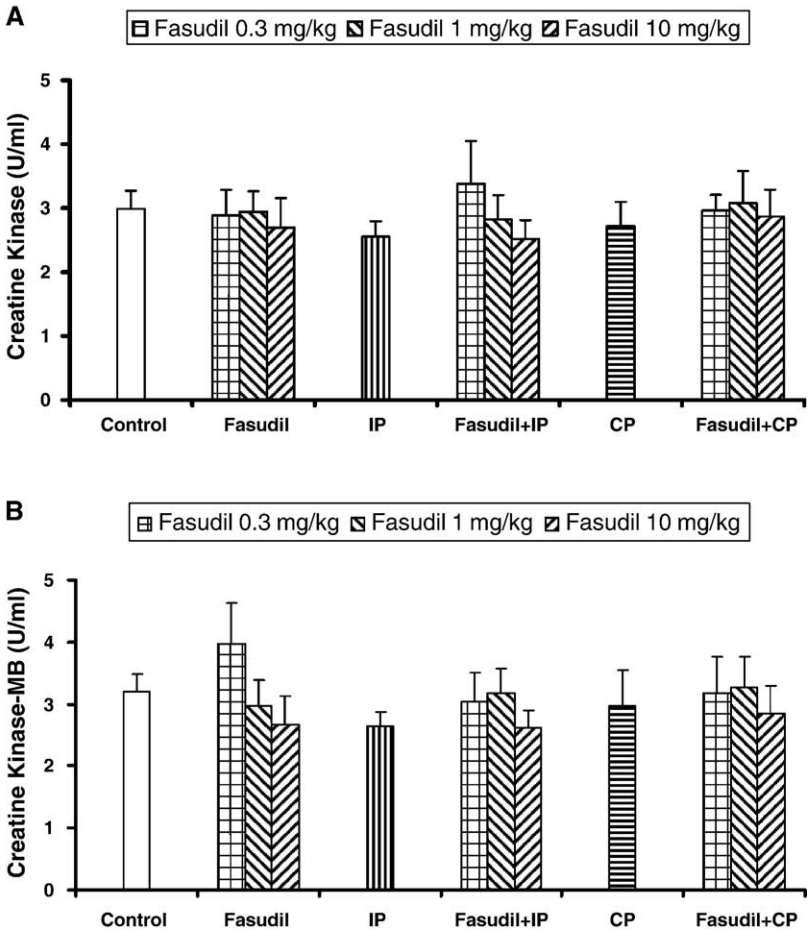

Fig. 3. Effects of fasudil on creatine kinase (CK) (A) and creatine kinase–MB (CK-MB) activity (B) in plasma of the anesthetized rats. All values are the mean±S.E.M., $n=5–25$. IP, ischemic preconditioning; CP, carbachol preconditioning.

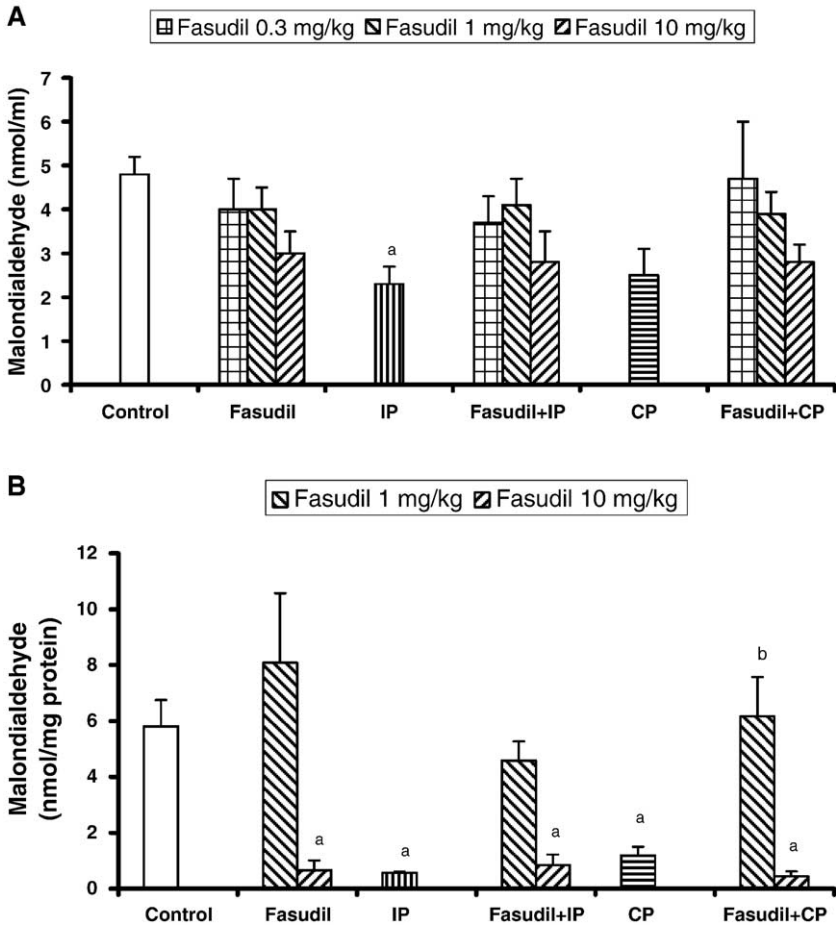

Fig. 4. Effects of fasudil on plasma (A) (n=5–25), and ischemic myocardial (B) (n=5–10) malondialdehyde levels of the anesthetized rats. All values are the mean±S.E.M. [a]$P<0.05$ vs. control group, [b]$P<0.05$ compared to carbachol preconditioning. IP, ischemic preconditioning; CP, carbachol preconditioning.

that the observed effects at low doses may be related to K_{ATP} channel inhibition (Nishizawa et al., 2005). The data presented in this study showed that fasudil at 10 mg/kg dose is able to mimic ischemic preconditioning in rats. These results support our previous report (Demiryurek et al., 2005) demonstrating that a Rho-kinase inhibitor at a high dose on its own can mimic preconditioning and does not inhibit ischemic preconditioning or carbachol preconditioning in anesthetized rats.

4.2. Effects of fasudil on infarct size

Although low doses of fasudil (0.3 and 1 mg/kg) abolished the beneficial effects of ischemic preconditioning and carbachol preconditioning, a high dose of fasudil (10 mg/kg) elicited marked reductions in infarct size measurement when compared to control group in our experimental model. Our results may support the recently published findings that treatment with fasudil decreased myocardial infarct size in anesthetized rats subjected to transient coronary artery occlusion and reperfusion (Wolfrum et al., 2004). Yada et al. (2005) also observed a significant reduction in infarct size following intracoronary administration of hydroxyfasudil in anesthetized dogs.

4.3. Effects of fasudil on arrhythmias

This is the first study examining the effects of fasudil on ventricular arrhythmias in a coronary occlusion and reperfusion model. Although fasudil caused a reduction in mean arterial blood pressure in the present study, it appeared to modify the number of ventricular ectopic beats and incidence of ventricular tachycardia. Fasudil may have direct effects on myocardium. However, hydroxyfasudil has been shown to have no inotropic or chronotropic effect on the isolated hearts of guinea pigs and does not affect the P-R or QTc interval in anesthetized dogs (Utsunomiya et al., 2001). It is likely that the effects of fasudil in our experiments are not related to alteration in calcium flux, since it has been shown that fasudil does not have a blocking action on slow myocardial Ca^{2+} channels (Asano et al., 1987).

4.4. Coronary vasodilator effect of fasudil

Since Rho-kinase inhibitors have vasodilator effects, these drugs may increase regional myocardial blood flow (Shimokawa, 2002). However, it has been reported that the infarct-limiting effects of Rho-kinase inhibition could be independent of either a change in systemic hemodynamics or the

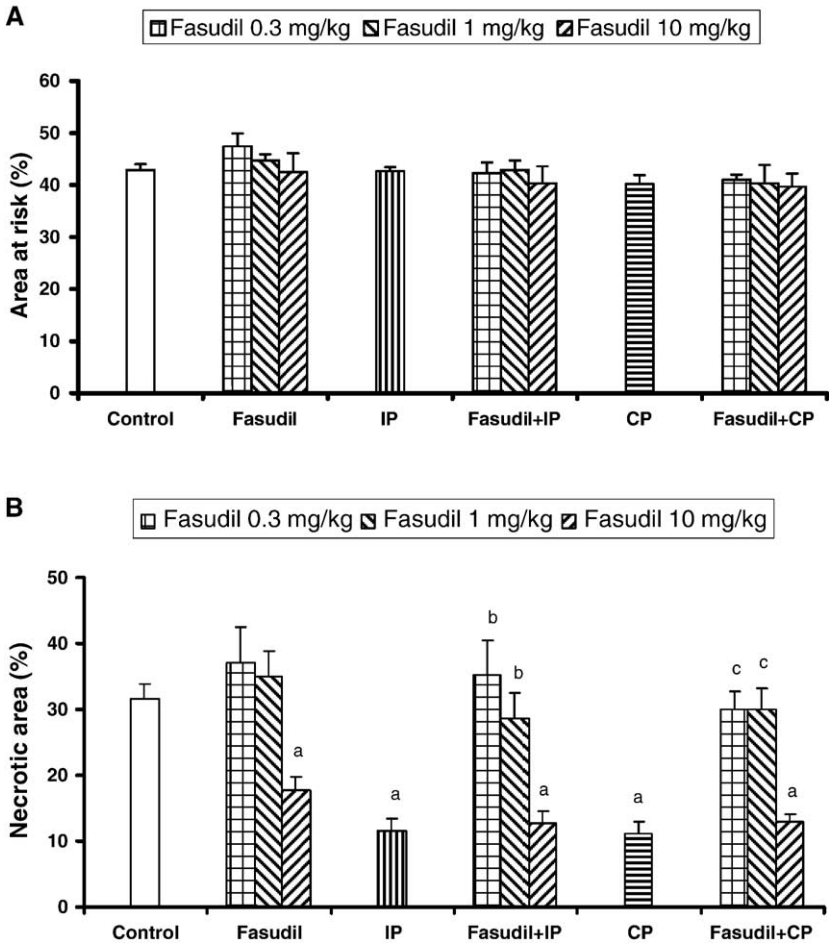

Fig. 5. Effects of fasudil on area at risk (A) ($n=5$–25), and infarct size (B) ($n=5$–16). Area at risk indexed to total left ventricle (area at risk/total left ventricle × 100) and necrotic area indexed to area at risk (necrotic area/area at risk × 100) in percentage of wet weight. All values are the mean ± S.E.M. [a]$P<0.05$ vs. control group, [b]$P<0.05$ compared to IP group, [c]$P<0.05$ compared to carbachol preconditioning. IP, ischemic preconditioning; CP, carbachol preconditioning.

recruitment of collateral blood flow in dogs (Sanada et al., 2004). Although it has been demonstrated that fasudil significantly prevented the reduction in the coronary flow by vasopressin in isolated rat hearts (Satoh et al., 2001a), it is not known whether fasudil has any in vivo acute effects on coronary circulation in rats. It has been shown that inhibition of Rho-kinase with hydroxyfasudil (0.1 and 0.3 mg/kg) and fasudil (0.3 mg/kg) protects myocardium subjected to pacing-induced ischemia through the increase in coronary blood flow in anesthetized open-chest dogs (Utsunomiya et al., 2001). Infusion of the Rho-kinase inhibitor fasudil (300 μg/min for 15 min into the left coronary artery) markedly suppressed coronary artery spasm in patients with vasospastic angina (Shimokawa, 2002; Masumoto et al., 2002). Fasudil (1.5 mg/min for 15 min) successfully resolved the spasm and improved myocardial ischemia in patients with severe coronary spasm resistant to intensive conventional vasodilator therapy after coronary artery bypass grafting (Inokuchi et al., 2004). Collectively, these data may imply that during coronary vasoconstriction, Rho-kinase is activated and Rho-kinase inhibition can cause coronary vasodilation.

4.5. Effects of fasudil on carbachol preconditioning

Carbachol may increase regional myocardial blood flow which may contribute to cardioprotection seen in the present study. Ischemic preconditioning is mediated via several sarcolemmal receptors, which are mostly linked to inhibitory G-protein as seen with the muscarinic M_2 receptor agonist carbachol (Yamaguchi et al., 1997). Pretreatment with carbachol causes a delay in cell necrosis similar to ischemic preconditioning (Thornton et al., 1993; Pisarenko et al., 1999). Carbachol is able to mimic ischemic preconditioning by reducing infarct size or improving contractile function after periods of regional or global ischemia in rat and rabbit hearts (Thornton et al., 1993; Yamaguchi et al., 1997). It has been demonstrated that both carbachol and acetylcholine activate K_{ATP} channels in myocytes via M_2 muscarinic receptors, which leads to acute preconditioning (Qian et al., 1996). Miyazaki et al. (2002) recently showed that carbachol activates Rho-kinase through stimulation of RhoA activity in smooth muscle. We have shown that administration of fasudil prior to carbachol was effective in reducing both arrhythmia score and ventricular fibrillation incidence in rats.

4.6. Effects of fasudil on biochemical parameters

In the present study, occlusion of the left main coronary artery for a period of 30 min resulted in substantial injury to the myocardium. Both fasudil and preconditioning markedly reduced the myocardial injury in rats. The reduction in infarct size in the preconditioning groups was accompanied by a decrease in circulating levels of lactate and malondialdehyde suggesting that ischemic preconditioning was able to attenuate the myocardial injury and inhibit lipid peroxidation. We have found that fasudil did not significantly modify the circulating levels of creatine kinase, creatine kinase-MB and troponin T in our experiments. Troponin T, creatine kinase and creatine kinase-MB levels were found to be detectable levels, but these values were not affected by preconditioning. This may be related to the fact that our experimental period is not long enough to detect the marked changes, since it is reported that levels of these markers start to increase a few hours after the onset of myocardial damage and remain increased for several days (Mair et al., 1992). However, fasudil at 10 mg/kg reduced ischemic myocardial malondialdehyde levels in our experiments.

4.7. Cardioprotective mechanisms of fasudil

Our results may support the recent findings obtained in mice by Bao et al. (2004) who showed that 30 min of coronary occlusion and 24 h reperfusion upregulated expression of RhoA, and subsequently activated Rho-kinase in ischemic myocardium. Sanada et al. (2004) also showed that a 60-min period of ischemia caused Rho-kinase activation and this activation was attenuated by ischemic preconditioning in anesthetized dogs. Our results support the conclusion that inhibition of Rho-kinase may be involved in the signaling pathway of ischemic preconditioning. There are pieces of evidence that the Rho/Rho-kinase pathway is independent of protein kinase C (PKC) in generating cardioprotection (Sanada et al., 2004). It has been reported that the Rho/Rho-kinase pathway is independent of PKC stimulated by phorbol esters in smooth muscle (Jensen et al., 1996; Fu et al., 1998). However, it has been recently shown in a porcine model that the phorbol ester-induced spasm at the chronically IL-1β-treated coronary segment in vivo was significantly inhibited by hydroxyfasudil (Kandabashi et al., 2003). These studies suggest that PKC and Rho-kinase coexist on the same intracellular signalling pathway, with PKC located upstream on Rho-kinase. However, ischemic preconditioning and preischemic PKA activation, but not PKC activation, have been shown to cause a substantial decrease of Rho-kinase activation during sustained ischemia in a recent study (Sanada et al., 2004). It has been proposed that transient preischemic activation of PKA reduces infarct size through Rho-kinase inhibition and actin cytoskeletal deactivation during sustained ischemia, implicating a novel mechanism for cardioprotection by ischemic preconditioning independent of PKC (Sanada et al., 2004). Moreover, fasudil is able to inhibit other protein kinases, most notably PKC-related kinase and PKCδ (Davies et al., 2000; Eto et al., 2001). PKC-related kinase is activated by RhoA, which is increased after ischemia/reperfusion (Bao et al., 2004). Cardioprotective effects of Rho-kinase inhibition against ischemia–reperfusion injury may also be related to activation of endothelial NO synthase (Wolfrum et al., 2004) or Rho-mediated ecto-5'-nucleotidase activation (Ledoux et al., 2002). It has been recently shown that the hydroxyfasudil and Y-27632 increased Akt phosphorylation, leading to increase in Akt kinase activity and nitric oxide release in human saphenous endothelial cells (Wolfrum et al., 2004). Additionally, cotreatment with phosphatidylinositol 3-kinase inhibitors or an eNOS inhibitor has been demonstrated to block the cardiovascular protective effects of fasudil in rats (Wolfrum et al., 2004). These mechanisms may be synergistically responsible for mediating a variety of cardioprotective pathways triggered by preconditioning.

4.8. Effects of fasudil on neutrophils

Neutrophils can cause reperfusion injury by obstruction of capillary vessels, production of vasoactive substances, and release of reactive oxygen species. Neutrophils are a major source of oxidants in hearts reperfused in vivo after prolonged ischemia (Duilio et al., 2001). The cardioprotective effect of Rho-kinase inhibition may be related to neutrophil accumulation in the ischemic myocardium, since treatment with Y-27632 has been shown to result in a significant reduction in the accumulation of neutrophils in ischemic myocardium (Bao et al., 2004). Although fasudil has no direct superoxide scavenger properties as assessed in a cell-free (hypoxanthine-xanthine oxidase) system, it inhibits the superoxide production from *N*-formyl-methionyl-leucyl-phenylalanine or phorbol-12-myristate-13 acetate-stimulated human polymorphonuclear neutrophils (Siomboing et al., 2001) and neutrophil accumulation in rats (Satoh et al., 1999). There is also evidence that fasudil decreases leukocyte recruitment and adhesion to the mesenteric endothelium after I/R injury in wild-type but not $eNOS^{-/-}$ mice (Wolfrum et al., 2004). Interestingly, it has been observed that Y-27632 is not protective in isolated perfused rat heart (Bao et al., 2004), which may further suggest that neutrophils play an important role in the effects of Rho-kinase inhibitors in vivo.

In conclusion, the results of this study showed that low doses of fasudil prevented the cardioprotective effects of ischemic preconditioning or pharmacological preconditioning and a high dose of fasudil was able to mimic the beneficial effects of ischemic and pharmacological preconditioning in anesthetized rats. Therefore, our data suggest that the high dose of fasudil can be used to induce pharmacological preconditioning. The present study may provide new insights into the underlying mechanisms of myocardial preconditioning.

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