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Effects of Y-27632, a selective Rho-kinase inhibitor, on myocardial preconditioning in anesthetized rats

Şeniz Demiryürek[a], Ali F. Kara[b], Ahmet Çelik[c], Mehmet Tarakçıoğlu[c], Cahit Bağcı[d], Abdullah T. Demiryürek[b,*]

[a]*Department of Physiology, Faculty of Medicine, Gazi University, Besevler, 06510 Ankara, Turkey*
[b]*Department of Pharmacology, Faculty of Medicine, University of Gaziantep, 27310 Gaziantep, Turkey*
[c]*Department of Biochemistry and Clinical Biochemistry, Faculty of Medicine, University of Gaziantep, 27310 Gaziantep, Turkey*
[d]*Department of Physiology, Faculty of Medicine, University of Gaziantep, 27310 Gaziantep, Turkey*

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Abstract

The objective of this study was to examine the effects of Y-27632, a selective Rho-kinase inhibitor, on ischemic preconditioning (IP) and carbachol preconditioning (CP) in anesthetized rats. Administration of Y-27632 (0.1 mg/kg) produced slight, but not significant, reduction in mean arterial blood pressure and suppressed the total number of ventricular ectopic beats (VEBs). IP, induced by 5 min coronary artery occlusion and 5 min reperfusion, decreased the incidence of ventricular tachycardia (VT) from 100 ($n = 30$) to 25% ($n = 24$) and abolished the occurrence of ventricular fibrillation (VF) (40% in control group) during 30 min of ischemia. The incidences of VT and VF in Y-27632 + IP group were found to be similar to IP group. Carbachol (4 μg/kg/min for 5 min) induced marked depressions in mean arterial blood pressure, heart rate and attenuated the total number of VEBs, but significant reductions in VT and VF incidences were noted in Y-27632 + CP group. Y-27632 infusion for 5 min abolished VF occurrence. Marked reductions in plasma lactate levels were observed in all treatment and preconditioning groups. IP led to marked decrease in malondialdehyde levels. Decreases in infarct size were also observed with all groups when compared to control. These results suggest that infusion of Y-27632 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. Therefore, it is likely that inhibition of Rho-kinase is involved in the signaling cascade of myocardial preconditioning.

Keywords: Arrhythmias; Cardioprotection; Infarct size; Preconditioning; Rho-kinase; Y-27632

1. Introduction

Rho-kinase phosphorylates the regulatory subunit of the myosin light chain phosphatase and inhibits its catalytic activity, causing an increase in regulatory light chain phosphorylation and contraction [1]. Rho/Rho-kinase-mediated pathway is involved in the physiological as well as pathophysiological regulation of the cardiovascular system [2]. Rho/Rho-kinase pathway is known to play an important role in the pathogenesis of cardiac dysfunction and cardiovascular remodeling [3]. The availability of specific Rho-kinase inhibitors, such as Y-27632, fasudil (HA-1077) and hydroxyfasudil, the main active metabolite of fasudil, has enabled the evaluation of the physiological and pathological roles of Rho-kinase. Y-27632 has been shown to act as a vasodilator in vivo when administered in animals [4]. Intracoronary administration of Y-27632 has been shown to exert the positive chronotropic, negative inotropic, negative dromotropic and repolarization-promoting effects at doses that could induce the coronary vasodilator actions in canine isolated hearts [5]. Recent studies suggest that inhibition of Rho-kinase protects the myocardium from pacing, vasopressin, or endothelin-induced ischemic injury [6,7]. In addition, chronic inhibi-

Abbreviations: CK-MB, creatine kinase-MB; CP, carbachol preconditioning; IP, ischemic preconditioning; LAD, left anterior descending; MDA, malondialdehyde; PKC, protein kinase C; PMA, phorbol-12-myristate-13 acetate; TBARS, thiobarbituric acid-reacting substances; VEBs, ventricular ectopic beats; VF, ventricular fibrillation; VT, ventricular tachycardia

* Corresponding author. Tel.: +90 342 3606060x77740; fax: +90 342 3601617.
E-mail address: demiryurek@gantep.edu.tr (A.T. Demiryürek).

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tion of Rho-kinase blunts the process of left ventricular hypertrophy leading to cardiac contractile dysfunction in hypertension-induced heart failure [8].

Preconditioning with brief periods of ischemia and reperfusion is considered the most potent anti-ischemic intervention known to date. Several studies have shown that ischemic preconditioning (IP), in addition to limiting infarct size [9], also generates antiarrhythmic effect [10], and protects coronary endothelial cells against reperfusion injury [11]. Numerous studies have demonstrated a role for various kinases including phosphoinositide-3-kinase or protein kinase C (PKC) in cardioprotective effect of preconditioning [12]. Recently, it has been reported that ischemia/reperfusion upregulated expression of Rho A, and subsequently activated Rho-kinase in ischemic myocardium from the mice [13]. However, the contribution of Rho-kinase in pharmacological preconditioning or on ischemia/reperfusion-induced arrhythmias has not been investigated. Therefore, the aim of this study was to elucidate the effects of Rho-kinase inhibitor Y-27632 on cardiac effects of IP and carbachol preconditioning (CP) 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) and anesthesia was maintained by supplementary injections (~10 mg/kg, i.v.) of thiopental sodium as required. The rats were intubated and ventilated with room air by means of a small animal ventilator (SAR-830, IITC Life Science) with a rate of 70 strokes/min. A standard limb lead II ECG was continuously monitored and recorded on a computer throughout the experiment by using a computerized data acquisition system (MP30, BIOPAC Systems, Inc.). Body temperature was measured via a rectal probe (TSD202A) 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 (BPT 300) 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) 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 micropoint reverse cutting needle, was placed around the left anterior descending (LAD) 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 mmHg, was discarded from the study at this point. Following a stabilization period of 15 min, the snare around the LAD 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. Successful reperfusion was confirmed by the return of the arterial blood pressure 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 [14], as ventricular tachycardia (VT), ventricular fibrillation (VF), and ventricular ectopic beat (VEB). VEB is defined as a discrete and identifiable premature QRS complex. VT was diagnosed as four or more consecutive VEBs. VF 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 VEB and not analyzed separately. VF may be sustained or may revert spontaneously to a normal sinus rhythm in the rat. Irreversible VF was defined as VF, 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 [15]. The following values were given:

1 0–50 VEBs with no VT or VF over the 30 min ischemia period;
2 50–500 VEBs only;
3 more than 500 VEBs, or one episode of spontaneously reversible VT or VF;
4 spontaneously reversible VT and/or VF for 2–30 episodes;

5 spontaneously reversible VT and/or VF for more than 30 episodes;
6 occurrence of irreversible VF.

2.3. Cardiac area at risk and infarct size determination

At the end of experiments, the LAD 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 [9,16]. The Evans blue solution stains the perfused myocardium, while the occluded vascular bed remains uncoloured. Then the heart was excised. Both atria and the roots of the great vessels were removed. 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 (TTC, in 20 mM phosphate buffer, pH 7.4) stain for a period of 30 min at 37 °C to visualize the infarct area. The area at risk of infarction was coloured brick red, due to the formation of a precipitate that results from the reaction of TTC with dehydrogenase enzymes. The loss of these enzymes from the infarcted myocardium prevents formation of the precipitate; thus the infarcted area within the risk region remains pale yellow (i.e. necrotic 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.

2.4. Exclusion criteria

Experiments were terminated or excluded from the final data analysis, if any of the following occurred: arrhythmias prior to coronary artery occlusion; mean arterial pressure less than 60 mmHg prior to drug administration and atrioventricular block during the first 5 min of ischemia (probably caused by ligature occluding the septal branch of the left coronary artery). In this study, one rat were excluded for absence of signs of ischemia, one for low blood pressure before occlusion and one for atrioventricular block during the 5 min of ischemia.

2.5. 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, $n = 30$), hearts were subjected to 30 min LAD coronary artery occlusion and 30 min reperfusion. In the second group of experiments (protocol 2, Y-27632, $n = 9$), hearts were subjected to Y-27632 at 0.1 mg/kg i.v. bolus dose [4] followed by 20 min washout and then 30 min occlusion and reperfusion. In the third group of experiments (protocol 3, IP, $n = 24$), hearts were preconditioned against 30 min ischemia and reperfusion by 5 min occlusion and 5 min reperfusion. In protocol 4 (Y-27632 + IP, $n = 8$), hearts were preconditioned with 5 min ischemia as

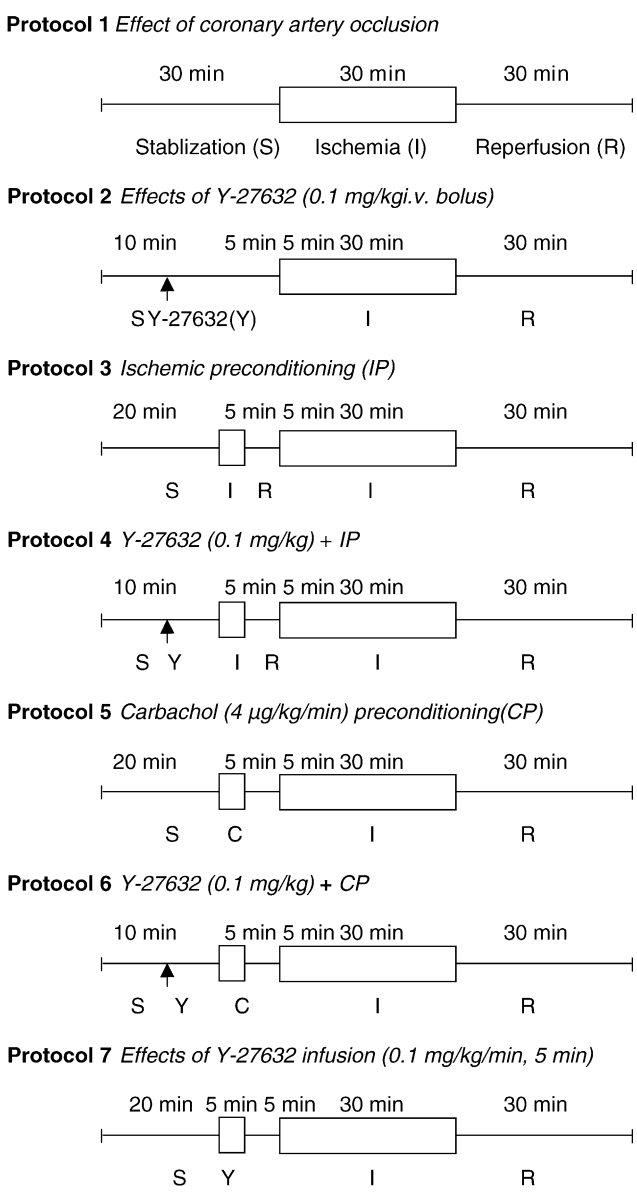

Fig. 1. Experimental protocol for the study. Rat hearts were subjected to 30 min coronary artery occlusion and 30 min reperfusion (protocol 1). Hearts were subjected to Y-27632 at the dose of 0.1 mg/kg 20 min prior to 30 min occlusion and 30 min reperfusion (protocol 2). Hearts were preconditioned against 30 min ischemia and reperfusion by 5 min occlusion and 5 min reperfusion (protocol 3). Hearts were preconditioned with 5 min ischemia as in protocol 2, but received bolus administration of Y-27632 (0.1 mg/kg) 10 min prior to preconditioning ischemia (protocol 4). Hearts were preconditioned against 30 min ischemia and reperfusion by 5 min carbachol infusion (4 μg/kg/min) (protocol 5). Hearts were preconditioned with 5 min carbachol infusion as in protocol 5, but received bolus administration of Y-27632 (0.1 mg/kg) 10 min prior to carbachol infusion (protocol 6). Hearts were subjected to 5 min i.v. infusion of Y-27632 (0.1 mg/kg/min) followed by 5 min stabilization and then 30 min occlusion and reperfusion (protocol 7).

in protocol 2, but received i.v. bolus administration of Y-27632 (0.1 mg/kg) 10 min prior to preconditioning ischemia. In the fifth group of experiments (protocol 5, CP, $n = 11$), hearts were preconditioned against 30 min ischemia and reperfusion by 5 min carbachol infusion (4 μg/kg/min, i.v.). This dose of carbachol has been shown to induce pharmacological preconditioning in rat heart [17]. In the sixth group of experiments (protocol 6, Y-27632 + CP, $n = 8$), hearts were preconditioned with 5 min carbachol infusion as in protocol 5, but received i.v. bolus administration of Y-27632 (0.1 mg/kg) 10 min prior to carbachol infusion. For the last series of experiments (protocol 7), the effects of i.v. infusion of Y-27632 on IP were investigated. In this group of experiments (Y-27632 infusion, $n = 8$), hearts were subjected to 5 min i.v. infusion of Y-27632 (0.1 mg/kg/min) followed by 5 min washout and then 30 min occlusion and reperfusion.

2.6. Biochemical analysis

Blood samples were collected at the end of the experiment. Then samples were promptly centrifuged at 5000 rpm, 4 °C, for 15 min, the plasma removed, and stored at −40 °C until assayed.

2.6.1. Measurement of lactate

For lactate measurement, blood samples were collected in tubes containing sodium fluoride/potassium oxalate. Quantitative measurement of lactate concentration in plasma was performed with the Vitros LAC Kit (Ortho-Clinical Diagnostics). The lower detection limit of the assay is 0.5 mM.

2.6.2. Assays for cardiac troponin T

Plasma cardiac troponin T levels were determined with the Elecsys Troponin T Kit (Roche Diagnostics), an automated two-site immunoassay using monoclonal antibodies specific for the cardiac troponin T isoenzyme, by using Elecsys 2010 immunoanalyzer. The lower detection limit of the assay is 0.01 ng/ml.

2.6.3. Measurements of creatine kinase-MB (CK-MB) activity

Plasma CK-MB activity was measured with a CK-MB Kit (Roche Diagnostics) by using an autoanalyzer (Roche Hitachi Modular DP Systems). The lower detection limit of the assay is 5 U/l.

2.6.4. Malondialdehyde (MDA) measurements

MDA was measured from plasma by a method as previously described [18]. MDA generation was evaluated by the assay of thiobarbituric acid-reacting substances (TBARS). In particular, the addition of a solution of 0.2 ml of sodium dodecyl sulphate (8.1%), 1.5 ml of 20% acetic acid solution (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid (pH 3.5), 0.1 ml of 90 mM butylated hydroxytoluene (for preventing the formation of TBARS in vitro) [19] and 0.6 ml of distilled water produced a chromogenic product which was extracted in *n*-butanol and pyridine. The organic layer was removed and MDA was read at 532 nm in a spectrophotometer and the results expressed as nM. The amount of TBARS was calculated as MDA equivalents using 1,1,3,3-tetramethoxypropane as standard.

2.7. Materials

Y-27632 dihydrochloride was purchased from Tocris Cookson Ltd. Evans blue, carbachol, and 2,3,5-triphenyltetrazolium chloride were obtained from Sigma Chemical Co. All the other materials used were in analytical grade and all stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Eczacıbası-Baxter) 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 ANOVA followed by Student–Newman–Keuls multiple comparisons test. A Fisher's exact test was used to detect significant differences in the incidence of VT and VF between groups. The Mann–Whitney *U*-test was used to detect significant differences between arrhythmia scores. In all tests, P values less than 0.05 was considered to be statistically significant.

3. Results

3.1. Hemodynamics

Table 1 summarizes mean arterial blood pressure in all groups. Occlusion of LAD produced a marked decrease in blood pressure and reperfusion partly restored these values in control group. Y-27632 caused slight, but not significant, decrease in mean arterial blood pressure. Blood pressure changes with this dose were similar to control group during occlusion and reperfusion periods. IP and CP produced marked decreases in blood pressure, which were not modified with Y-27632 administration. Five-minute Y-27632 infusion at 0.1 mg/kg/min dose led to marked reduction in blood pressure. 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

Ischemic preconditioning produced marked antiarrhythmic effects in anesthetized rats as shown in Table 3. Thirty minutes of coronary artery occlusion caused pronounced

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

	n	Baseline	i.v. bolus Y-27632		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	30	136 ± 5	–	–	–	–	–	–	107 ± 5*	96 ± 5*	112 ± 7*	104 ± 7*
Y-27632 (0.1 mg/kg)	9	131 ± 6	122 ± 7	116 ± 5	–	–	–	–	97 ± 7*	91 ± 6*	89 ± 5*	89 ± 6*
IP	24	130 ± 4	–	–	108 ± 3*	102 ± 4*	117 ± 4	120 ± 4	104 ± 4*	99 ± 4*	111 ± 4*	108 ± 5*
Y-27632 + IP	8	131 ± 4	122 ± 4	121 ± 7[a]	108 ± 6	103 ± 6*	110 ± 6	113 ± 7	102 ± 6*	88 ± 5*	86 ± 8*	84 ± 8*
CP	11	137 ± 7	–	–	109 ± 7*	98 ± 7*	104 ± 7*	107 ± 6*	94 ± 6*	88 ± 4*	95 ± 7*	95 ± 6*
Y-27632 + CP	8	136 ± 4	128 ± 3	121 ± 4[a]	114 ± 3*	105 ± 6*	106 ± 6*	105 ± 7*	102 ± 7*	88 ± 4*	86 ± 4*	84 ± 4*
Y-27632 infusion (0.1 mg/kg/min for 5 min)	8	131 ± 2	–	–	127 ± 3	116 ± 4	114 ± 4	111 ± 5*	105 ± 6*	99 ± 6*	98 ± 6*	88 ± 5*

[a] 10 min after i.v. bolus Y-27632. IP, ischemic preconditioning; CP, carbachol preconditioning.
* $P < 0.05$ when compared to baseline values.

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

	n	Baseline	i.v. bolus Y-27632		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	30	384 ± 8	–	–	–	–	–	–	378 ± 11	376 ± 12	387 ± 11	379 ± 12
Y-27632 (0.1 mg/kg)	9	373 ± 10	367 ± 13	358 ± 10	–	–	–	–	363 ± 9	359 ± 6	366 ± 11	376 ± 13
IP	24	382 ± 9	–	–	368 ± 7	373 ± 9	375 ± 9	378 ± 7	385 ± 8	381 ± 8	379 ± 10	375 ± 12
Y-27632 + IP	8	362 ± 14	357 ± 13	360 ± 11[a]	361 ± 12	366 ± 6	366 ± 7	366 ± 9	363 ± 9	376 ± 14	356 ± 9	357 ± 10
CP	11	395 ± 8	–	–	352 ± 19	204 ± 21*	213 ± 23*	316 ± 18*	301 ± 11*	329 ± 19*	320 ± 17*	323 ± 18*
Y-27632 + CP	8	411 ± 13	419 ± 12	416 ± 8[a]	401 ± 8	283 ± 11*	248 ± 18*	313 ± 17*	310 ± 17*	270 ± 16*	275 ± 16*	275 ± 15*
Y-27632 infusion (0.1 mg/kg/min for 5 min)	8	393 ± 8	–	–	381 ± 8	388 ± 8	382 ± 12	385 ± 12	380 ± 11	347 ± 22	347 ± 22	336 ± 19

[a] Ten minutes after i.v. bolus Y-27632. IP, ischemic preconditioning; CP, carbachol preconditioning. Values were given as mean ± S.E.M.
* $P < 0.05$ significantly different when compared to baseline values.

arrhythmogenic activity with 100% VT and 40% VF ($n = 30$). Preconditioning the hearts with 5 min of ischemia suppressed arrhythmias during the 30 min of occlusion period. In IP group, total number of VEBs (28 ± 9, $n = 24$, $P < 0.05$) was markedly lower than controls (713 ± 149, $n = 30$). The incidence of VT was markedly reduced (from 100 ($n = 30$) to 25% ($n = 24$), $P < 0.05$) and no VF was observed in IP group. Y-27632 (0.1 mg/kg, i.v.) on its own attenuated VEB numbers, but had no significant effect on VF or VT incidences. Similar to IP group, marked reductions in VEB numbers and VT incidences were recorded in Y-27632 + IP group. There was also no VF in this group. Although carbachol infusion did not produce strong preconditioning effect in anesthetized rats, marked reduction was observed in the number of VEBs (to 270 ± 131, $n = 11$, $P < 0.05$). Pretreatment with Y-27632 generated marked suppressions in the VEB numbers (to 67 ± 12, $n = 8$, $P < 0.05$) and the VT and VF incidences in carbachol preconditioning group. In order to examine whether or not Y-27632 itself is able to induce preconditioning-like effect, this drug was infused for 5 min prior to 30 min ischemia. Y-27632 infusion for 5 min generated cardio-

Table 3
Effects of Y-27632 on the severity of arrhythmias induced by 30 min of coronary artery occlusion in anesthetized rats

	n	Total VEBs	% VT	% Total VF	% Irreversible VF
Control	30	713 ± 149 (30)	100 (30)	40 (12)	10 (3)
Y-27632 (0.1 mg/kg)	9	192 ± 60* (9)	88.9 (8)	22.2 (2)	0 (0)
IP	24	28 ± 9* (23)	25* (6)	0* (0)	0 (0)
Y-27632 + IP	8	32 ± 11* (7)	25* (2)	0* (0)	0 (0)
CP	11	270 ± 131* (11)	81.8 (9)	9.1 (1)	0 (0)
Y-27632 + CP	8	67 ± 12* (8)	62.5* (5)	0* (0)	0 (0)
Y-27632 infusion (0.1 mg/kg/min for 5 min)	8	139 ± 57* (8)	87.5 (7)	0* (0)	0 (0)

VEBs, ventricular ectopic beats defined as discrete and identifiable premature QRS complexes; VT, ventricular tachycardia; VF, ventricular fibrillation. IP, ischemic preconditioning; CP, carbachol preconditioning. Numbers in parentheses are the number of hearts that exhibited that particular type of arrhythmia.
* $P < 0.05$ compared to control group.

Table 4
Effects of ischemic preconditioning, carbachol preconditioning, and Y-27632 on the time of onset of first arrhythmias, durations of VT and VF, and on arrhythmia scores in anesthetized rats

	n	Time of onset of first arrhythmias (s)	Duration of VT (s)	Duration of VF (s)	Arrhythmia scores
Control	30	36.0 ± 11.1	79.1 ± 17.5	19.6 ± 4.0	3.3 ± 0.1
Y-27632 (0.1 mg/kg)	9	132.2 ± 69.6	23.4 ± 8.0	101.5 ± 5.5*	2.6 ± 0.3
IP	24	136.2 ± 36.7	7.8 ± 4.3*	0	0.6 ± 0.2*
Y-27632 + IP	8	248.7 ± 129.5*	3.5 ± 1.5*	0	0.5 ± 0.3*
CP	11	78.8 ± 50.0	33.8 ± 15.8	37	2.3 ± 0.4*
Y-27632 + CP	8	221.8 ± 76.3	8.6 ± 1.7*	0	1.9 ± 0.5*
Y-27632 infusion (0.1 mg/kg/min for 5 min)	8	78.1 ± 32.1	21.4 ± 10.8	0	2.3 ± 0.4*

VT, ventricular tachycardia; VF, ventricular fibrillation. IP, ischemic preconditioning; CP, carbachol preconditioning.
* $P < 0.05$ compared to control group.

protective effect with significant reductions in the number of VEBs and VF incidence (Table 3).

Effects of IP, CP and Y-27632 on the time of onset of first arrhythmias, durations of VT and VF and on arrhythmia scores are shown in Table 4. The time of onset of first arrhythmias was markedly delayed in Y-27632 + IP group. There were no significant differences on this parameter in other groups. Durations of VT were significantly reduced in IP, Y-27632 + IP and Y-27632 + CP groups. The most marked reductions in the arrhythmia scores were observed in Y-27632 + IP (from 3.3 ± 0.1 ($n = 30$) to 0.5 ± 0.3 ($n = 8$), $P < 0.05$) and IP groups (to 0.6 ± 0.2 ($n = 24$), $P < 0.05$). Marked reductions in arrhythmia scores were found in CP and Y-27932 + CP groups when compared to control group. Although bolus administration of Y-27632 tended to decrease arrhythmia score, marked reduction was observed with Y-27632 infusion (0.1 mg/kg/min for 5 min) (Table 4).

3.3. Biochemical analysis

Lactate levels were markedly reduced in all treatment groups when compared to control (Fig. 2A). MDA levels were markedly attenuated only in IP group (Fig. 2B). Decreases in MDA levels were also noted in other treat-

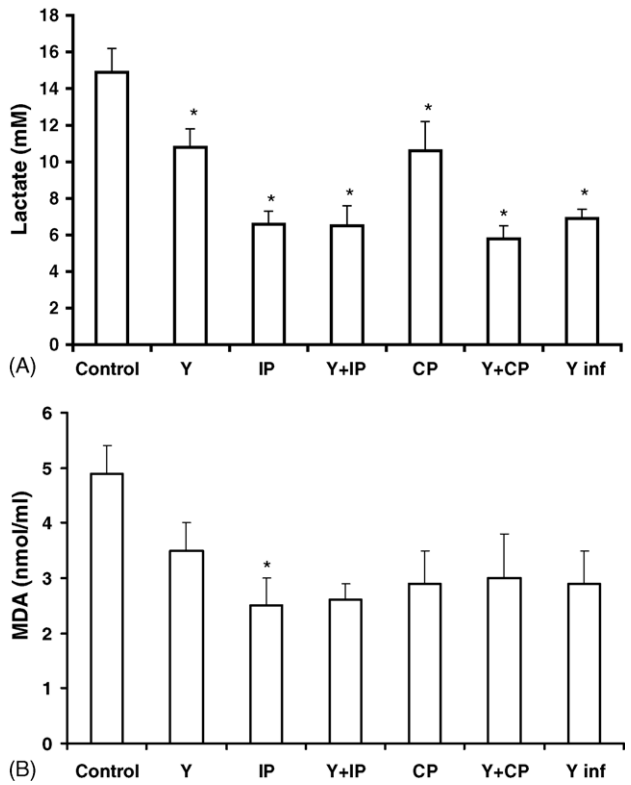

Fig. 2. Effects of Y-27632 on lactate (A) and malondialdehyde (MDA) (B) levels in plasma of the anesthetized rats. All values are the mean ± S.E.M., $n = 8$–30. *: $P < 0.05$ vs. control, and +: $P < 0.05$ significantly different when compared to IP group. IP, ischemic preconditioning; Y, Y-27632 at 0.1 mg/kg; Y+IP, Y-27632 (0.1 mg/kg) plus ischemic preconditioning group; CP, carbachol preconditioning; Y+CP; Y-27632 (0.1 mg/kg) plus carbachol preconditioning, Y inf, Y-27632 infusion (0.1 mg/kg/min for 5 min).

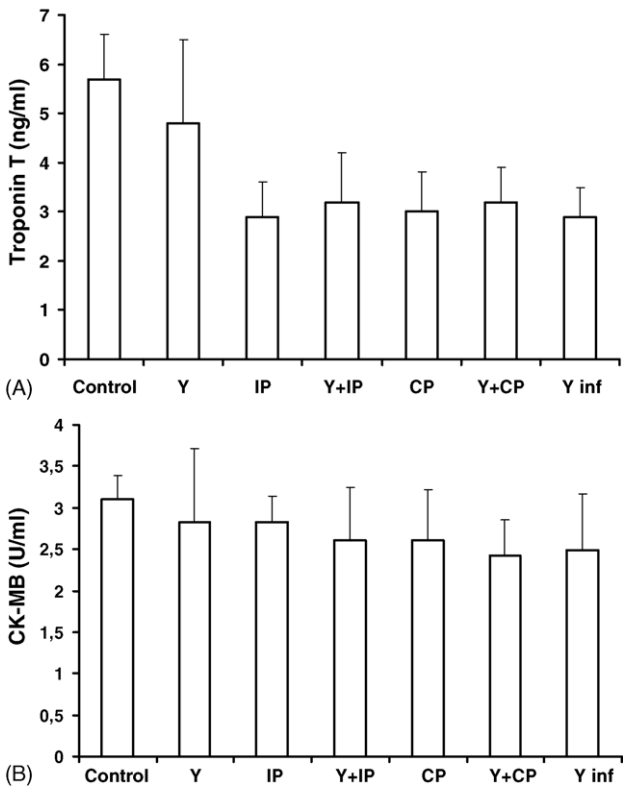

Fig. 3. Effects of Y-27632 on cardiac troponin T levels (A), and creatine kinase MB (CK-MB) activity (B) in plasma of the anesthetized rats. All values are the mean ± S.E.M., $n = 8$–30. Abbreviations are as in the legend of Fig. 2.

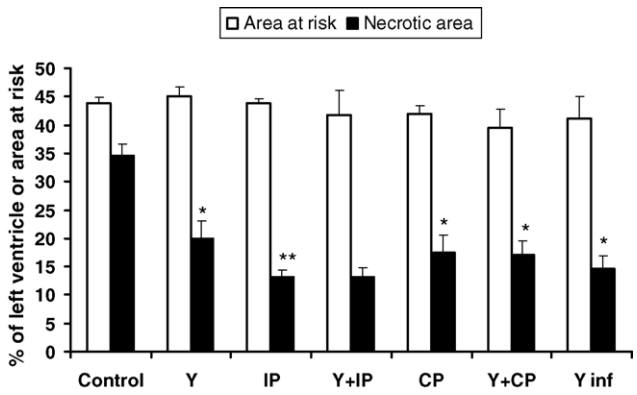

Fig. 4. Effects of Y-27632 on area at risk and infarct size. Area at risk indexed to total left ventricle (area at risk/total left ventricle × 100) and necrotic area indexed to area at risk (necrotic area/area at risk × 100) in percentage of wet weight. All values are the mean ± S.E.M., $n = 8–30$. *: $P < 0.05$ vs. control group and +: $P < 0.05$ significantly different when compared to IP group. Abbreviations are as in the legend of Fig. 2.

ment groups, but these reductions were not significant. There appear to be a slight decrease in troponin T levels between groups (Fig. 3A), but these changes did not reach a significance level. None of the groups had any significant effects on CK-MB levels (Fig. 3B).

3.4. Area at risk and infarct size measurements

No significant differences were noted in the left ventricular area at risk between the groups. However, necrotic area was markedly reduced in all treatment groups when compared to control group (Fig. 4).

4. Discussion

In this study we have shown that Y-27632, a Rho-kinase inhibitor, on its own produced cardioprotective effects in anesthetized rats. We have demonstrated that ischemic preconditioning and pharmacological preconditioning were present in the Y-27632-pretreated rats. Our results also provided first experimental evidence that infusion of Y-27632 generated an antiarrhythmic effect against coronary artery occlusion, and was able to mimic preconditioning. Preconditioning generated by 5 min coronary artery occlusion diminished the incidence of VT and abolished the occurrence of VF. Administration of Y-27632 appeared to increase the antiarrhythmic effects of CP in rats. Arrhythmia scores were also significantly depressed with IP or Y-27632 infusion. Marked decreases in lactate levels and infarct size in preconditioning and Y-27632 treated rats showed that Y-27632 may inhibit anaerobic respiration and/or myocardial injury. Our results may support the recent findings that ischemia/reperfusion upregulated RhoA expression in ischemic myocardium, and increased Rho-kinase activity in mice [13]. Recently, Sanada et al. [20] also showed that a 60 min period of ischemia caused Rho-kinase activation and this activation was attenuated by IP in anesthetized dogs. Our results support the conclusion that inhibition of Rho-kinase involves in the signaling pathway in ischemic preconditioning and extends this conclusion with pharmacological preconditioning.

Since Rho-kinase inhibitors have vasodilator effects, these drugs may increase regional myocardial blood flow [1,2]. It is likely that this vasorelaxant effect of Y-27632 may contribute to cardioprotection seen in the present study. 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 recruitment of collateral blood flow in dogs [20]. There are studies showing that administration of Y-27632 resulted in the AV nodal conduction delay, enhanced the repolarization process of the ventricle, and exerted modest positive chronotropic action in dog hearts [5,21]. These direct actions of Y-27632 may contribute the antiarrhythmic effects observed in the present study. Inhibition of Rho-kinase has been shown to protect myocardium subjected to pacing-induced ischemia through the increase in coronary blood flow in anesthetized open-chest dogs [6]. It is known that Rho-kinase inhibiton significantly dilated spastic arteries in a swine model of coronary artery spasm and prevented myocardial injury after ischemia [22,23]. In addition, Rho-kinase inhibitor fasudil markedly suppresses coronary artery spasm in patients with vasospastic angina [2,24], implying that Rho-kinase may substantially be involved in enhanced coronary vasoconstriction in animals and humans.

Muscarinic acetylcholine receptors are coupled to G_i-proteins and may reduce adenylyl cyclase activity in sarcolemmal membranes. Carbachol improves functional recovery and confers cellular protection, and this protection depends mainly but not entirely on its bradycardic effect [25]. Carbachol inhibits the cAMP-mediated positive inotropic effect by activation of G_i and subsequent lowering of tissue cAMP levels or activation of phosphatase that may lead to dephosphorylation of regulatory proteins that have been phosphorylated by PKA [26]. 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 muscarinic M_2 receptor agonist carbachol [17]. Pretreatment with carbachol causes a delay in cell necrosis similar to ischemic preconditioning [27,28]. Carbachol is able to mimic IP by reducing, infarct size or improving contractile function after periods of regional or global ischemia in rat and rabbit hearts [17,27]. 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 [29]. We have shown that administration of Y-

27632 prior to carbachol was effective in reducing both arrhythmia score and VF incidence and duration in rats.

In the present study, occlusion of left main coronary artery for a period of 30 min resulted in substantial injury to the myocardium. The reduction in infarct size in preconditioning groups was accompanied by a decrease in circulating levels of lactate and MDA suggesting that IP was able to attenuate the myocardial injury and inhibit lipid peroxidation. To determine the efficacy of Y-27632 for the prevention of myocardial injury, it may be useful to examine the effects of Y-27632 on the indicators of myocardial cell damage, for example creatine kinase and troponin T. However, we have found that Y-27632 did not significantly modify the circulating levels of these markers in our experiments. There was a tendency towards reduction in troponin T levels in the present study, but these changes did not reach statistically significance level. 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 troponin T levels start to increase a few hours after the onset of myocardial damage and remain increased for several days [30]. A longer period of reperfusion would have probably made marked differences in troponin T and CK-MB levels among groups.

Jensen et al. [31] have reported that the Rho/Rho-kinase pathway is independent of PKC stimulated by phorbol esters, although both converge on inhibiting myosin phosphatase. Moreover, it has been stated that Rho-kinase is not required for phorbol ester-induced Ca^{2+}-sensitization in smooth muscle [32]. However, it has been recently shown in a porcine model that phorbol ester caused coronary spasm in vivo was significantly inhibited by hydroxyfasudil [33]. Furthermore, it has been also demonstrated in cultured human umbilical vein endothelial cells that PKC functions upstream of Rho-kinase and PKC is required for Rho-kinase activation by RhoA [34]. These studies suggest that PKC and Rho-kinase coexist on the same intracellular signaling pathway, with PKC located upstream on Rho-kinase. There is evidence that tyrosine kinase(s) are also involved in the regulation of RhoA activity [35]. Since both PKC and tyrosine kinase(s) are known to be involved in preconditioning [12,36], our results may suggest that Rho and inhibition of Rho-kinase play an important role to confer cardioprotection of preconditioning in addition to other kinases. However, IP and pre-ischemic 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 [20]. It has been proposed that transient pre-ischemic 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 [20]. Moreover, Y-27632 inhibits other protein kinases, most notably PKC-related kinase and PKCδ [37,38]. PKC-related kinase is activated by RhoA, which is increased after ischemia/reperfusion [13]. Cardioprotective effects of Rho-kinase inhibition against ischemia–reperfusion injury may also be related to activation of endothelial NO synthase [39] or Rho-mediated ecto-5′-nucleotidase activation [40]. These mechanisms may be synergistically responsible for mediating a variety of cardioprotective pathways triggered by preconditioning.

Our results may support the data obtained in mice by Bao et al. [13] who showed that 30 min of coronary occlusion and 24 h reperfusion upregulated expression of RhoA, and subsequently activated Rho-kinase in ischemic myocardium. In addition to ischemia, hypoxia has been recently shown to upregulate Rho-kinase expression in endothelial cells [41]. Oral administration of a Rho-kinase inhibitor Y-27632 has been shown to inhibit the activation of Rho-kinase following ischemia/reperfusion and reduce infarct size [13]. Sanada et al. [20] demonstrated that Rho-kinase inhibitors, hydroxyfasudil and Y-27632, were given as intracoronary infusion during 60 min ischemic period decreased Rho-kinase activation and in fact size in dogs. However, we have applied Y-27632 either as a single bolus dose or 5 min i.v. infusion before sustained ischemia and observed reduction in infarct size in our experiments.

Neutrophils 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 [42]. The cardioprotective effect of Rho-kinase inhibition may be related to neutrophil accumulation, since treatment with Y-27632 has been shown to result in a significant reduction in the accumulation of neutrophils in ischemic myocardium [13]. Y-27632 has been shown to inhibit the superoxide production from phorbol-12-myristate-13 acetate (PMA), but not *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), stimulated human polymorphonuclear neutrophils [43] and suppresses neutrophil accumulation in mice [13]. Although polymorphonuclear leukocytes adhesion induced by PMA or FMLP was not influenced by Y-27632, polymorphonuclear leukocytes aggregation induced by PMA was dose-dependently decreased by Y-27632 [43]. Interestingly, it has been observed that there is no evidence for the protective effect of Y-27632 in isolated perfused rat heart [13], which may further suggest that neutrophils play an important role in the effects of Rho-kinase inhibitors in vivo. In addition, Y-27632 has been shown to depress in remarkable elevation in serum levels of proinflammatory cytokines; interleukin-6, keratinocyte chemoattractant and granulocyte colony-stimulating factor [13]. Collectively, these effects may lead to the inhibition of stress-induced regional inflammatory responses and diminishes myocardial ischemia–reperfusion injury.

In conclusion, the data obtained in this study showed that Y-27632 was able to mimic the cardioprotective effect of preconditioning in anesthetized rats. Therefore, our data

suggest that Y-27632 is a potential candidate drug for pharmacological preconditioning. The present study provides new insights into the mechanism of myocardial preconditioning.

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References

[1] Somlyo AP, Somlyo AV. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J Physiol 2000;522:177–85.
[2] Shimokawa H. Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. J Cardiovasc Pharmacol 2002;39:319–27.
[3] Kobayashi N, Horinaka S, Mita S, Nakano S, Honda T, Yoshida K, et al. Critical role of Rho-kinase pathway for cardiac performance and remodeling in failing rat hearts. Cardiovasc Res 2002;55: 757–67.
[4] Takahara A, Sugiyama A, Satoh Y, Yoneyama M, Hashimoto K. Cardiovascular effects of Y-27632, a selective Rho-associated kinase inhibitor, assessed in the halothane-anesthetized canine model. Eur J Pharmacol 2003;460:51–7.
[5] Sugiyama A, Yatomi Y, Takahara A, Satoh Y, Hashimoto K. Cardiac effects of a selective rho-associated kinase inhibitor, Y-27632, assessed in canine isolated, blood-perfused heart preparations. Jpn J Pharmacol 2002;88:359–61.
[6] Utsunomiya T, Satoh S, Ikegaki I, Toshima Y, Asano T, Shimokawa H. Antianginal effects of hydroxyfasudil, a Rho-kinase inhibitor, in a canine model of effort angina. Brit J Pharmacol 2001;134:1724–30.
[7] Sato S, Ikegaki I, Asano T, Shimokawa H. Antiischemic properties of fasudil in experimental models of vasospastic angina. Jpn J Pharmacol 2001;87:34–40.
[8] Satoh S, Ueda Y, Koyanagi M, Kadokami T, Sugano M, Yoshikawa Y, et al. Chronic inhibition of Rho kinase blunts the process of left ventricular hypertrophy leading to cardiac contractile dysfunction in hypertension-induced heart failure. J Mol Cell Cardiol 2003;35:59–70.
[9] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124–36.
[10] Altug S, Demiryurek AT, Kane KA, Kanzik I. Evidence for the involvement of peroxynitrite in ischaemic preconditioning in rat isolated hearts. Brit J Pharmacol 2000;130:125–31.
[11] Richard V, Kaeffer N, Tron C, Thuillez C. Ischemic preconditioning protects against coronary endothelial dysfunction induced by ischemia and reperfusion. Circulation 1994;89:1254–61.
[12] Murphy E. Primary and secondary signaling pathways in early preconditioning that converge on the mitochondria to produce cardioprotection. Circ Res 2004;94:7–16.
[13] Bao W, Hu E, Tao L, Boyce R, Mirabile R, Thudium DT, et al. Inhibition of Rho-kinase protects the heart against ischemia/reperfusion injury. Cardiovasc Res 2004;61:548–58.
[14] Walker MJA, Curtis MJ, Hearse DJ, Campbell RWF, Janse MJ, Yellon DM, et al. The Lambeth conventions: guidelines for the study of arrhythmias in ischemia, infarction and reperfusion. Cardiovasc Res 1988;22:447–55.
[15] Demiryurek AT, Yildiz G, Esiyok S, Altug S. Protective effects of poly (ADP-ribose) synthase inhibitors on digoxin-induced cardiotoxicity in guinea-pig isolated hearts. Pharmacol Res 2002;45:189–94.
[16] Ytrehus K, Liu Y, Tsuchida A, Miura T, Liu GS, Yang XM, et al. Rat and rabbit heart infarction: effects of anesthesia, perfusate, risk zone, and method of infarct sizing. Am J Physiol 1994;267:H2383–90.
[17] Yamaguchi F, Nasa Y, Yabe K, Ohba S, Hashizume Y, Ohaku H, et al. Activation of cardiac muscarinic receptor and ischemic preconditioning effects in in situ rat heart. Heart Vessels 1997;12:74–83.
[18] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8.
[19] Jentzsch AM, Bachmann H, Furst P, Biesalski HK. Improved analysis of malondialdehyde in human body fluids. Free Radic Biol Med 1996;20:251–6.
[20] Sanada S, Asanuma H, Tsukamoto O, Minamino T, Node K, Takashima S, et al. Protein kinase a as another mediator of ischemic preconditioning independent of protein kinase C. Circulation 2004;110:51–7.
[21] Sugiyama A, Takahara A, Yatomi Y, Satoh Y, Nakamura Y, Hashimoto K. Constitutional rho-kinase regulates atrioventricular nodal conduction and ventricular repolarization of the canine heart. J Cardiovasc Pharmacol 2003;41:930–3.
[22] Katsumata N, Shimokawa H, Seto M, Kozai T, Yamawaki T, Kuwata K, et al. Enhanced myosin light chain phosphorylations as a central mechanism for coronary artery spasm in a swine model with interleukin-1β. Circulation 1997;96:4357–63.
[23] Yamamoto Y, Ikegaki I, Sasaki Y, Uchida T. The protein kinase inhibitor fasudil protects against ischemic myocardial injury induced by endothelin-1 in the rabbit. J Cardiovasc Pharmacol 2000;35:203–11.
[24] Masumoto A, Mohri M, Shimokawa H, Urakami L, Usui M, Takeshita A. Suppression of coronary artery spasm by the Rho-kinase inhibitor fasudil in patients with vasospastic angina. Circulation 2002;105:1545–7.
[25] Hendrikx M, Toshima Y, Mubagwa K, Flameng W. Muscarinic receptor stimulation by carbachol improves functional recovery in isolated, blood perfused rabbit heart. Cardiovasc Res 1993;27:980–9.
[26] Endoh M. Dual inhibition of myocardial function through muscarinic and adenosine receptors in the mammalian heart. J Appl Cardiol 1987;2:213–30.
[27] Thornton JD, Liu GS, Downey JM. Pretreatment with pertussis toxin blocks the protective effects of preconditioning: evidence for a G-protein mechanism. J Mol Cell Cardiol 1993;25:311–20.
[28] Pisarenko OI, Shulzhenko VS, Studneva IM. Metabolic and functional effects of carbachol and ischaemic preconditioning in rat isolated heart. Clin Exp Pharmacol Physiol 1999;26:26–31.
[29] Qian YZ, Levasseur JE, Yoshida K, Kukreja RC. K_{ATP} channels in rat heart: blockade of ischemic and acetylcholine-mediated preconditioning by glibenclamide. Am J Physiol 1996;271:H23–8.
[30] Mair J, Dienstl F, Puschendorf B. Cardiac troponin T in the diagnosis of myocardial injury. Crit Rev Clin Lab Sci 1992;29:31–57.
[31] Jensen PE, Gong MC, Somlyo AV, Somlyo AP. Separate upstream and convergent downstream pathways of G-protein- and phorbol ester-mediated Ca^{2+} sensitization of myosin light chain phosphorylation in smooth muscle. Biochem J 1996;318:469–75.
[32] Fu X, Gong MC, Jia T, Somlyo AV, Somlyo AP. The effects of the Rho-kinase inhibitor Y-27632 on arachidonic acid-, GTPγS-, and phorbol ester-induced Ca^{2+}-sensitization of smooth muscle. FEBS Lett 1998;440:183–7.
[33] Kandabashi T, Shimokawa H, Miyata K, Kunihiro I, Eto Y, Morishige K, et al. Evidence for protein kinase C-mediated activation of Rho-kinase in a porcine model of coronary artery spasm. Arterioscler Thromb Vasc Biol 2003;23:2209–14.
[34] Barandier C, Ming XF, Rusconi S, Yang Z. PKC is required for activation of ROCK by RhoA in human endothelial cells. Biochem Biophys Res Commun 2003;304:714–9.
[35] Sakurada S, Okamoto H, Takuwa N, Sugimoto N, Takuwa Y. Rho activation in excitatory agonist-stimulated vascular smooth muscle. Am J Physiol 2001;281:C571–8.

[36] Soylemez S, Demiryurek AT, Kanzik I. Involvement of tyrosine kinase in peroxynitrite-induced preconditioning in rat isolated heart. Eur J Pharmacol 2003;464:163–9.
[37] Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000;351:95–105.
[38] Eto M, Kitazawa T, Yazawa M, Mukai H, Ono Y, Brautigan DL. Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase C alpha and delta isoforms. J Biol Chem 2001; 276:29072–8.
[39] Bivalacqua TJ, Champion HC, Usta MF, Cellek S, Chitaley K, Webb RC, et al. RhoA/Rho-kinase suppresses endothelial nitric oxide synthase in the penis: a mechanism for diabetes-associated erectile dysfunction. Proc Natl Acad Sci USA 2004;101:9121–6.
[40] Ledoux S, Laouari D, Essig M, Runembert I, Trugnan G, Michel JB, et al. Lovastatin enhances ecto-5′-nucleotidase activity and cell surface expression in endothelial cells: implication of rho-family GTPases. Circ Res 2002;90:420–7.
[41] Takemoto M, Sun J, Hiroki J, Shimokawa H, Liao JK. Rho-kinase mediates hypoxia-induced downregulation of endothelial nitric oxide synthase. Circulation 2002;106:57–62.
[42] Duilio C, Ambrosio G, Kuppusamy P, DiPaula A, Becker LC, Zweier JL. Neutrophils are primary source of O_2 radicals during reperfusion after prolonged myocardial ischemia. Am J Physiol 2001;280:2649–57.
[43] Kawaguchi A, Ohmori M, Harada K, Tsuruoka S, Sugimoto K, Fujimura A. The effect of a Rho kinase inhibitor Y-27632 on superoxide production, aggregation and adhesion in human polymorphonuclear leukocytes. Eur J Pharmacol 2000;403:203–8.