Free Radical Research, July 2007; 41(7): 757–769

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healthcare

Landiolol has cardioprotective effects against reperfusion injury in the rat heart via the PKCε signaling pathway

YOSUKE TAKAHASHI, SHIGEKAZU TAKEMURA, YUKIKO MINAMIYAMA, TOHIHIKO SHIBATA, HIDEKAZU HIRAI, YASUYUKI SASAKI, MASANORI SAKAGUCHI, & SHIGEFUMI SUEHIRO

Department of Cardiovascular Surgery, Osaka City University Graduate School of Medicine, Osaka, Japan

Accepted by Professor G. Mann

(Received 21 November 2006; in revised form 17 February 2007)

Abstract
Landiolol, a highly cardioselective β1-blocker, has cardioprotective effects against ischemia-reperfusion injury, although the precise mechanism is still unclear. The aim of this study was to clarify the cardioprotective mechanism of landiolol. Experiments were performed on Langendorff-perfused rat hearts undergoing 20 min stabilization, and 45 min of ischemia followed by 60 min of reperfusion. Various drugs with or without landiolol (100 μM) were administered before ischemia for 20 min. Preischemic administration of landiolol reduced cardiac cellular damage and improved the recovery of cardiac function by about 40%. The α1 blocker prazosin, the protein kinase C (PKC) inhibitor chelerythrine or the K_{ATP} channel blocker glibenclamide, but not the selective mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate abrogated the cardioprotective effect induced by landiolol. Following landiolol pretreatment the activation of PKCε and heat shock protein 27 were significantly higher than that in control. These data indicate that preischemic application of landiolol induces cardioprotective effects through PKCε-mediated pathway, similar to that afforded by ischemic preconditioning.

Keywords: *Protein kinase C, mitogen-activated protein kinase, heart rate*

Introduction

Cardioprotective strategies for attenuating ischemia-reperfusion (I/R) injury have important clinical implications. Murry and colleagues first described that an inherent protective mechanism, designated ischemic preconditioning (IP), protects the heart against prolonged ischemic damage [1]. Cardioprotection induced by repeated short episodes of I/R prior to sustained ischemia is termed IP, while cardioprotection afforded by brief administration of substances prior to sustained ischemia is known as pharmacological preconditioning. Many preconditioning agents have been described to date, including adrenergic receptor agonists, B2 bradykinin receptor agonists, A1 adenosine receptor agonists, opioid receptor agonists, protein kinase C (PKC) activators and mitochondrial ATP-sensitive potassium (K_{ATP}) channel openers. The beneficial effects of preconditioning are mediated by PKC, p38 mitogen-activated protein kinase (MAPK) and/or K_{ATP} channels [2–4]. Furthermore, MAPK-activated protein kinase-2 located downstream of p38 MAPK [5,6] is known to phosphorylate the 27-kDa small heat-shock protein (HSP27) [7–9], which plays a protective role against ischemic or oxidative stress [10,11].

Landiolol hydrochloride is a highly cardioselective β1-blocker (β1/β2 = 255) with little α-blocking action. It is nine times more potent in its β1-blocking activity and eight times more cardioselective than esmolol *in vivo*. In addition, its activity is ultra-short-acting and disappears after cessation of administration

Correspondence: Y. Takahashi, Department of Cardiovascular Surgery, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. Tel: 81 6 6645 3980. Fax: 81 6 6646 3071. E-mail: ysk@msic.med.osaka-cu.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760701338810

in vivo. Landiolol has neither intrinsic sympathomimetic activity nor significant membrane-stabilizing activity, and its cardiodepressive effect is lower than those of other β-blockers including esmolol [12,13]. Landiolol has been reported to have cardioprotective effects [14,15].

In the human myocardium, there are predominantly three adrenergic receptors (α1, β1 and β2). The ratios of these α1, β1 and β2 adrenergic receptors are about 1:8:2 [16,17]. The crosstalk between α1, β1 and β2 adrenergic receptors stimulation in the cardiac inotropic response has been only partially understood. Schäfer et al. [18] demonstrated that β1 blocker augmented the α1 adrenergic receptor induced activation of PKC. Thus, we also considered that landiolol might enhance PKC signaling in the myocardium.

Recently, we showed that administration of landiolol before, but not during, ischemia improved postischemic cardiac function and reduced cardiac cellular damage after reperfusion [19]. These results suggested that landiolol may have cardioprotective effects via enhancement of PKC signaling. Hence, the aim of this study was to determine the optimal concentration of landiolol for cardioprotection and investigate the mechanism of landiolol against I/R injury in the Langendorff-perfused heart

Materials and methods

Chemicals

Prazosin chloride, chelerythrine chloride, glibenclamide and 5-hydroxydecanoate (5-HD) were purchased from Sigma (St Louis, MO). All other reagents used were of analytical grade.

Isolated heart preparation and I/R protocol

Male Wistar rats weighing 270–320 g were purchased from SLC Japan Inc. (Shizuoka, Japan). All animals were housed and allowed free access to tap water and a standard rodent diet. The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals approved by the Local Ethics Committee of Osaka City University. Rats were anesthetized with diethyl ether, followed by injection of heparin (100 IU/kg) into the femoral vein. Diethyl ether was chosen on the basis that it affected neither rate-pressure product (RPP) nor post-ischemic cardiac function. The heart was rapidly harvested and immersed in ice-cold Krebs-Henseleit bicarbonate buffer (KHBB) containing (in mM) 118.5 NaCl, 25.0 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose and 2.5 $CaCl_2$, adjusted to pH 7.4. The aorta was rapidly attached to a stainless steel apparatus and retrogradely perfused with KHBB equilibrated with 95% O_2 and 5% CO_2 at 37°C at a constant perfusion pressure of 100 cmH_2O during the first 20 min of stabilization. Through a left atrial incision, a latex balloon connected to a pressure transducer was inserted into the left ventricle (LV) for measurement of the LV isovolumic pressure. The balloon was inflated to obtain an LV end-diastolic pressure (LVEDP) of 4–8 mm Hg as previously described [20]. The LV developed pressure (LVDP), maximum first derivative of the LV pressure during systole (max dP/dt), LVEDP, and heart rate (HR) were continuously monitored using a polygraph with a computer analysis system (LEG-1000; Nihon Kohden, Tokyo, Japan). All hearts were spontaneously beating without pacing.

Experimental protocol

Experimental protocol was shown in Figure 1. Each heart was allowed to stabilize during the initial 20 min. Subsequently, cardiac arrest was achieved by clamping the aortic cannula and injecting St Thomas' Hospital cardioplegic solution containing (in mM) 110 NaCl, 10 $NaHCO_3$, 18 KCl, 1.2 $MgCl_2$ and 1.2 $CaCl_2$, adjusted to pH 7.8. The cardioplegic solution was infused at 37°C at a constant perfusion pressure of 60 cmH_2O for 3 min. The hearts were subjected to global ischemia for 45 min at 37°C and then reperfused with KHBB for 60 min at a constant pressure of 100 cmH_2O to obtain about 50% recovery of cardiac function after 45 min ischemia at 37°C in control group.

Assessment of ventricular function

The LVDP, max dP/dt and LVEDP were measured every 5 min before cardiac arrest and after reperfusion. The postischemic recoveries of LVDP and max dP/dt after 60 min of reperfusion were expressed as the percentages of the respective preischemic values. The RPP was expressed as HR × LVDP. Experiment 1 was designed to determine the optimal concentration of landiolol for cardioprotection against I/R injury. Various concentrations of landiolol (0, 5, 50, 100 and 200 μM) were infused for 20 min before ischemia in each group ($n = 6$). Experiment 2 was designed to examine the cardioprotective mechanism of landiolol. Prazosin (1 μM; a selective α1-blocker), Chelerythrine (6 μM; a non-selective PKC inhibitor), glibenclamide (0.25 μM; a non-selective K_{ATP} channel blocker) and 5-HD (100 μM; a mitochondrial K_{ATP} channel blocker) were individually infused with landiolol (100 μM) before ischemia for 20 min ($n = 4$). The concentrations of prazosin, chelerythrine and 5-HD were selected by reference to previous reports [21–26], while the concentration of glibenclamide (>0.25 μM) decreased both the RPP and post-ischemic cardiac function in Experiment 2.

Assessment of myocardial injury

Cellular damage was assessed by measuring creatine kinase (CK) release into the coronary effluent collected

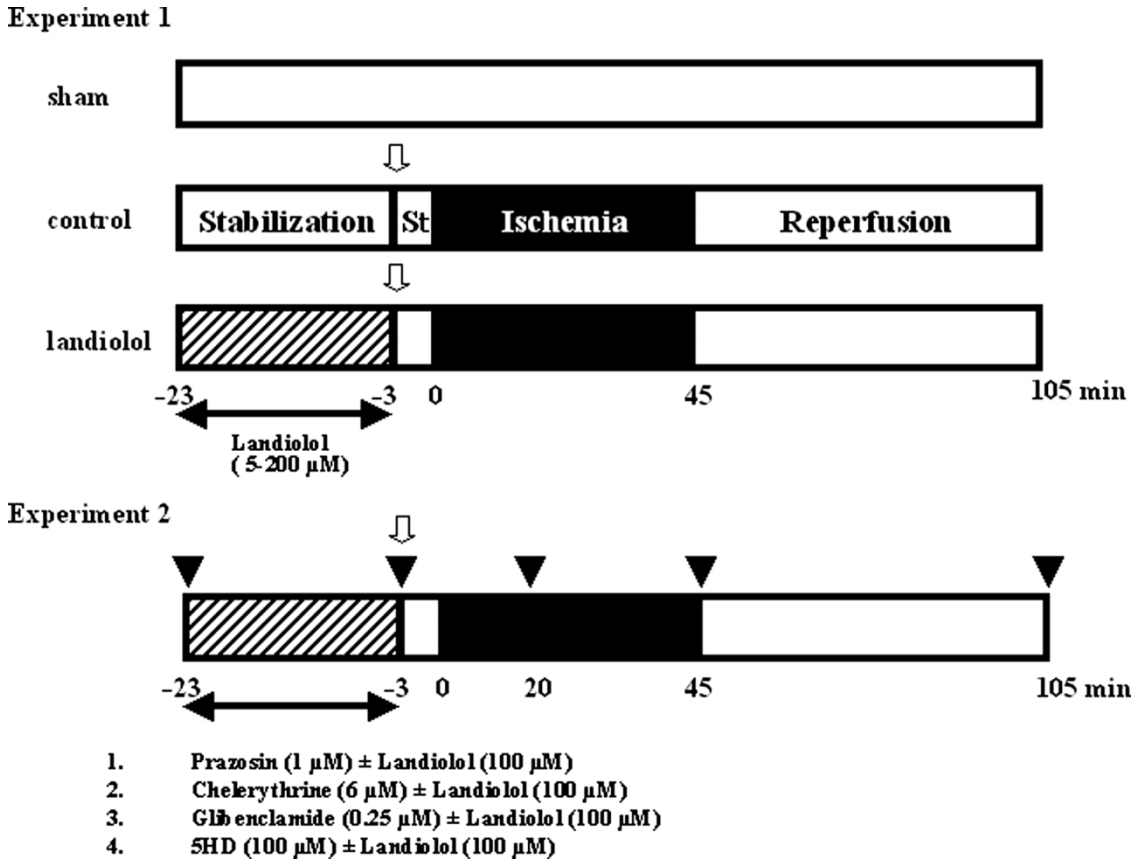

Figure 1. Schematic diagram illustrating the experimental protocol. In control group, hearts were sequentially subjected to 20 min of perfusion under preischemic stabilization, 3 min of cardioplegic infusion with St Thomas' Hospital cardioplegic solution, 45 min of global ischemia at 37°C and 60 min of reperfusion. Experiment 1 was designed to determine the optimal concentration of landiolol for cardioprotection against I/R injury. In landiolol group, various concentrations of landiolol (5, 50, 100 and 200 μM) were infused for 20 min before ischemia ($n = 6$). Cardiac functions were recorded after equilibration for 20 min (Arrow). Experiment 2 was designed to examine the cardioprotective mechanism of landiolol. The hearts were sequentially subjected to 20 min of perfusion with or without various drugs, 3 min of cardioplegic infusion with St. Thomas' Hospital cardioplegic solution, 45 min of global ischemia at 37°C, and 60 min of reperfusion. Various drugs were individually infused with or without landiolol (100 μM) before ischemia for 20 min ($n = 4$). Cardiac functions were recorded after infusion of various drugs for 20 min (Arrow). Samples were analyzed by western blotting at the indicated time (Arrow head). St, 3 min infusion of St Thomas' Hospital cardioplegic solution.

during the first 20 min of reperfusion. The CK content in each sample was measured as previously described [20]. The samples were mixed with N-acetylcysteine (20 μM) and stored at 4°C until use. The total CK leakage was calculated according to the CK activity and coronary effluent volume over the first 20 min.

Tissue sample preparation and subcellular fractionation

The excised hearts were rapidly removed from the perfusion system and frozen in liquid nitrogen at the indicated times (pre-Langendorff perfusion, preischemia, 20 min postischemia, 45 min postischemia and 60 min after reperfusion). The LV tissue was homogenized in lysis buffer containing (in mM) 10 Tris–HCl (pH 7.4), 320 sucrose, 1 EGTA, 2 EDTA, 5 NaN_3, 10 β-mercaptoethanol, 50 NaF, 0.02 leupeptin, 0.01 E64, 0.12 pepstatin, 1 sodium orthovanadate and 0.2 PMSF. The homogenates were mixed with two volumes of lysis buffer and centrifuged (1000*g*, 10 min, 4°C). For fractionation, the pellet was washed once and suspended in lysis buffer to obtain the nuclear fraction. The supernatant was centrifuged at 100,000*g* for 60 min, and the resulting pellet was the membrane-particulate fraction, while the supernatant was the cytosolic fraction. The membrane-particulate fraction was resuspended in lysis buffer containing 0.5% Triton X-100 and centrifuged at 100,000*g* for 60 min, and the resulting detergent-treated supernatant was the membrane fraction. The nuclear fraction was solubilized in 1% Triton X-100 buffer containing (in mM) 150 NaCl, 10 Tris-HCl (pH 7.4), 1 EGTA, 1 EDTA, 0.2 sodium orthovanadate, 50 NaF, 0.02 leupeptin, 0.01 E64 and 0.12 pepstatin, and then centrifuged (15,000*g*, 30 min) to obtain the soluble nuclear fraction.

Western blot analysis for PKCε, and phosphorylation of PKCε PKCδ, and HSP27

The subcellular localization of PKCε and phosphorylation of PKCε, PKCδ and HSP27 were examined

by western blot analysis. Aliquots (20 μg protein) of the total cytosolic, membranous, and whole protein extracts of each sample were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 3% nonfat dry milk and incubated overnight with anti-PKCε (mouse monoclonal antibody: Transduction Laboratories, Lexington, KY), anti-phospho-PKCε (rabbit polyclonal antibody: Upstate, Lake Placid, NY), anti-phospho PKCδ (rabbit polyclonal antibody: Abcam, Tokyo, JPN), and anti-phospho HSP27 (rabbit polyclonal antibody: R&D Systems, Inc. Minneapolis, MN) primary antibodies at 4°C. After washing, the membranes were incubated with secondary antibodies for 90 min and visualized using an enhanced chemiluminescence ECL reagent (Amersham Biosciences, Buckinghamshire, UK). The band intensities were quantified using image analysis computer software (Scion Image Beta 4.03).

Statistical analysis

All data are expressed as means ± SD. Statistical analyses were performed by ANOVA and Dunnett's test. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Effects of landiolol on cardiac function in the preischemic condition

The preischemic parameters of cardiac function are summarized in Table I. None of the concentrations (0–200 μM) of landiolol affected any of the baseline functional parameters.

Effects of landiolol on cardiac function and cellular injury after reperfusion

The cardiac function and CK leakage after reperfusion are shown in Figure 2. All the groups in Experiment 1 showed similarly marked reductions in LVDP (Figure 2A) and max dP/dt after ischemia, while LVEDP slowly increased. The percent recoveries of LVDP (Figure 2B) and max dP/dt (Figure 2C) were significantly decreased after 60 min of reperfusion. These parameters were significantly improved by pretreatment with 100 μM landiolol (LVDP: 54.5 ± 10.5% in the control group vs. 76.1 ± 6.6% in the landiolol group, $p < 0.05$; max dP/dt: 47.5 ± 14.9% in the control group vs. 71.4 ± 6.9% in the landiolol group, $p < 0.05$). In the 100 μM landiolol group, the increase in LVEDP after reperfusion was attenuated compared with that in control hearts (48.0 ± 10.8 mm Hg in the control group vs. 29.4 ± 3.8 mm Hg in the landiolol group, $p < 0.05$) (Figure 2D). CK leakage increased after reperfusion, and administration of landiolol at more 50 μM significantly reduced this effect (Figure 2E).

Effects of various agents on cardiac function in the preischemic condition

The preischemic parameters of cardiac function in the *prazosin*, chelerythrine, glibenclamide and 5-HD groups are summarized in Table II. No significant differences were detected for any of the parameters of cardiac function in any of the groups with or without landiolol.

Effect of prazosin on the cardioprotective effects of landiolol

Prazosin alone did not affect the percent recovery of LVDP (Figure 3A), percent recovery of max dP/dt (Figure 3B), LVEDP (Figure 3C), or CK leakage (Table III) after I/R injury. The protective effects of landiolol on these parameters were abolished following the addition of prazosin (LVDP: 76.1 ± 6.6% in the landiolol group vs. 53.5 ± 5.2% in the landiolol plus prazosin group, $p < 0.05$; max dP/dt: 71.4 ± 6.9% in the landiolol group vs. 51.9 ± 1.6% in the landiolol plus prazosin group, $p < 0.05$) (Figure 3). The addition of prazosin markedly abolished the landiolol-induced reduction of LVEDP

Table I. Effects of landiolol on cardiac function in the preischemic condition.

	Experiment 1				
	Landiolol (μM)				
	0 (control)	5	50	100	200
LVDP (mm Hg)	153 ± 12	152 ± 13	152 ± 16	151 ± 17	142 ± 27
max dP/dt (mm Hg/s)	4500 ± 320	4800 ± 700	5100 ± 920	5000 ± 970	4600 ± 750
LVEDP (mm Hg)	5.1 ± 1.2	5.4 ± 1.5	6.7 ± 2.1	5.1 ± 0.8	6.8 ± 1.8
HR (bpm)	255 ± 21	258 ± 25	265 ± 12	241 ± 19	253 ± 47
RPP (mm Hg bpm)	37800 ± 5120	39200 ± 6230	40220 ± 3940	38700 ± 5630	38900 ± 2230

All parameters were measured after 20 min of Langendorff perfusion with or without landiolol (0–200 μM). Data are expressed as the means ± SD ($n = 6$). LVDP, left ventricular developed pressure; max dP/dt, maximum first derivative of the left ventricular pressure during systole; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; RPP, rate-pressure product.

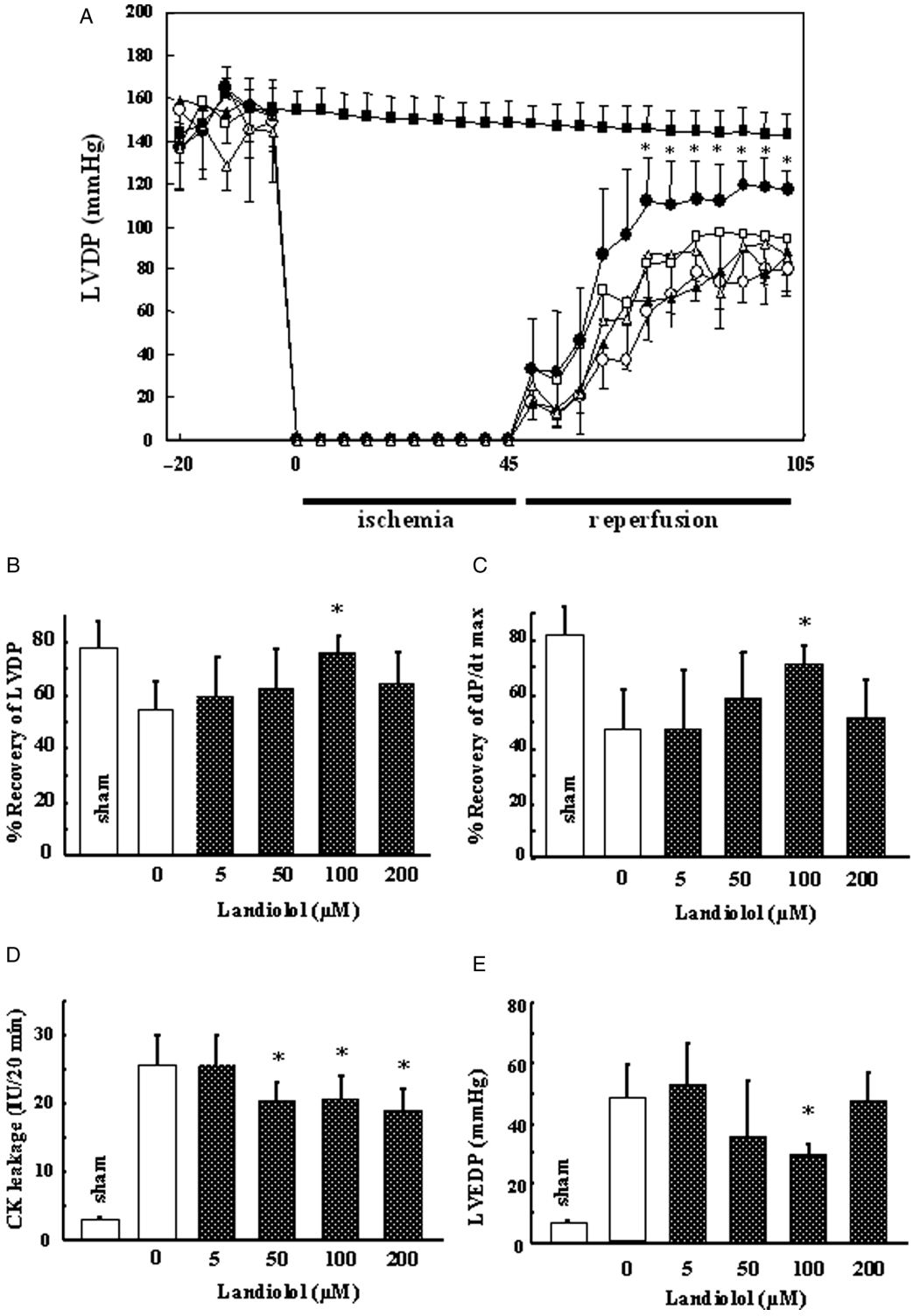

Figure 2. Effects of landiolol on the functional recovery of the LV and CK leakage after reperfusion. The cardiac function was measured every 5 min before cardiac arrest and after reperfusion. The postischemic recoveries of cardiac function after 60 min of reperfusion were expressed as percentages of the respective preischemic values. CK leakage was assessed in coronary effluent samples collected during the first 20 min of reperfusion. (A) Left ventricular developed pressure (LVDP). Closed squares, sham; Open circles, control; closed triangles, 5 μM landiolol; open squares, 50 μM landiolol; closed circles, 100 μM landiolol; open triangles, 200 μM landiolol. (B) Percentage of recovery of left ventricular developed pressure (LVDP). (C) % recovery of maximum first derivative of the left ventricular pressure during systole (max dP/dt). (D) Left ventricular end-diastolic pressure (LVEDP). (E) CK. In *B–E*: open bars, control (vehicle) group; dotted bars, landiolol groups (0–200 μM). Data are expressed as means ± SD ($n = 6$), $*p < 0.05$ vs. the control group.

Table II. Effects of various drugs on cardiac function in the preischemic condition.

Drug (μM)	Landiolol (100 μM)	Experiment 2 LVDP (mm Hg)	max dP/dt (mm Hg/s)	LVEDP (mm Hg)	HR (bpm)	RPP (mm Hg bpm)
Prazosin (1)	(−)	142 ± 8.7	3800 ± 600	5.7 ± 1.0	256 ± 17	36,000 ± 2300
	(+)	147 ± 23	3600 ± 200	6.9 ± 1.3	220 ± 20	32,000 ± 4100
Chelerythrine (6)	(−)	159 ± 14	5000 ± 600	5.8 ± 0.5	285 ± 19	45,000 ± 3100
	(+)	154 ± 12	5400 ± 870	6.0 ± 1.0	289 ± 2.0	45,000 ± 3100
Glibenclamide (0.25)	(−)	154 ± 17	3800 ± 520	6.4 ± 0.9	245 ± 23	38,000 ± 2500
	(+)	150 ± 22	4000 ± 930	7.2 ± 1.6	252 ± 8.3	38,000 ± 4700
5-HD (100)	(−)	145 ± 16	4400 ± 580	6.3 ± 2.4	273 ± 18	40,000 ± 3200
	(+)	155 ± 14	3900 ± 420	5.8 ± 1.5	285 ± 19	44,000 ± 3600

All parameters were measured after 20 min of Langendorff perfusion with or without landiolol (100 μM). Various drugs were infused before ischemia. Data are means ± SD ($n = 4$). LVDP, left ventricular developed pressure; max dP/dt, maximum first derivative of the left ventricular pressure during systole; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; RPP, rate-pressure product.

(29.4 ± 3.8 mm Hg in the landiolol group vs. 48.0 ± 14.9 mm Hg in the landiolol plus prazosin group, $p < 0.05$) (Figure 3). The addition of prazosin markedly abolished the landiolol-induced attenuation of CK leakage (17.8 ± 5.4 IU in the landiolol group vs. 26.0 ± 5.4 IU in the landiolol plus prazosin group, $p < 0.05$) (Table III).

Effect of chelerythrine on the cardioprotective effects of landiolol

Chelerythrine alone did not affect the percent recovery of LVDP (Figure 4A), percent recovery of max dP/dt (Figure 4B), LVEDP (Figure 4C), or CK leakage (Table III) after I/R injury. The protective

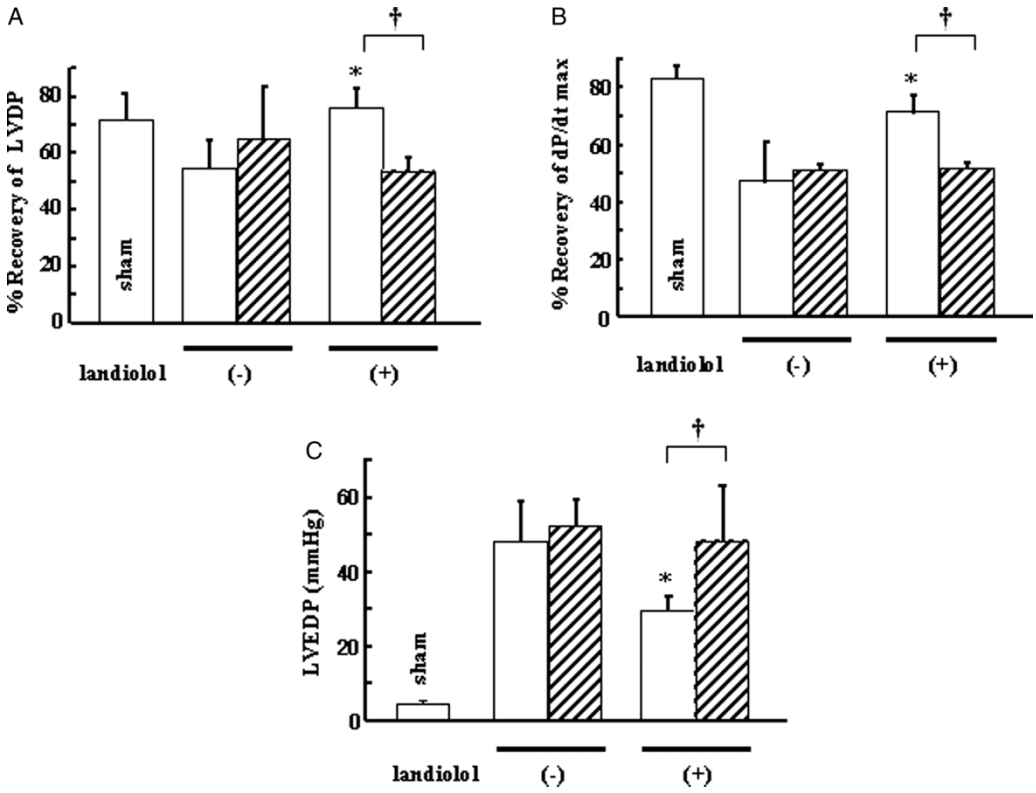

Figure 3. Effects of prazosin on the functional recovery of the LV with or without landiolol. Rats were prepared as described in the legend for Figure 1. Prazosin (1 μM), a α1-blocker, was administered before ischemia. The postischemic recoveries of cardiac function after 60 min of reperfusion were expressed as percentages of the respective preischemic values. (A) LVDP, left ventricular developed pressure; (B) max dP/dt, maximum first derivative of the left ventricular pressure during systole; (C) LVEDP, left ventricular end diastolic pressure. Open bars, vehicle with or without landiolol groups; hatched bars, prazosin with or without landiolol groups. Data are expressed as means ± SD ($n = 4$), $^{\star}p < 0.05$ vs. the vehicle without landiolol group, $^{\dagger}p < 0.05$.

Table III. Effects of various drugs on CK leakage during the first 20 min of reperfusion.

	CK (IU)		
	Landiolol (100 μM)		
Drug (μM)	(−)	(+)	
Vehicle	25.3 ± 4.2	17.8 ± 5.4⋆	#
Prazosin (1)	28.7 ± 5.1	26.0 ± 5.4	#
Chelerythrine (6)	23.7 ± 2.2	25.3 ± 2.3	
Glibenclamide (0.25)	24.8 ± 3.1	21.8 ± 3.1	
5-HD (100)	29.6 ± 1.8	22.5 ± 4.6	

The CK content in the effluent was measured during the first 20 min of reperfusion with or without landiolol (100 μM) in each group. Various drugs were infused before ischemia. Data are means ± SD ($n = 4$). ⋆$p < 0.05$ vs. the vehicle without landiolol group #$p < 0.05$.

effects of landiolol on these parameters were abolished following the addition of chelerythrine (LVDP: 76.1 ± 6.6% in the landiolol group vs. 62.3 ± 4.0% in the landiolol plus chelerythrine group, $p < 0.05$; max dP/dt: 71.4 ± 6.9% in the landiolol group vs. 53.1 ± 2.7% in the landiolol plus chelerythrine group, $p < 0.05$) (Figure 4). The addition of chelerythrine markedly abolished the landiolol-induced reduction of LVEDP (29.4 ± 3.8 mm Hg in the landiolol group vs. 41.3 ± 3.2 mm Hg in the landiolol plus chelerythrine group, $p < 0.05$) (Figure 4). The addition of chelerythrine markedly abolished the landiolol-induced attenuation of CK leakage (17.8 ± 5.4 IU in the landiolol group vs. 25.3 ± 2.3 IU in the landiolol plus chelerythrine group, $p < 0.05$) (Table III).

Effects of K_{ATP} channel blockers on the cardioprotective effects of landiolol

To clarify the involvement of K_{ATP} channels in the preconditioning effects, we used different types of K_{ATP} channel blockers. Neither glibenclamide, a non-selective K_{ATP} channel blocker, nor 5-HD, a selective

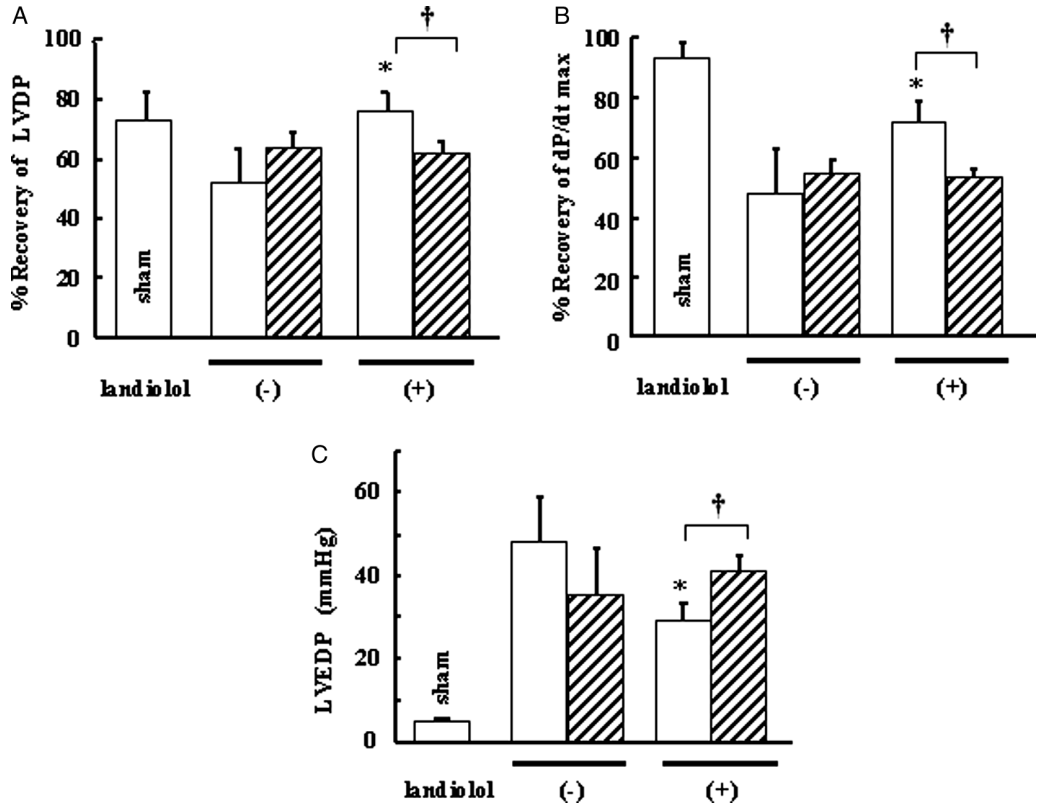

Figure 4. Effects of chelerythrine on the functional recovery of the LV with or without landiolol. Rats were prepared as described in the legend for Figure 1. Chelerythrine (6 μM), a non-selective PKC inhibitor, was administered before ischemia. The postischemic recoveries of cardiac function after 60 min of reperfusion were expressed as percentages of the respective preischemic values. (A) LVDP, left ventricular developed pressure; (B) max dP/dt, maximum first derivative of the left ventricular pressure during systole; (C) LVEDP, left ventricular end diastolic pressure. Open bars, vehicle with or without landiolol groups; hatched bars, chelerythrine with or without landiolol groups. Data are expressed as means ± SD ($n = 4$), ⋆$p < 0.05$ vs. the vehicle without landiolol group, †$p < 0.05$.

mitochondrial K_{ATP} channel blocker, affected the percent recoveries of LVDP (Figure 5A) and max dP/dt (Figure 5B), and LVEDP (Figure 5C) after I/R injury. Although 100 μM landiolol improved the postischemic LV functional recovery compared with the control group, glibenclamide abolished the protective effects of landiolol (LVDP: 76.1 ± 6.6% in the landiolol group vs. 60.2 ± 6.4% in the landiolol plus glibenclamide group, $p < 0.05$; max dP/dt: 71.4 ± 6.9% in the landiolol group vs. 51.9 ± 4.8% in the landiolol plus glibenclamide group, $p < 0.05$; LVEDP: 29.4 ± 3.8 mm Hg in the landiolol group vs. 52.0 ± 13.4 mm Hg in the landiolol plus glibenclamide group, $p < 0.05$) (Figure 5). On the other hand, 5-HD did not affect the beneficial effects of landiolol on the postischemic LV function (Figure 5). Neither glibenclamide nor 5-HD inhibited the protective effect of landiolol on cellular damage (CK leakage: 17.8 ± 5.4 IU in the landiolol group vs. 21.5 ± 3.6 IU and 22.5 ± 4.6 IU in the landiolol plus glibenclamide and landiolol plus 5-HD groups, respectively) (Table III).

Subcellular distribution of PKCε

A western blot analysis was performed to investigate the distribution of PKCε (Figure 6). The ratio of membranous PKCε in the both sham and control groups showed no remarkable change at every time points. Continuous infusion of landiolol increased the amount of membranous PKCε and this increase rapidly return to the basal level following discontinuation of landiolol. The ratio of membranous PKCε in the landiolol group gradually increased after reperfusion (Figure 6). The ratios of membranous PKCε before ischemia and after 60 min of reperfusion in the landiolol group were significantly higher than those in the control group (Figure 6).

Phosphorylation of PKCε

The expression of phospho-PKCε in the control group increased before ischemia and decreased to the basal level after 60 min of reperfusion in whole tissue lysates (Figure 7A). Landiolol significantly increased

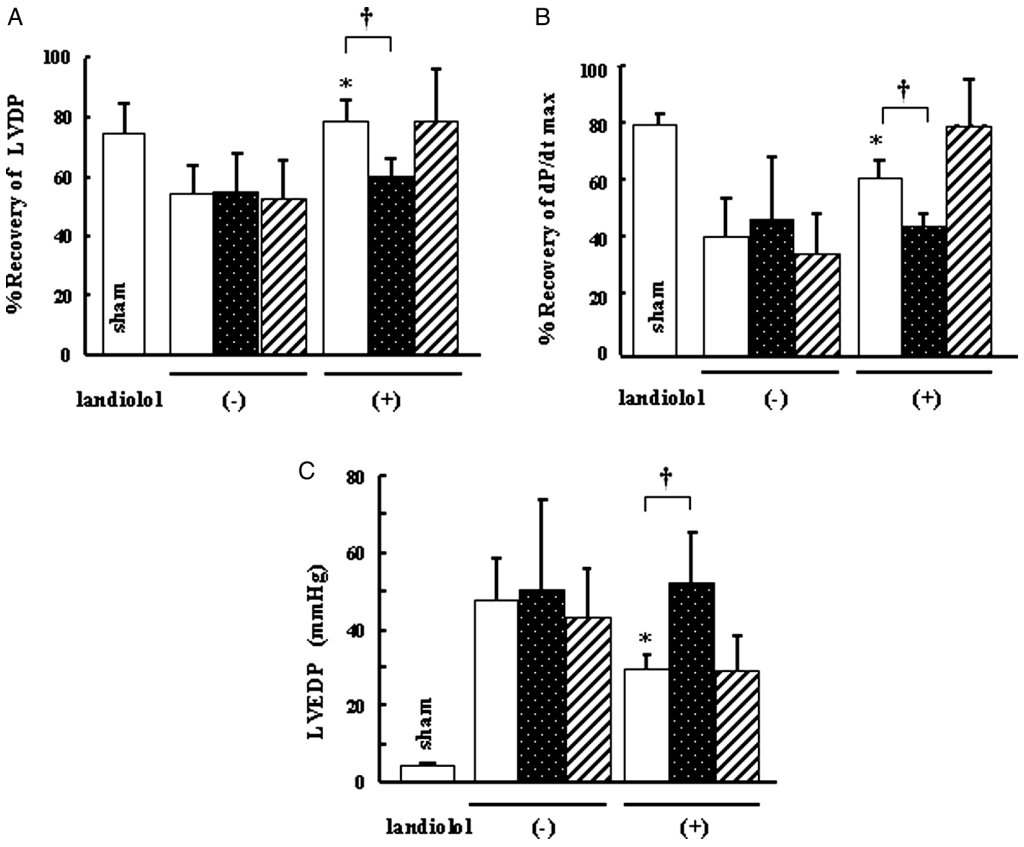

Figure 5. Effects of K_{ATP} channel blockers on the functional recovery of the LV with or without landiolol. 5-hydroxydecanoate (5-HD; 100 μM), a mitochondrial K_{ATP} channel blocker, or glibenclamide (0.25 μM), a non-selective K_{ATP} channel blocker, were administered before ischemia. (A) LVDP, left ventricular developed pressure; (B) max dP/dt, maximum first derivative of the left ventricular pressure during systole; (C) LVEDP, left ventricular end diastolic pressure. Open bars, vehicle with or without landiolol groups; dotted bars, glibenclamide with or without landiolol groups; hatched bars, 5-HD with or without landiolol groups. Data are means ± SD ($n = 4$), $*p < 0.05$ vs. the vehicle without landiolol group, $^{\dagger}p < 0.05$.

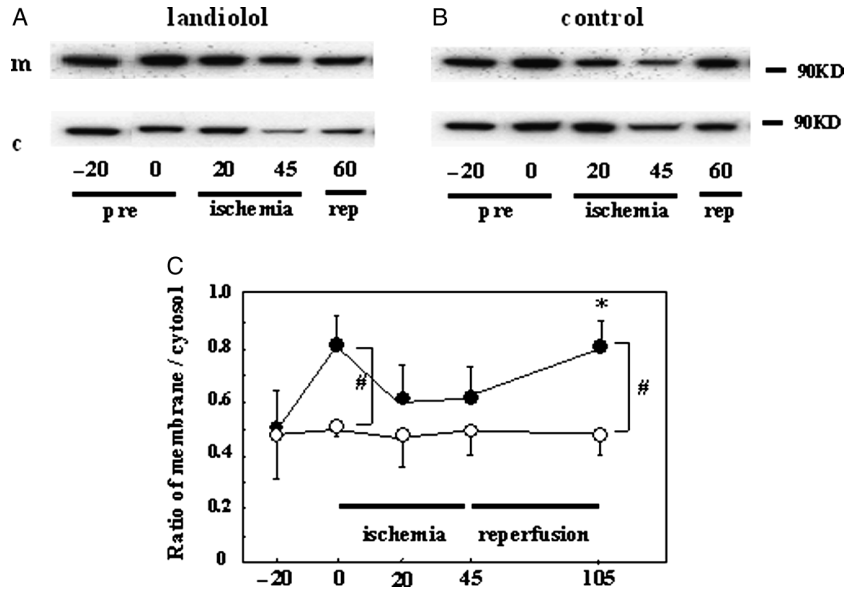

Figure 6. Effects of landiolol on the expression of PKCε in the cytosol and membrane. At the indicated time points, hearts were frozen, homogenized and analyzed by western blotting to evaluate the translocation of PKCε from the cytosol to the membrane. (A) Representative western blotting image. (B) Densitometric evaluation of the ratios of membranous/cytosolic PKCε. Open circles, ratios of PKCε in the control (vehicle) group; closed circles, ratios of PKCε in the landiolol group. M, membrane; C, cytosol; Pre, preischemia; Rep, reperfusion. Data are means ± SD ($n = 3$), $\star p < 0.05$ vs. the landiolol group at the basal level, $\#p < 0.05$.

phospho-PKCε expression before ischemia and preserved after reperfusion compared with the basal level. Prazosin significantly decreased phospho-PKCε expression before ischemia and returned to the basal level after reperfusion (Figure 7A). In landiolol group, phospho-PKCε was significant higher than that of control group before ischemia and after reperfusion. Prazosin abolished the effect of landiolol before ischemia and after reperfusion (Figure 7A).

Phosphorylation of PKCδ

The expression of phospho-PKCδ in whole tissue lysates did not change at the indicated time in all groups (Figure 7B).

HSP27 phosphorylation

The expression of nuclear phospho-HSP27 in the control group increased after 60 min of reperfusion (Figure 8). Landiolol significantly increased phosho-HSP27 expression before ischemia and preserved after reperfusion compared with the basal level (Figure 8). In landiolol group, phospho-HSP27 was significantly higher than that of control group before ischemia and after reperfusion (Figure 8).

Discussion

In the present study, landiolol was found to improve postischemic cardiac function and to reduce cellular injury. The recovery of cardiac function by landiolol was mediated by the α1 adrenoreceptor-induced PKC signaling pathway, resulted in the opening of sarcolemmal K_{ATP} channels. The attenuation of cardiac cellular injury was mediated by α1 adrenoreceptor-induced PKC signaling pathway, rather than by the opening of the K_{ATP} channels.

In the guinea pig Langendorff-perfused heart, Kurosawa et al. [14] demonstrated that 20 μM landiolol had no cardioprotective effects, while 500 μM landiolol showed cardioprotection with significant myocardial depression before ischemia. From these results, we chose to administer doses of landiolol ranging from 5 to 200 μM in the preischemic period. In the present study, we found that the optimal dose of landiolol for cardioprotection was 100 μM without myocardial depression. Although the cardiac cellular injury was reduced by 200 μM landiolol after reperfusion, the cardiac function failed to improve, indicating that landiolol has cardioprotective effect on cellular injury dose-dependently and high doses of landiolol may induce negative inotropic effects after reperfusion. Since red blood cell's esterase is not included in Langendorff model, the biological half lives of landiolol might be prolonged and negative inotropic effect of landiolol might be persisting after reperfusion.

In general, PKC signal transduction has been reported to be important for preconditioning. Endogenous catecholamines cause α1 stimulation, followed by PKC activation and opening of its downstream targets, K_{ATP} channels, with a consequent reduction in the intracellular Ca^{2+} overload [27–29]. Sanada et al. [30] and Arnaud et al. [31] reported that PKC activation during preconditioning induces HSP27 expression, thereby contributing to the

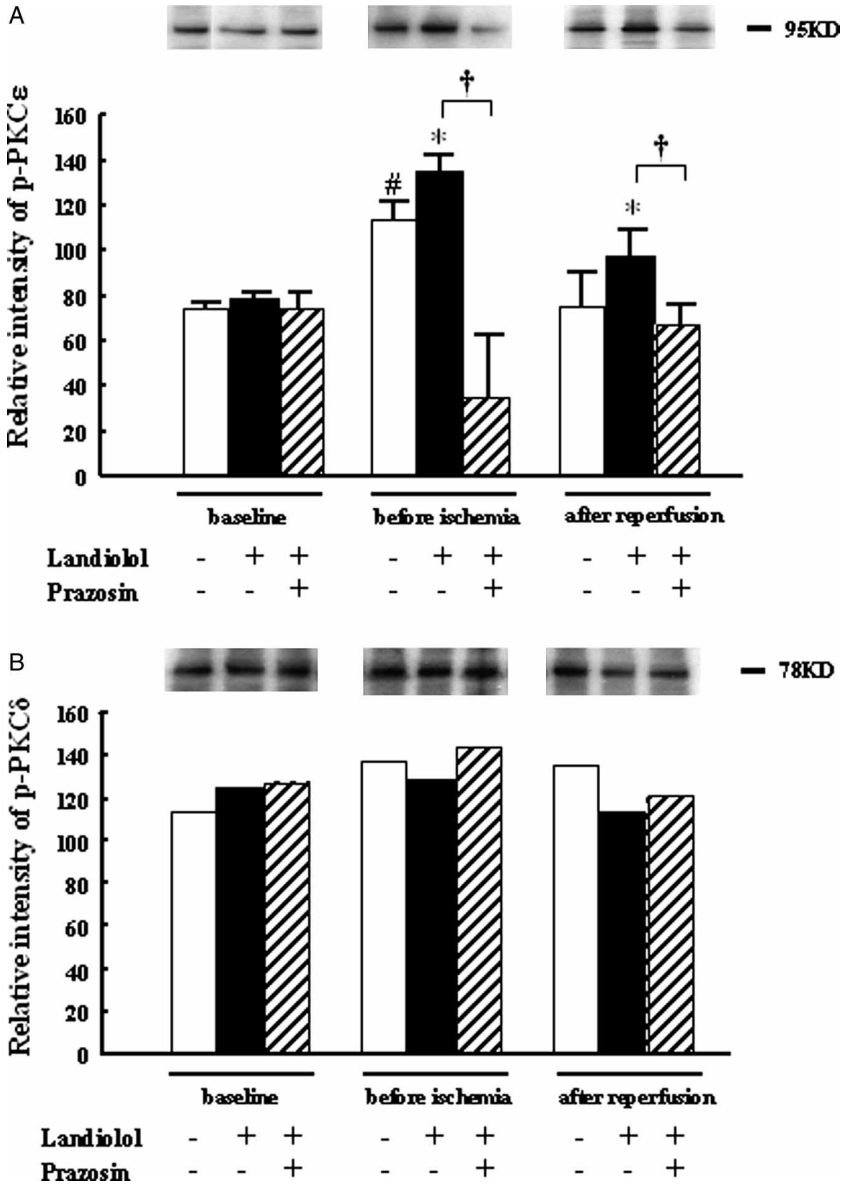

Figure 7. Effects of landiolol on the phosphorylation of PKCε and PKCε Hearts were prepared as described in the legend for Figure 6. (A) Representative western blotting image and densitometric evaluation of phospho PKCε expression from western blots of the whole fraction. (B) Representative western blotting image and densitometric evaluation of phospho PKCε expression from western blots of the whole fraction. Open bars, vehicle without landiolol groups; closed bars, vehicle with landiolol groups; hatched bars, prazosin with landiolol groups. Data are means ± SD ($n = 3$), $\star p < 0.05$ vs. the landiolol group at the basal level, $\#p < 0.05$ vs. the control group at the basal level, $^{\dagger}p < 0.05$.

functional improvement after reperfusion by stabilizing the actin cytoskeleton and inducing antiapoptotic effects. Recently, there have been a few reports concerning preconditioning via the PKA pathway [32]. Endogenous catecholamines cause cAMP elevation and PKA activation, resulting in down-regulation of the β-adrenergic signal transduction pathway, thus contributing to the attenuation of cAMP generation during sustained ischemia and functional improvement during reperfusion [33]. In fact, prazosin (α1-blocker), chelerythrine (a non-selective PKC inhibitor), and glibenclamide (a non-selective K_{ATP} channel blocker) abolished the cardioprotective effects of landiolol in the present study. Therefore, we consider that the cardioprotective effects of landiolol may be concerned with pharmacological preconditioning mainly mediated via the α1-induced PKC pathway.

The ratio of membranous PKCε in the landiolol group was increased before ischemia and after reperfusion compared to the control group. We found that the former elevation of the membranous PKCε ratio might be trigger of preconditioning by landiolol, while the latter might be due to cardioprotection by landiolol. As a consequence, the level of phospho-HSP27 in the landiolol group might be higher levels before ischemia and after reperfusion compared to the control group. Furthermore, we showed that prazosin

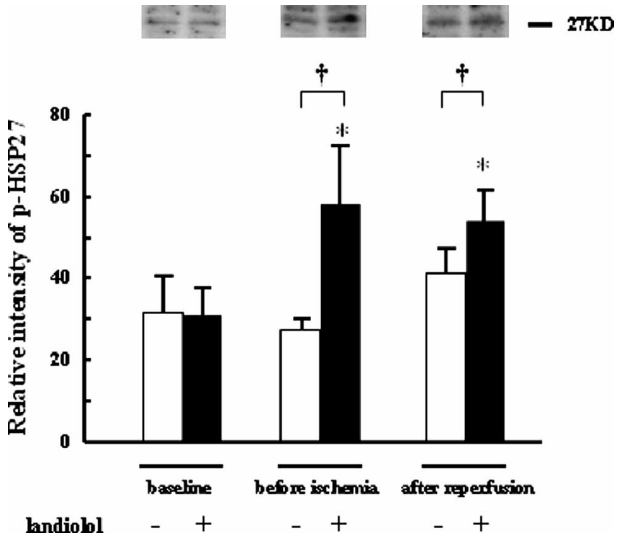

Figure 8. Effects of landiolol on the phosphorylation of HSP27 in the nuclear fraction. Hearts were prepared as described in the legend for Figure 6. Representative western blotting image and densitometric evaluation of phospho-HSP27 expression from western blots of the nuclear fraction. Open bars, vehicle without landiolol groups; closed bars, vehicle with landiolol groups. Data are means ± SD ($n = 3$), $\star p < 0.05$ vs. the landiolol group at the basal level, $^{\dagger}p < 0.05$.

abolished the activation of PKCε by landiolol, indicating that activation of PKCε was due to the augmentation of α1 signaling.

Over recent years, it has been reported that one of the mechanisms of IP was involved in adrenaline induced PKC and PKA signaling. Therefore, the effect of β-blocker on IP has been topic until today. There are controversial studies about the effect of selective β1-blocker against IP. Lange et al. [34] reported that esmolol (30 mg/kg/h) attenuate IP-induced cardioprotection *in vivo* rabbit heart, whereas Iliodromitis et al. [35] (30 mg/kg/h + 3 mg/kg/h) reported that esmolol maintain the IP-induced cardioprotection *in vivo* rabbit heart. Furthermore, Mieno et al. [36] demonstrated that landiolol (3 μM) enhances IP-induced cardioprotection in Langendorff rabbit heart. These conflicting results might be explained by the difference in study protocols (the frequency of IP), drugs concentration, and species.

Since the 1980s, the cardioprotective effects of preischemic administration of long-acting β-blockers, such as propranolol, metoprolol and atenolol, have been known to prevent I/R injury. The mechanisms underlying these cardioprotective effects involve energy-sparing effects, antioxidation and preservation of sarcoplasmic reticular function [37–40]. Recently, several reports have shown that preischemic administration of esmolol, an ultra-short-acting β1 selective-blocker, had cardioprotective effects in the prevention of I/R injury [15,41,42], although the cardioprotective mechanism of esmolol has been not fully elucidated. The present study has provided new insights into β1 adrenergic antagonists that induce cardioprotection via PKC pathway similar to afforded by IP. To the best of our knowledge, there have been no studies on adrenergic antagonist-induced preconditioning effects.

Recently, postconditioning effect has been topic and novel strategy to protect myocardium from I/R injury. Similar to preconditioning, postconditioning has been reported to involve PKC signaling pathway [43,44], We preliminary demonstrated that post-ischemic administration of 100 μM landiolol tended to have cardioprotective effects on cardiac functional recovery and cellular injury after reperfusion (percentage of recovery of LVDP: 54.5 ± 10.5% in the control group vs. 65.8 ± 4.5% in the post-ischemic landiolol administration). Therefore, landiolol may have possibility to induce postconditioning effect.

We propose that the present study on the cardioprotective activity of landiolol has the following clinical significance. It is important that landiolol shows low cardiodepression and is an ultra-short-acting β-blocker. During cardiac surgery, administration of long-acting β-blockers is not suitable due to their prolonged negative inotropic effects. In this study, the dose of 100 μM landiolol was 10 ~ 30 folds higher than that used in clinical use. However, in on-pump cardiac surgery, high dose of landiolol can be available even if negative inotropic effect of β-blocker is caused. In fact, significant high dose of esmolol, that is about 15 ~ 100 times higher, has been useful as cardioplegic arrest in cardiac surgery today [45]. Thus, administration of high dose of short-acting β-blockers, such as esmolol and landiolol, before ischemia is preferable during cardiac surgery.

The limitation of this study was described below. First, we did not study whether other β1-blockers have the cardioprotective effect mediated by PKC pathway as landiolol had. In fact, we consider that these β1-blockers including esmolol may have so less β1 selectivity than landiolol as to induce α1 adrenergic augmentation. Since we consider that high β1 selectivity of β blocker is essential to induce cardioprotection, further studies are needed. Second, we used chelerythrine that was a broad-spectrum PKC inhibitor in this study. The amelioration of the protective effects of landiolol following I/R by chelerythrine may have been caused by antagonism of the other PKC isozymes. Yabe et al. [46] reported that pharmacological preconditioning by adrenergic stimulation is mediated by activation of PKCδ in the rat heart. On the other hand, Arnaud et al. [31] reported that heat stress-induced preconditioning is mediated by PKCε in the rat heart. Since the role of PKC isoforms in the preconditioning has been controversial, we should measure other phenotype of PKC. Third, Landiolol activated HSP27 before ischemia and after reperfusion in this study. However we did not study whether HSP27 activation was

abolished in association with PKC inhibition. Thus, further studies are needed.

In conclusion, the present study has demonstrated that the cardioselective β1-blocker landiolol has cardioprotective effects against I/R injury. The mechanism for this phenomenon was explained by activation of the PKCε-mediated pathway. This cardioprotective effect by landiolol may have implications for new therapies aimed at minimizing reperfusion injury in cardiac surgery.

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