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Cardioprotective effect and mechanism of action of landiolol on the ischemic reperfused heart

Saori Kimura-Kurosawa, Noriaki Kanaya, Noriko Kamada, Naoyuki Hirata, Masayasu Nakayama, and Akiyoshi Namiki

Department of Anesthesiology, Sapporo Medical University, School of Medicine, S-1, W-16, Chuo-ku, Sapporo 060-8543, Japan

Abstract
Purpose. The authors examined the cardioprotective effect of landiolol, an ultra short-acting, highly selective β1-blocker, and its role in cardiac work, antioxidative effect, and sarcoplasmic reticulum (SR) function in hearts subjected to ischemia-reperfusion.
Methods. Isolated guinea pig hearts were subjected to ischemia-reperfusion by stopping the perfusion for 45 min and reperfusing. Before the ischemia, hearts were treated with landiolol (20, 100, or 500 μM) for 15 min (LAN group). In another set of experiments, before ischemia, hearts were washed out for 15 min after treatment with landiolol (WO group). In other hearts, the tissue concentration of malondialdehyde was measured after reperfusion. We also examined the phosphorylation of phospholamban at Ser^{16} and Thr^{17} residues to evaluate the SR function.
Results. After 90 min of reperfusion, left ventricular pressure (LVP) was restored significantly in the LAN-500 μM group regardless of heart rate. However, the improvement in recovery in LVP disappeared in the WO group. The tissue malondialdehyde levels were decreased in the LAN group compared with those in the control group. In the control group, the phosphorylation of phospholamban at Ser^{16} and Thr^{17} residues was markedly increased after reperfusion. Landiolol at 500 μM suppressed the increase of phosphorylation at Ser^{16} residues.
Conclusion. The present study demonstrated that landiolol had a lipid peroxidation-reducing effect and suppressed the increase in phospholamban phosphorylation at the Ser^{16} residue in hearts subjected to ischemia-reperfusion. These findings indicate that landiolol may have an anti-ischemic effect, via an antioxidant effect and/or via preserving SR function during the ischemic period.

Key words β-Blockers · Ischemic heart · Lipid peroxidation · Phospholamban phosphorylation

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Introduction

Landiolol hydrochloride is a newly developed highly cardioselective β-blocker with a potency ratio (β1/β2) of 255, compared with 33 for esmolol and 0.68 for propranolol [1]. We have previously reported that landiolol hydrochloride, as well as propranolol and esmolol, has cardioprotective effects on isolated guinea pig hearts subjected to ischemia-reperfusion (I/R) injury [2]. However, the exact mechanisms by which cardioprotective agents act are not yet fully understood. β-Blockers are thought to exert beneficial effects on the ischemic heart by lowering myocardial oxygen consumption as a consequence of reduced contractility and heart rate (HR) [3].

In previous studies, nicorandil, volatile anesthetics, and other drugs had a pharmacological preconditioning effect with a brief period of exposure, which improved the cardiac function of I/R hearts, similar to ischemic preconditioning [4,5]. The cardioprotective effect of pharmacological preconditioning is characterized by a short-term memory phase similar to that observed during ischemic preconditioning [5]. It is not yet known whether the cardioprotective effect of a β-blocker is associated with pharmacological preconditioning.

The beneficial effects of β-blockers are also thought to be due to their antioxidant effect [6,7]. β-Blockers can scavenge free radicals by binding to hydrophobic sites within the biological membrane. Also, they can reduce the lipid peroxidation that occurs during reperfusion of the ischemic myocardium, simultaneously decreasing the saturated fatty acids in the membrane phospholipids [8]. On the basis of these findings, β blockers can ameliorate reperfusion injury and preserve membrane phospholipids.

Recently, β-blockers have also been reported to preserve sarcoplasmic reticulum (SR) function and gene expression in rat hearts subjected to I/R [9]. Because the SR is known to play a crucial role in the regulation of

intercellular Ca^{2+} mobilization, β-blockers may have cardioprotective effects by preserving the SR function in hearts subjected to ischemia-reperfusion. Ca^{2+} uptake in the SR occurs via an SR Ca^{2+}-ATPase (SERCA2a), which is regulated by its interaction with phospholamban (PLB) [9]. In the dephosphorylated state, PLB inhibits SERCA2a activity and SR Ca^{2+} transport. Phosphorylation of PLB by either cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) at the Ser^{16} residue or Ca^{2+}-calmodulin-dependent protein kinase (CaMKII) at the Thr^{17} residue relieves this inhibition, thus increasing SERCA2a activity and the rate of SR Ca^{2+} uptake [10,11]. Thus, the status of PLB phosphorylation may vary during ischemia and reperfusion, with consequent changes in SERCA2a activity and SR Ca^{2+} uptake.

Based on this background, the present study was performed, by investigating PLB phosphorylation in isolated guinea pig hearts, to determine whether: (1) the cardioprotective effect of landiolol in hearts subjected to I/R is due to an energy-sparing mechanism, (2) landiolol has a pharmacological preconditioning effect, (3) the cardioprotective effect of landiolol in hearts subjected to I/R is due to an antioxidant mechanism, or (4) landiolol has an effect on SR function.

Materials and methods

Langendorff heart preparation

This investigation conformed to the guidelines for care and use of laboratory animals published by the National Institutes of Health and was approved by our institutional animal care committee. As described previously [12], male English short-haired guinea pigs (250–300 g) were each intraperitoneally injected with 20 mg of ketamine and 1000 U of heparin and were decapitated after they had become unresponsive to noxious stimulation. After thoracotomy, the inferior vena cava and superior vena cava were sectioned and the aorta was cannulated distal to the aortic valve. Each heart was perfused in a retrograde fashion via the aorta with a cold, oxygenated, modified Krebs-Ringer's (K-R) solution equilibrated with 95% oxygen and 5% carbon dioxide and was then rapidly excised. The perfusate was disk-filtered (5 μM bore size) in-line and had the following composition (in mM): 137 Na^+, 5 K^+, 1.2 Mg^+, 2.5 Ca^{2+}, 134 Cl^-, 15.5 HCO_3^-, 1.2 $H2PO_4^-$, 11.5 glucose, 2 pyruvate, 16 mannitol, and 0.05 ethylenediamine tetraacetic acid. Perfusate and bath temperatures were maintained at 37°C by a thermostatically controlled water circulator (Thermo pump; Taitec, Koshiya, Japan). Isovolemic development (systolic-diastolic) of left ventricular pressure (LVP), spontaneous atrial heart rate (HR), and aortic flow, which indicates coronary flow (CF), were measured continuously. LVP was measured by using a pressure transducer (DTX plus press; Ohmeda, Madison, WI, USA) connected to a thin, saline-filled latex balloon (LB-3; MARS, Sapporo, Japan) inserted into the left ventricle (LV) through the left atrium. The balloon volume was adjusted to maintain a diastolic LVP of 10 mmHg during the initial control period so that any increase in diastolic LVP reflected an increase in left-ventricular wall stiffness or diastolic contracture. The volume of the balloon was unchanged during the experiment. Coronary (aortic) inflow was measured continuously at a constant temperature and at a normal aortic perfusion (gravity) pressure of 55 mmHg by a transit-time, self-calibrating, in-line ultrasonic flowmeter (Research Flowmeter Transonic T106X; Transonic System, Ithaca, NY, USA) placed directly into the aortic inflow line.

Experimental protocol

Experiment 1: Does landiolol have a cardioprotective effect by a reduction in cardiac work during the preischemic period?

This experiment was performed to determine whether landiolol has a cardioprotective effect via a reduction in cardiac work. Hearts were perfused with K-R solution for a 15-min stabilization period. The hearts were then subjected to 45 min of global ischemic arrest at 37°C by clamping the aortic cannula. Finally, the hearts were reperfused for 90 min (37°C) for postischemic functional measurement. Before ischemia, hearts (n = 7 in each group) were treated with landiolol (20, 100, or 500 μM) for 15 min. The concentrations of landiolol were determined on the basis of previous in vitro findings [2]. In the clinical setting, landiolol is injected at a rate of $0.125\,mg{\cdot}kg^{-1}{\cdot}min^{-1}$ for the first 1 min and then administered at a rate of $0.04\,mg{\cdot}kg^{-1}{\cdot}min^{-1}$ continuously. The maximum clinical concentration of landiolol (C_{max}) is $2.0 \pm 0.8\,\mu g{\cdot}ml^{-1}$ and that is within the range of doses used in the present study. The 500-μM landiolol group was divided into two groups. In one group, HR was kept constant by pacing the heart at 300 $beats{\cdot}min^{-1}$ throughout the study via stimuli (3F46; San-Ei Instruments, Tokyo, Japan) applied to the left ventricle (4V, two time threshold; 2-ms duration), and in the other group, pacing was not performed.

Cardiac mechanical function was estimated as LVP, HR, CF, and the rate-pressure product (RPP; LVP × HR).

Experiment 2: Does landiolol have a preconditioning effect on I/R injury?

To determine whether landiolol has a cardioprotective effect after washout on hearts subjected to I/R, the

cardioprotective effect of landiolol was examined after washout. After washout hearts (n = 7 in each group) were subjected to 45 min of global ischemic arrest at 37°C by clamping the aortic cannula. Finally, the hearts were reperfused for 90 min (37°C) for postischemic functional measurement. Before ischemia, the perfusion solution was switched to K-R solution for 15 min after treatment with landiolol (20, 100, or 500 μM) for 15 min. Cardiac mechanical function was estimated as LVP, HR, CF, and rate-pressure product (RPP; LVP × HR).

Experiment 3: Does landiolol prevent I/R injury by reducing lipid peroxidation?

To determine whether landiolol has a cardioprotective effect on hearts subjected to I/R by reducing lipid peroxidation, hearts were perfused with K-R solution for a 15-min stabilization period. Hearts were rendered globally ischemic for 45 min, and then perfusion was begun with K-R solution for 90 min. The hearts were randomly divided into four groups: a control group and landiolol (20, 100, and 500 μM) groups (n = 7 in each group). In the landiolol groups, hearts were treated before ischemia with 20, 100, and 500 μM landiolol for 15 min. After 90 min of reperfusion, the tissue concentration of malondialdehyde (MDA) was measured to evaluate lipid peroxidation.

Frozen left ventricular myocardial samples were stored at –80°C until biochemical analysis was performed. The MDA concentration was determined from the reaction of N-methyl-2-phenylindole with MDA [13]. Briefly, myocardial samples were homogenized with 20 mM phosphate buffer, pH 7.4, containing 5 mM butylated hydroxytoluene (BHT), to make a 20% homogenate. The homogenate was centrifuged at 3000 g for 10 min at 4°C. N-Methyl-2-phenylindole and 12 N HCl were then added to the supernatant and the mixture was incubated at 45°C for 60 min. Turbid samples were centrifuged at 15000 g for 10 min. Measurement of absorbance at 586 nm was performed. Final MDA levels are reported as μg of MDA per milligram protein.

Experiment 4: Does landiolol have an effect on the phosphorylation of phospholamban in hearts subjected to I/R?

The effect of landiolol on the phosphorylation of PLB in hearts subjected to I/R was investigated. Hearts were perfused with K-R solution for a 15-min stabilization period. Hearts were rendered globally ischemic for 45 min, and then perfusion was begun with K-R solution. The hearts were randomly divided into four groups: a control group and landiolol (20, 100, and 500 μM) groups (n = 7 in each group). Hearts were freeze-clamped at preischemia (baseline value), after 45 min of ischemia, and after 1 min of reperfusion. Frozen left ventricular myocardial samples were stored at –80°C until biochemical analysis was performed.

The ventricular tissue from guinea pig hearts was homogenized in nine volumes of homogenization buffer containing 250 mM sucrose, 1 mM ethylenediamine tetraacetic acid (EDTA), and 50 mM Tris. The homogenate buffer also contained a protease inhibitor cocktail. The homogenate was then centrifuged at 600 g for 10 min, and the supernatant was further centrifuged at 8000 g for 10 min. The supernatant was then centrifuged at 15000 g for 60 min. The resulting supernatant was subjected to sodium dodecylsufate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Protein samples (25 μg of total protein/lane) were separated by electrophoresis through SDS-PAGE in 10% to 20% gradient slab gels. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA) and probed with polyclonal antibodies raised to a PLB peptide (residues 9-19) phosphorylated at Ser^{16} (1:500, upstate) or at Thr^{17} (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactivity was visualized with a peroxidase-linked anti-rabbit IgG antibody (1:1000; Amersham Life Science, Buckinghamshire, UK), using a chemiluminescence ECL plus kit (Amersham Life Science). The signal intensity of the bands was quantified by optical densitometric analysis.

Landiolol was donated by Ono Pharmaceutical (Osaka, Japan).

Statistical analysis

Values for results are expressed as means ± SEM. To determine differences in recovery of function between the groups, data were subjected to analyses by analysis of variance with repeated measures. Post hoc analyses were performed with Fisher's protected least significant difference test. A P value of less than 0.05 was considered statistically significant.

Results

Landiolol has a cardioprotective effect independent of a reduction in cardiac work in the preischemic period

Table 1 shows the effects of landiolol on LVP, HR, RPP, and CF in hearts exposed to ischemia for 45 min followed by 90 min of reperfusion. The highest dose of landiolol caused a significant decrease in RPP at 15 min after drug administration. Figure 1 shows the changes in LVP before, during, and after global ischemia. After 90 min of reperfusion, LVP was restored to 65 ± 7% of the baseline value in the control group. In hearts pretreated with 500 μM of landiolol, LVP was restored to

Table 1. Hemodynamic effects of landiolol in experiment 1

	Landiolol				
	CONT	20 μM	100 μM	500 μM	500 μM-Pacing
LVP (mmHg)					
BL	42 ± 2	47 ± 5	41 ± 1	42 ± 3	43 ± 3
PRE	42 ± 2	46 ± 5	36 ± 1*	22 ± 3*,**	28 ± 4*,**
R-90	27 ± 3*	31 ± 3*	29 ± 1*	43 ± 4**	44 ± 3**
HR (bpm)					
BL	225 ± 18	231 ± 22	255 ± 7	194 ± 26	300 ± 1**
PRE	225 ± 18	216 ± 19	189 ± 5*	125 ± 20*,**	300 ± 1**
R-90	190 ± 12	205 ± 14	197 ± 12*	173 ± 24	301 ± 0**
RPP ($mmHg \cdot min^{-1} \cdot 1000^{-1}$)					
BL	9.2 ± 0.9	10.4 ± 0.5	10.4 ± 0.4	7.9 ± 0.7	12.9 ± 0.8**
PRE	9.2 ± 0.9	9.7 ± 0.6	6.7 ± 0.1*	2.6 ± 0.3*,**	8.5 ± 1.3*
R-90	4.8 ± 0.5*	6.3 ± 0.8*	5.8 ± 0.5*	7.3 ± 1.0	13.4 ± 0.9**
CF ($ml \cdot min^{-1}$)					
BL	18.3 ± 0.3	14.8 ± 1.4	17.2 ± 0.6	16.0 ± 1.5	14.1 ± 1.0**
PRE	18.3 ± 0.3	15.3 ± 1.8	16.1 ± 0.7	14.8 ± 1.8	13.4 ± 1.3**
R-90	12.9 ± 4.3	7.5 ± 1.2*	10.0 ± 1.4*	12.8 ± 2.3	13.6 ± 1.4

* $P < 0.05$ versus the corresponding baseline value; ** $P < 0.05$ versus CONT
Values are means ± SEM; $n = 7$ in each group
LVP, left ventricular pressure; HR, heart rate; RPP, rate-pressure product; CF, coronary flow; BL, baseline; PRE, preischemia (after 15 min of drug or control, equilibration); R-90, 90 min after reperfusion; CONT, control

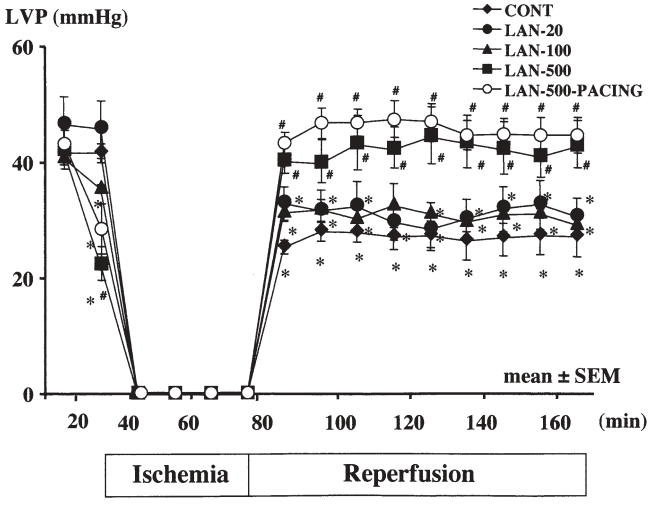

Fig. 1. Effects of landiolol (20, 100, 500 μM) on left ventricular pressure (*LVP*) in hearts subjected to ischemia/reperfusion (I/R). Hearts were perfused with Krebs-Ringer's (K-R) solution for a 15-min stabilization period. Hearts were then subjected to 45 min of global ischemic arrest. Finally, the hearts were reperfused for 90 min for postischemic functional measurement. Before ischemia, hearts ($n = 7$ in each group) were treated with landiolol (20, 100, or 500 μM) for 15 min. The 500-μM landiolol group was divided into two groups. In one group, HR was kept constant by pacing the heart at 300 beats·min^{-1} throughout the study, via stimuli. *CONT*, control group; *LAN-20*, 20-μM landiolol group; *LAN-100*, 100-μM landiolol group; *LAN-500*, 500-μM landiolol group. *LAN-500-PACING*, 500-μM landiolol-with-pacing group. Values are expressed as means ± SEM. * $P < 0.05$ versus corresponding baseline value; # $P < 0.05$ versus control group

103 ± 9% of the baseline value. However, landiolol at doses of 20 and 100 μM did not significantly improve postischemic recovery in LVP. In the pacing group, RPP was not significantly different from that in the control group before ischemia. However, LVP was restored to 103 ± 3% of the baseline value after 90 min of reperfusion. After 90 min of reperfusion in the control group, HR was restored to 83 ± 13% of the baseline value in the control group (Table 1). After 90 min of reperfusion, HR was restored to 91 ± 10%, 78 ± 6%, and 91 ± 12% of baseline values at 20, 100, and 500 μM of landiolol, respectively. There was no significant difference among the groups in the magnitude of recovery in HR at 90 min after reperfusion.

Landiolol has a cardioprotective effect independent of cardiac preconditioning in the preischemic period

Table 2 shows the hemodynamic effects of landiolol on LVP, HR, RPP, and CF in hearts exposed to ischemia for 45 min followed by 90 min of reperfusion after landiolol washout for 15 min. Figure 2 shows the changes in LVP before, during, and after global ischemia. Treatment with 20 and 100 μM of landiolol induced no significant changes in LVP before ischemia. After treatment with 500 μM of landiolol, LVP decreased to 48 ± 15% of its corresponding baseline level. After washout, LVP was restored to 84 ± 18% of its corresponding baseline level. In the control group, LVP was restored to 66 ± 6% of the baseline value after 90 min of reperfusion. After

Table 2. Hemodynamic effects of landiolol in experiment 2

		Landiolol		
	CONT	20 μM	100 μM	500 μM
LVP (mmHg)				
BL	40 ± 1	46 ± 5	39 ± 3	37 ± 3
PRE	40 ± 1	46 ± 4	33 ± 5	19 ± 7*,**
WO	40 ± 1	46 ± 4	36 ± 1	29 ± 6
R-90	26 ± 3*	31 ± 3	27 ± 5*	29 ± 5
HR (bpm)				
BL	223 ± 3	205 ± 22	241 ± 12	211 ± 5
PRE	223 ± 3	193 ± 22	204 ± 11	135 ± 10*,**
WO	223 ± 3	190 ± 26	243 ± 14	180 ± 20
R-90	200 ± 16	168 ± 30*	224 ± 12	195 ± 27
RPP ($mmHg \cdot min^{-1} \cdot 1000^{-1}$)				
BL	8.9 ± 0.6	9.8 ± 2.0	9.2 ± 0.5	7.7 ± 0.5
PRE	8.9 ± 0.6	9.3 ± 1.9	6.9 ± 1.2*	2.7 ± 1.0*,**
WO	8.9 ± 0.6	9.2 ± 1.8	8.6 ± 0.3	5.7 ± 1.4
R-90	5.0 ± 0.5*	5.6 ± 1.5	6.0 ± 1.2*	5.7 ± 1.3
CF ($ml \cdot min^{-1}$)				
BL	18.0 ± 0.9	16.3 ± 0.5	17.5 ± 1.3	17.4 ± 1.1
PRE	18.0 ± 0.9	16.1 ± 1.4	15.8 ± 2.3	17.3 ± 1.5
WO	18.0 ± 0.9	14.7 ± 1.9	17.4 ± 1.5	18.8 ± 0.6
R-90	10.4 ± 1.3*	9.4 ± 1.2*	13.7 ± 1.5	13.4 ± 2.4

* $P < 0.05$ versus the corresponding baseline value, ** $P < 0.05$ versus CONT
Values are means ± SEM; $n = 7$ in each group
LVP, left ventricular pressure; HR, heart rate; RPP, rate-pressure product; CF, coronary flow; BL, baseline; PRE, preischemia (after 15 min of drug or control, equilibration); WO, washout (after 15 min of washout); R-90, 90 min after reperfusion; CONT, control

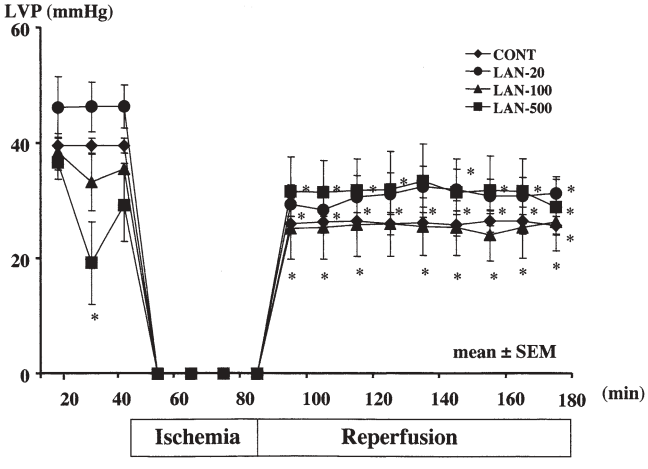

Fig. 2. Effects of landiolol (20, 100, 500 μM) on the LVP in hearts subjected to I/R. Hearts were perfused with K-R solution for a 15-min stabilization period. Hearts were then subjected to 45 min of global ischemic arrest. Finally, the hearts were reperfused for 90 min for postischemic functional measurement. Before ischemia, hearts ($n = 7$ in each group) were washed out with K-R solution for 15 min after treatment with landiolol (20, 100, or 500 μM) for 15 min. *CONT*, control group; *LAN-20*, 20-μM landiolol group; *LAN-100*, 100-μM landiolol group; *LAN-500*, 500-μM landiolol group. Values are expressed as means ± SEM. *$P < 0.05$ versus corresponding baseline value

90 min of reperfusion, LVP was restored to 70 ± 7%, 68 ± 9%, and 77 ± 7% of the baseline values at 20, 100, and 500 μM of landiolol, respectively. Although treatment with 500 μM of landiolol seemed to result in a better recovery in LVP after reperfusion, there was no significant difference between the groups in the magnitudes of LVP recovery after 90-min reperfusion. Only 500 μM landiolol caused a significant decrease in RPP at 15 min after drug administration; however, RPP recovered to 76 ± 18% of its corresponding baseline level after washout. HR was restored to 91 ± 8%, 80 ± 8%, 93 ± 5%, and 92 ± 12% of the corresponding baseline values in the control, 20-μM, 100-μM, and 500-μM landiolol groups, respectively, after 90 min of reperfusion. Although the highest dose of landiolol caused a significant decrease in HR, there was no significant difference between the groups in the magnitudes of recovery in HR at 90 min after reperfusion. In the control group, CF was restored to 57 ± 5% of the baseline value after 90 min of reperfusion. After 90 min of reperfusion, CF was restored to 58 ± 7%, 79 ± 7%, and 75 ± 9% of the baseline values at 20, 100, and 500 μM landiolol, respectively. Although treatment with 100 and 500 μM landiolol seemed to result in a better recovery in CF after reperfusion, there was no significant difference between the groups in the magnitudes of CF recovery after 90 min of reperfusion.

Pretreatment with landiolol decreased I/R-induced MDA production

Figure 3 shows the tissue concentrations of MDA after 90 min of reperfusion in the isolated hearts subjected to I/R. The tissue MDA level was 1.16 ± 0.12 μg·mg^{-1} protein in the control group. Tissue MDA levels were 0.52 ± 0.03, 0.66 ± 0.09, and 0.57 ± 0.11 μg·mg^{-1} protein in the landiolol 20-, 100-, and 500-μM groups, respectively. The tissue MDA levels decreased significantly in the hearts treated with landiolol, regardless of dose, in comparison with the level in the control group.

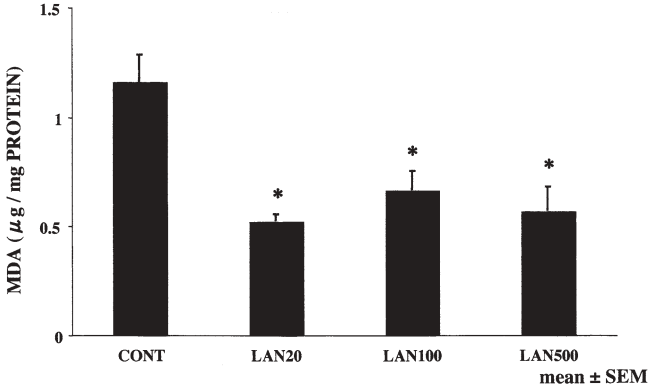

Fig. 3. Effect of landiolol on the tissue concentrations of malondialdehyde (*MDA*) in hearts subjected to I/R. Hearts were perfused with K-R solution for a 15-min stabilization period. Hearts were then subjected to 45 min of global ischemic arrest. Finally, the hearts were reperfused for 90 min. Before ischemia, hearts (n = 7 in each group) were treated with landiolol (20, 100, or 500 μM) for 15 min. MDA was measured 90 min after the onset of reperfusion. *CONT*, control group; *LAN20*, 20-μM landiolol group; *LAN100*, 100-μM landiolol group; *LAN500*, 500-μM landiolol group. Values are expressed as means ± SEM. *P < 0.05 versus corresponding baseline value

Landiolol suppressed the I/R-induced phosphorylation of the Ser^{16} residue but not that of the Thr^{17} residue of PLB

Figure 4 shows representative immunoblots and the results of phosphorylation of the Ser^{16} residue of PLB in hearts subjected to I/R. In the control group, phosphorylation of Ser^{16} residues at 45 min after ischemia was 79 ± 20% of the baseline value. Treatment with 20, 100, and 500 μM landiolol decreased the phosphorylation of Ser^{16} residues by 58 ± 9%, 44 ± 6%, and 75 ± 10% compared with the corresponding baseline values, respectively; there was no significant difference among the groups. In the control group, phosphorylation of Ser^{16} residues was markedly increased at 1 min after reperfusion, by 380 ± 38% compared with the baseline value. Treatment with 500 μM landiolol significantly suppressed the increase in the phosphorylation of Ser^{16} residues, by 40 ± 11%. Although 20 and 100 μM landiolol did not prevent the phosphorylation of Ser^{16} residues in the I/R hearts, the magnitude of the increase in phosphorylation after reperfusion was suppressed compared to that in the control group.

Figure 5 shows representative immunoblots and the results of phosphorylation of Thr^{17} residues of PLB in

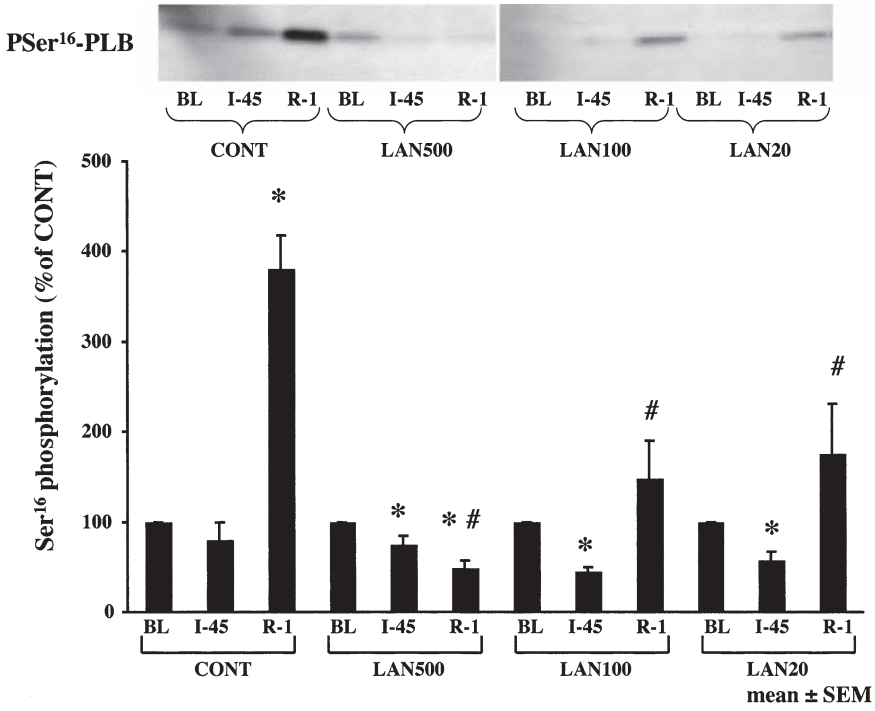

Fig. 4. Effects of landiolol on phosphorylation of Ser^{16} residues of phospholamban (*PLB*) in hearts subjected to I/R. *Upper panel*, representative immunoblots; *lower panel*, average results of the densitometric analysis of phosphorylation of Ser^{16} residues of PLB under baseline conditions (*BL*), 45 min after ischemia (*I-45*), and 1 min after reperfusion (*R-1*). Hearts were perfused with K-R solution for a 15-min stabilization period. Hearts were rendered globally ischemic for 45 min, and then perfusion was begun with K-R solution. Before ischemia, hearts (n = 7 in each group) were treated with landiolol (20, 100, or 500 μM) for 15 min. Hearts were freeze-clamped at preischemia (baseline value), after 45 min of ischemia and after 1 min of reperfusion. *CONT*, control group; *LAN20*, 20-μM landiolol group; *LAN100*, 100-μM landiolol group; *LAN500*, 500-μM landiolol group. Values are expressed as means ± SEM. *P < 0.05 versus corresponding baseline value; $^{\#}P$ < 0.05 versus control group

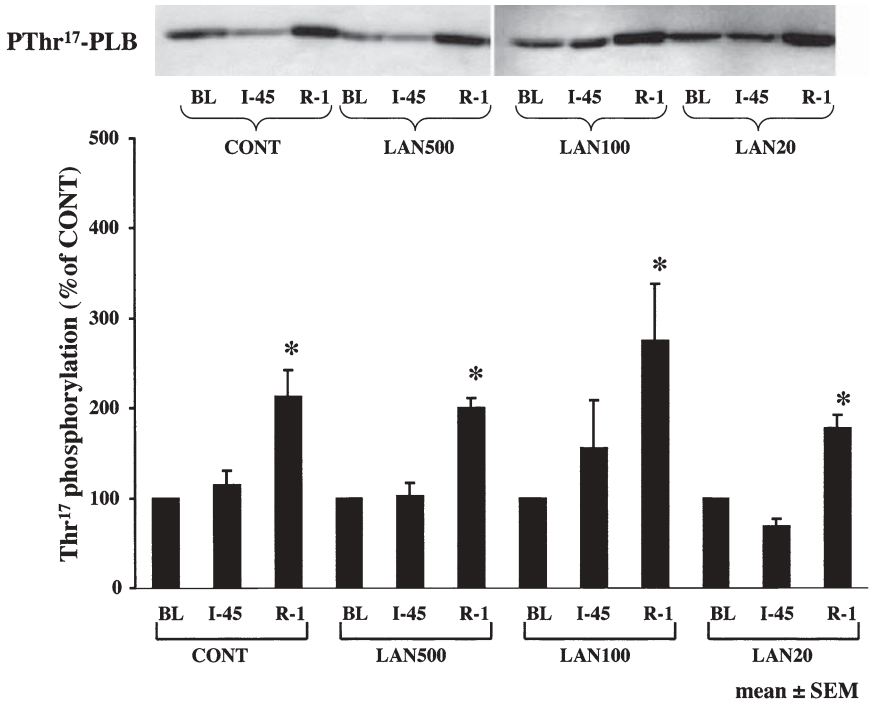

Fig. 5. Effects of landiolol on phosphorylation of Thr[17] residues of PLB in hearts subjected to I/R. *Upper panel*, representative immunoblots; *lower panel*, average results of the densitometric analysis of phosphorylation of Thr[17] residues of PLB under baseline conditions (*BL*), 45 min after ischemia (*I-45*), and 1 min after reperfusion (*R-1*). Hearts were perfused with K-R solution for a 15-min stabilization period. Hearts were rendered globally ischemic for 45 min, and then perfusion was begun with K-R solution. Before ischemia, hearts (n = 7 in each group) were treated with landiolol (20, 100, or 500 μM) for 15 min. Hearts were freeze-clamped at preischemia (baseline value), after 45 min of ischemia and after 1 min of reperfusion. *CONT*, control group; *LAN20*, 20-μM landiolol group; *LAN100*, 100-μM landiolol group; *LAN500*, 500-μM landiolol group. Values are expressed as means ± SEM. *$P < 0.05$ versus corresponding baseline value

hearts subjected to I/R. Phosphorylation of Thr[17] residues at 45 min after ischemia was 115 ± 16%, 69 ± 8%, 155 ± 54%, and 103 ± 15% of the corresponding baseline values in the control, 20-μM, 100-μM, and 500-μM landiolol groups, respectively. In the control group, phosphorylation of Thr[17] residues was markedly increased at 1 min after reperfusion, by 213 ± 30% compared with the baseline value. However, pretreatment with 20, 100, and 500 μM landiolol did not suppress the increases in phosphorylation of Thr[17] residues (178 ± 15%, 275 ± 63%, and 201 ± 12% compared with the corresponding baseline values, respectively) in the I/R hearts.

Discussion

We previously reported that landiolol, a new ultra-short-acting, highly selective β_1-blocker, had a cardioprotective effect in the ischemic heart when it was administered before ischemia [2]. The present study demonstrated that: (1) landiolol, only at the highest dose used, had a cardioprotective effect in hearts subjected to I/R, (2) landiolol also had a cardioprotective effect in hearts subjected to I/R independent of a reduction in cardiac work, (3) landiolol had a cardioprotective effect only when it was administered just before ischemia, and the cardioprotective effect disappeared after washout, (4) landiolol had a lipid peroxidation-reducing effect in hearts subjected to I/R although no dose-dependency was shown, and (5) landiolol suppressed I/R-induced PLB phosphorylation at the Ser[16] residue in a dose-dependent manner. Our results indicate that the beneficial effect of landiolol may be a result of the inhibition of lipid peroxidation and/or the preservation of SR function, but that the effect is not a result of an energy-sparing or a preconditioning effect.

β-Blockers are known to reduce myocardial oxygen consumption by decreasing both heart rate and myocardial contractility, which improves the oxygen supply/demand ratio [14]. Their beneficial effects are also thought to be due to their antioxidant effects, antiperoxidative activity [6,7], and, in the case of propranolol, membrane-stabilizing properties [15]. Recently, β-blockers have also been reported to preserve SR function and gene expression in rat hearts subjected to I/R [9].

Landiolol hydrochloride is a newly developed highly cardioselective β-blocker with a potency ratio (β1 / β2) of 255, compared with 33 for esmolol and 0.68 for propranolol [1]. It has a short duration (half-life, 4 min) of activity, enabling rapid recovery after cessation of administration by rapid hydrolysis of its ester link [1]. It has neither intrinsic sympathomimetic activity nor significant membrane-stabilizing activity [1]. We have previously reported that landiolol hydrochloride, as well as propranolol and esmolol, has cardioprotective effects on isolated guinea pig hearts subjected to I/R [2].

Wallace et al. [16] reported that perioperative administration of atenolol reduced the incidence of cardiac events in a randomized, double-blinded, placebo-controlled study. They found that perioperative atenolol given for 1 week after operation to patients at high risk

for coronary artery disease reduced the incidence of postoperative myocardial ischemia and the risk of death at 2 years. HR before an episode of ischemia and during the entire postoperative week was lower in the atenolol group. Such preischemic energy-sparing effects, due to the depression of cardiodynamic function, have been suggested as mechanisms by which β-blockers provide postischemic functional recovery of the myocardium [17]. However, the cardioprotective effects cannot be explained only by energy-sparing mechanisms in the preischemic period [15]. In fact, Wallace et al. [16] found that 15% or more of ischemic episodes were independent of HR. In the present study, landiolol (500 μM) had a protective effect on myocardium subjected to ischemia-reperfusion. In hearts in which HR was kept constant by pacing, landiolol (500 μM) also had a protective effect without causing a reduction in RPP in the pre- and post-ischemic periods. These findings indicate that β-blockers may exert anti-ischemic effects independent of their effects on reducing cardiac work.

Ischemic preconditioning is the phenomenon by which a brief period of ischemia and reperfusion protects the myocardium from subsequent sustained ischemia and reperfusion [18]. It has been reported that nicorandil [4], volatile anesthetics [5], and several other drugs have a pharmacological preconditioning effect, with a brief period of exposure improving the cardiac function of I/R hearts, similar to ischemic preconditioning. The cardioprotective effect of ischemic preconditioning is characterized by an acute memory phase, such that the preconditioning stimulus may precede the prolonged period of ischemia by 30 min to 2 h, during which time the heart remains resistant to infarction [5]. The present study demonstrated that landiolol had a cardioprotective effect only when it was administered just before ischemia and that its cardioprotective effect disappeared after washout. Therefore, our results indicate that the cardioprotective effect of landiolol is not a pharmacological preconditioning effect.

Reactive oxygen species, such as superoxide anions, hydroxyl radicals, and hydrogen peroxide, are some of the factors involved in the production of I/R damage in the heart [19,20]. These reactive oxygen species are generated intra- and extracellularly in the myocardium and endothelium during postischemic reperfusion, inducing lipid peroxidation of the cell membrane, which can then contribute to intracellular Ca^{2+} overload and, hence, mechanical and metabolic derangements [20,21]. A previous study showed that metoprolol prevented myocardial I/R injury and enhanced myocardial recovery via the prevention of lipid peroxidation [22]. Therefore, we investigated whether the cardioprotective effect of landiolol in hearts subjected to I/R was due to a lipid peroxidation-reducing mechanism. The role of reactive oxygen species in ischemia-reperfusion injury has been examined by detecting the byproducts of target molecule oxidation (lipid peroxidation and protein oxidation) and by determining the consumption of tissue antioxidants [23]. To examine the possibility, of landiolol acting via a lipid peroxidation-reducing mechanism, we measured the tissue concentration of MDA, an indicator of lipid peroxidation-associated free-radical generation [22]. The present study demonstrated that the level of tissue MDA decreased significantly in hearts treated with landiolol (20, 100, and 500 μM), regardless of dose, in comparison with the level in the control group. The precise mechanism concerning the lack of dose-dependency of landiolol in its effects on MDA production was unexplained. Because we only measured the tissue concentration of MDA, and did not perform histological examination, the extent of tissue injury was not evaluated. Thus, it remains possible that a reduction in lipid peroxidation is one of mechanisms underlying the cardioprotective effects of landiolol.

The SR is known to play a crucial role in the regulation of intercellular Ca^{2+}. Ca^{2+} uptake in the SR occurs via SERCA2a, which is regulated by its interaction with PLB [9]. Phosphorylation of PLB by either cAMP-dependent protein kinase (PKA) at the Ser^{16} residue or Ca^{2+}-calmodulin-dependent protein kinase (CaMKII) at the Thr^{17} residue increases SERCA2a activity and the rate of SR Ca^{2+} uptake [24]. The present study demonstrated that phosphorylation of PLB at Ser^{16} and Thr^{17} residues was significantly increased in hearts subjected to I/R in comparison with baseline levels. Treatment of hearts with landiolol (500 μM) significantly suppressed the phosphorylation of Ser^{16} residues, but landiolol showed no effect on the phosphorylation of Thr^{17} residues when compared with the baseline values. Because the phosphorylation of Ser^{16} is regulated by PKA, interrupting the β-adrenergic signaling cascade can diminish this phosphorylation. In contrast, the phosphorylation of Thr^{17} is regulated by CaMKII, and interrupting the β-adrenergic signaling cascade has no effect on this phosphorylation. We found that landiolol suppressed the phosphorylation of Ser^{16} residues, but that it showed no effect on the phosphorylation of Thr^{17} residues.

An increased phosphorylation state of PLB increases the Ca^{2+} sensitivity of SERCA2a, and more Ca^{2+} is pumped into the SR [10]. This would decrease Ca^{2+} overload in the cytosol. However, an increase in SR Ca^{2+} uptake would produce an enhanced SR Ca^{2+} load and Ca^{2+} release [25], which would contribute to cytosolic Ca^{2+} overload and contractile dysfunction. Moreover, increased cytosolic Ca^{2+} would stimulate SR Ca^{2+} release, triggering a self-perpetuating cycle, which would further increase cytosolic Ca^{2+}. It is possible that this is one of the mechanisms underlying the cardioprotective effects of landiolol.

PKA-dependent phosphorylation of the L-type Ca^{2+} channel has been reported to increase Ca^{2+} influx through the sarcolemma [26]. Inhibition of the phosphorylation by β-adrenergic blockade might contribute to a decrease in cytosolic Ca^{2+} overload and contractile recovery. Tilisolol, a nonselective β-blocker, has been reported to have blocking effects, at high concentrations, on the transmembrane Ca^{2+} current via the L-type Ca^{2+} channel in single guinea pig ventricular myocytes [27]. Therefore, a reduction in transmembrane Ca^{2+} current is one potential mechanism of the cardioprotective effect of landiolol. Although it has been reported that the L-type Ca^{2+} current is not affected by 10–100 μM landiolol [28], a higher dose of landiolol may reduce transmembrane Ca^{2+} influx. Electrophysiological studies are required to confirm this hypothesis.

The present study demonstrated that landiolol (500 μM) had a cardioprotective effect on isolated guinea pig hearts subjected to I/R injury. The effect of landiolol on hearts subjected to I/R cannot be explained by an energy-sparing mechanism in the preischemic period, and landiolol had no pharmacological preconditioning effect. This study also showed that landiolol had a lipid peroxidation-reducing effect and that landiolol suppressed the increase in PLB phosphorylation in the SR at the Ser^{16} residue in ischemia-reperfusion hearts. These findings suggest that the lipid peroxidation-reducing effect and PLB phosphorylation-suppressive effect are some of the mechanisms underlying the cardioprotective effects of landiolol.

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