Exercise Preconditioning of Myocardial Infarct Size in Dogs Is Triggered by Calcium

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Abstract: We showed that exercise induces early and late myocardial preconditioning in dogs and that these effects are mediated through nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase activation. As the intracoronary administration of calcium induces preconditioning and exercise enhances the calcium inflow to the cell, we studied if this effect of exercise triggers exercise preconditioning independently of its hemodynamic effects. We analyzed in 81 dogs the effect of blocking sarcolemmal L-type Ca^{2+} channels with a low dose of verapamil on early and late preconditioning by exercise, and in other 50 dogs, we studied the effect of verapamil on NADPH oxidase activation in early exercise preconditioning. Exercise reduced myocardial infarct size by 76% and 52% (early and late windows respectively; $P < 0.001$ both), and these effects were abolished by a single low dose of verapamil given before exercise. This dose of verapamil did not modify the effect of exercise on metabolic and hemodynamic parameters. In addition, verapamil blocked the activation of NADPH oxidase during early preconditioning. The protective effect of exercise preconditioning on myocardial infarct size is triggered, at least in part, by calcium inflow increase to the cell during exercise and, during the early window, is mediated by NADPH oxidase activation.

Key Words: preconditioning, exercise, calcium, myocardial infarction

(*J Cardiovasc Pharmacol*™ 2015;65:276–281)

INTRODUCTION

We previously showed that exercise induces early preconditioning (EP) and late preconditioning (LP) on the myocardial infarct size (IS) induced by coronary occlusion in dogs[1] and that these effects are mediated through nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase activation and mitochondrial adenosine triphosphate–sensitive K^+ channels.[2,3] However, the triggering for this kind of preconditioning is not known. Because intracoronary administration of Ca^{2+} induces preconditioning[4–6] and this protection can be blocked by verapamil[5,6] and the activation of α-adrenoceptors and β-adrenoceptors during exercise[7] increases Ca^{2+} inflow to the cell,[8–10] we reasoned that this increase of Ca^{2+} inflow triggers exercise preconditioning. As catecholamine-induced increase in intracellular calcium is experimentally inhibited by verapamil,[9] a nondihydropyridine sarcolemmal L-type Ca^{2+} channel blocker,[11] we studied if EP and LP induced by exercise are prevented by the administration of verapamil before exercise (VE). Furthermore, as calcium, and more specifically the calcium-sensing receptor, has been involved as a mediator in cardiac ischemic preconditioning independently of its hemodynamic effects,[12,13] we used a single low dose of verapamil, which did not modify the cardiac hemodynamic effect of exercise, to test the hypothesis that the increase of Ca^{2+} inflow to the cell during exercise triggers the cardioprotection independently of its hemodynamic effects.

We also recently showed that EP by exercise increases NADPH oxidase activity and that its inhibition with apocinin reverts the protective effect of exercise on myocardial IS.[2] To assess if calcium is involved in this activation, we additionally studied the effect of verapamil on NADPH oxidase activation in early exercise preconditioning.

Received for publication July 27, 2014; accepted November 4, 2014.

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Supported by Fondo Nacional de Investigación Científica y Tecnológica (FONDECYT), grants 1030449 and 1030446, and by Fondo de Áreas Prioritarias (FONDAP), Center for Molecular Studies of the Cell, 15010006 from Chile.

The authors report no conflicts of interest.

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METHODS

This investigation conforms to 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) and was approved by our institutional animal care and use committee.

We followed 2 experimental protocols:

Protocol 1: Effect of Verapamil on the Preconditioning Effect of Exercise on IS

Eighty-six mongrel dogs were instrumented under aseptic surgery. Briefly, under anesthesia with intravenous (IV) pentobarbital (30 mg/kg) and mechanical ventilation of the lungs, the thorax was opened at the fifth left intercostal space, and Silastic catheters were implanted into the aortic root through its wall, into the coronary sinus through the great coronary vein, and into the left atrium through its appendage. An occluder (plastic snare) was implanted around the anterior descending coronary artery immediately distal to the emergence of the first diagonal branch. Epicardial pacing electrodes were sutured in the outflow tract of the right ventricle to

control heart rate. The tubings, wires, and the snare were tunneled out of the thoracic cavity through the subcutaneous tissue, and their proximal ends kept under the skin in the interscapular region. Prophylactic antibiotics and analgesia were provided for a few days after surgery. After 2 weeks of recovery, dogs were trained in the laboratory during 2 additional weeks to allow them to become accustomed to run on a treadmill at a speed of 5–9 km/h for 5 minutes, twice a day. The rest of the day the dogs were kept in comfortable cages at about 22°C with food and water ad libitum. Three dogs died during surgery, and 2 dogs died a few days afterward because of different complications of surgery. Previously, in 10 other dogs, we determined the proper verapamil dose that did not induce significant hemodynamic changes and allowed dogs to run on the treadmill. Therefore, 81 dogs were randomly assigned to the following 6 experimental groups (Fig. 1):

1. Nonpreconditioned dogs (NP, N = 14): Dogs were allowed to rest for 60 minutes in the laboratory and then they were anesthetized (intravenous pentobarbital, 30 mg/kg). The anterior descending coronary artery was occluded with the plastic snare for 1 hour and reperfused for 4.5 hours. To obtain a stable preparation and comparable hemodynamic conditions between groups, aortic pressure changes were damped by connecting the systemic arterial circulation, through the femoral artery, to a large reservoir filled with homologous, heparinized, and constantly stirred blood thermoregulated to 37°C. The height of the reservoir was frequently adjusted to maintain a mean arterial pressure of about 100 mm Hg in the aortic root during the experiments. Heart rate was kept constant at about 150 beats per minute by electrical stimulation. Dogs' rectal temperature was measured with an electric thermometer and maintained at about 37°C with a heather under the surface of the surgical table.

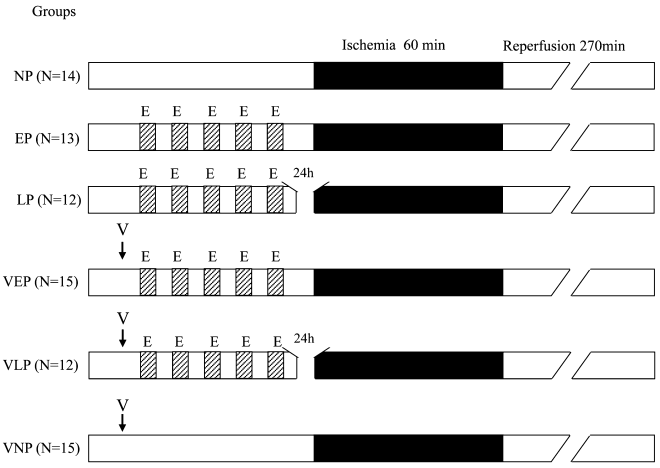

FIGURE 1. Protocol 1: experimental groups to study the effect of exercise and verapamil on IS. E, exercise; EP, dogs with early preconditioning; LP, dogs with late preconditioning; NP, nonpreconditioned dogs; VEP, verapamil before early preconditioning; VLP, verapamil before late preconditioning; VNP, verapamil in nonpreconditioned dogs.

2. EP by exercise (EP, N = 13): Same procedure as in group NP was followed, but dogs were allowed to run on the treadmill for 5 periods, 5 minutes each at 6 km/h with intervening 5-minute periods of rest. After the last exercise period, the animals rested for about 10 minutes to allow the heart rate and the aortic pressure to recover to basal values before inducing pacing at about 150 cycles per minute, connecting the arterial line with the reservoir and inducing the infarction as in group NP.
3. LP by exercise (LP, N = 13): Preconditioning was performed as in group EP, but infarct was induced 24 hours after the exercise.
4. Verapamil before EP (VEP, N = 15): Similar to group EP, but verapamil 0.15 mg/kg IV was administered about 5 minutes before exercise.
5. Verapamil before LP (VLP, N = 12): Similar to group LP, but verapamil 0.15 mg/kg IV was administered 5 minutes before exercise.
6. Verapamil in nonpreconditioned dogs (VNP, N = 15): Similar to group NP, but verapamil was administered at a time equivalent to group VEP. This group was a control for group VEP to determine the effects of verapamil on myocardial ischemia without preconditioning.

Aortic pressure and heart rate were continuously recorded during rest and exercise periods and during ischemia and reperfusion. Myocardial blood flow to the left ventricular wall was measured with the radioactive microsphere technique as previously described[14] during the last period of exercise and after recovery in all preconditioned dogs and before the coronary occlusion in nonpreconditioned dogs. Myocardial O_2 consumption was calculated as the product of myocardial flow times the coronary arteriovenous difference in O_2 content (between aortic root and coronary sinus blood samples). Collateral flow to the ischemic left ventricular wall, 30 minutes into the ischemic period, was also measured with the microspheres technique. The size of the infarction relative to the risk region was measured with the triphenyltetrazolium staining technique.[14] The magnitude of the infarction was expressed by the volume of the necrotic region as percent of the volume of the risk region. The risk region was expressed as percent of the total left ventricular volume.

Protocol 2: Effect of Exercise and Verapamil on NADPH Oxidase Activation

Fifty dogs, surgically instrumented and trained as indicated in the previous protocol, were randomly assigned to the following experimental groups to measure the activity of NADPH oxidase (Fig. 2):

1. Control (C, N = 19): After resting for 1 hour, the dogs were anesthetized (IV pentobarbital 30 mg/kg), the thorax was opened under mechanical ventilation of the lungs, and the heart was excised for analysis of the NADPH oxidase activity.
2. Exercise (E, N = 11): Dogs performed exercise as described above. After the last exercise period, the animals rested for 10 minutes to allow the heart rate and the aortic pressure to return to basal values, then they were

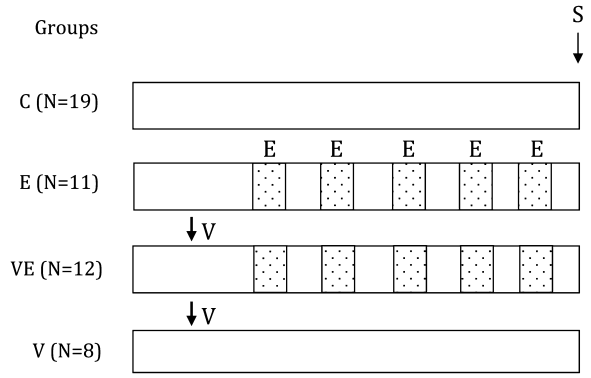

FIGURE 2. Protocol 2: experimental groups to study the effect of exercise and verapamil on NADPH oxidase activation. C, control; E, exercise; S, sampling; V, verapamil without exercise; VE, verapamil before exercise.

anesthetized, the lungs mechanically ventilated, and the heart excised for analysis.

3. Verapamil before exercise (VE, N = 12): Similar to group E, but verapamil, 0.15 mg/kg, was administered 5 minutes before exercise.
4. Verapamil (V, N = 8): As in group C, but verapamil, 0.15 mg/kg, was administered 1 hour before the animals were anesthetized for analysis.

Determination of NADPH Oxidase Activity

Superoxide production was measured by lucigenin chemiluminescence by incubating sarcoplasmic reticulum (SR) vesicles (0.2 mg/mL) in 100 mM MOPS–Tris, pH7.0, 100 μM NADPH, and 5 μM lucigenin at 25°C. Chemiluminescence was measured in a Berthold FB 12 luminometer and expressed as nanomoles of superoxide anion per milligram protein per minute. SR vesicles were isolated from the zone of the ventricular wall perfused by the anterior descending coronary artery as previously described in detail.[15] SR fractions were snap frozen in liquid nitrogen and kept at −80°C. Protein content was determined by the method of Hartree.[16]

Statistical Analysis

Multiple comparisons were performed with analysis of variance followed by Holm *t* test analysis. To discard the effect of collateral flow to the ischemic region from the effect of the maneuvers on the IS, we regressed ISs on collateral flow values and compared these regressions with analysis of covariance (ANCOVA). Results are expressed as mean ± SEM. The null hypothesis was discarded with a P value <0.05.

Criteria for Exclusion

To avoid variability in the IS because of different exposure to ischemia during ischemia/reperfusion (IR) period, we used 2 exclusion criteria, a collateral flow to the ischemic region >0.2 mL·g^{-1}·min^{-1} and >3 consecutive attempts required to convert ventricular fibrillation.

RESULTS

Protocol 1: Effect of Verapamil on the Preconditioning Effect of Exercise on Myocardial IS

Of the 81 dogs randomized to this protocol, 8 were discarded because of the exclusion criteria (2 dogs in groups NP and VLP and 1 dog in groups VEP, LP, EP, and VNP). The results correspond to 73 dogs.

The hemodynamic evolution during baseline, ischemia, and reperfusion is depicted in Table 1. No significant differences were observed across these periods or among the groups. The administration of verapamil, both in preconditioned and in nonpreconditioned dogs, did not induce any hemodynamic change. As there were no differences in the hemodynamic evolution between EP and LP, with and without verapamil, both groups were considered together as 1 group. The regression of ISs, in the different groups, on the values of collateral flow to the ischemic region showed an independence of IS from collateral flow (ANCOVA, $P < 0.02$, not shown). There were no significant differences in myocardial risk size between groups; the risk size/total area expressed as percent were: 48.9 ± 2.2, 47.7 ± 1.8, 46.3 ± 2.4, 49.8 ± 3.8, 50.3 ± 4.5, and 46.1 ± 5.8 for groups NP, EP, LP, VEP, VLP, and VNP, respectively ($P > 0.05$). Table 2 shows the hemodynamic and metabolic effects of exercise in preconditioned dogs (early and late) with and without verapamil. During exercise, there was a significant increase in heart rate (47%), mean aortic pressure (15%), coronary flow (61%), and myocardial O_2 consumption (62%) compared with basal, but no significant changes were observed in the arteriovenous difference of oxygen and lactate content. The electrocardiogram did not show repolarization changes. The administration of verapamil did not modify this response to exercise.

The effects of the different maneuvers on IS are shown in Figure 3. EP decreased IS from 25.3% ± 3.5% to 6.0% ± 2.7% ($P < 0.001$, EP vs. NP) and LP from 25.3% ± 3.5% to 12.1% ± 2.1% ($P < 0.001$, LP vs. NP). Both effects were abolished by the administration of VE ($P > 0.05$ for both). Verapamil by itself (in nonpreconditioned dogs) did not modify IS (from 25.3% ± 3.5% in group NP to 26.1% ± 2.7% in group VNP, $P > 0.05$).

Protocol 2: Effect of Exercise and Verapamil on NADPH Oxidase Activation

Figure 4 shows the effects of EP by exercise and verapamil on NADPH oxidase activation. Exercise increased the activation and verapamil, administered before exercise, reverted it. The drug by itself did not modify the NADPH oxidase activation.

DISCUSSION

Ischemic preconditioning, described in 1986,[17] is a mechanism by which brief periods of ischemia induce protection against subsequent longer ischemic periods. Since then, there has been an increasing understanding of its mechanisms[18–22] and other forms of cardioprotection,[23–27] including exercise preconditioning.[1–3]

TABLE 1. Hemodynamic Effects of Ischemia and Reperfusion

			Ischemia		Reperfusion		
	Maneuvre	Baseline	30 min	60 min	60 min	120 min	180 min
HR, cycles per minute	NP	153 ± 10	150 ± 12	155 ± 11	160 ± 13	158 ± 11	147 ± 9
	P	155 ± 14	160 ± 12	147 ± 10	155 ± 11	150 ± 9	149 ± 10
	VP	158 ± 7	169 ± 4	168 ± 4	167 ± 3	154 ± 6	150 ± 12
	VNP	147 ± 10	154 ± 7	158 ± 4	150 ± 5	150 ± 6	146 ± 6
SAP, mm Hg	NP	105 ± 8	110 ± 10	115 ± 7	109 ± 9	107 ± 11	110 ± 9
	P	100 ± 7	115 ± 8	120 ± 10	117 ± 11	110 ± 10	115 ± 8
	PV	98 ± 5	115 ± 4	111 ± 5	114 ± 4	111 ± 5	109 ± 8
	VNP	101 ± 4	113 ± 5	114 ± 5	110 ± 5	107 ± 4	104 ± 5
DAP, mm Hg	NP	80 ± 3	84 ± 6	85 ± 5	82 ± 5.0	80 ± 5	78 ± 8
	P	79 ± 6	87 ± 9	79 ± 7	83 ± 7.0	80 ± 5	79 ± 6
	VP	70 ± 5	85 ± 3	86 ± 4	84 ± 3	85 ± 5	80 ± 7
	VNP	75 ± 3	82 ± 4	83 ± 4	79 ± 5.0	77 ± 5	76 ± 5

Values are presented as mean ± SEM.

NP, nonpreconditioned; P, preconditioned with exercise (early and late considered together); VP, verapamil followed by preconditioning with exercise; VNP, verapamil in nonpreconditioned dogs.

Exercise Preconditioning on IS Is Triggered by Ca^{2+}

This study confirms our previous results in the sense that exercise induces early and late cardioprotection with a substantial decrease of the myocardial IS induced by coronary occlusion in dogs.[1,3] Furthermore, the results of this study add to the above findings and suggest, for the first time, that this protection by exercise is triggered or mediated by the calcium inflow to the cell, probably through sarcolemma L-type channels, because verapamil completely reversed the preconditioning effect of exercise.

The effect of verapamil cannot be explained by changes in collateral blood flow to the ischemic myocardium as revealed by the ANCOVA analysis nor by differences in risk region volumes between the groups. The absence of changes in the arteriovenous difference of O_2 and lactate contents and of ventricular repolarization changes argue against the occurrence of ischemia during exercise and thereby the possibility that preconditioning was induced by ischemia. The results cannot be attributed either to hemodynamic or myocardial O_2 consumption changes during the experiments because these changes during exercise were of similar magnitude in groups with and without verapamil. This is explained by the lower dose of verapamil we used compared with others studies in a similar canine model (0.15 vs. 0.8 mg/kg, respectively), who found only a slight reduction in mean blood pressure.[28] The dose we used was based on pilot studies to obtain the proper dose not producing a significant hemodynamic effect. Accordingly, we focused on the hypothesis that calcium inflow to the cell, but not the hemodynamic–metabolic changes produced by exercise, mediates the protection afforded by exercise.

Our results seem to disagree with those of Miyawaki et al[6] in Langendorff-perfused rat hearts. They found that only doses of verapamil, associated with a decrease in ventricular + dP/dt during calcium preconditioning, were able to block the protection elicited by calcium, suggesting that the protection was associated with an increase in myocardial activity induced by calcium. However, Sun and Murphy[12] recently found that the inhibition of calcium-sensing receptor abolished the ischemic preconditioning-induced cardioprotection in Langendorff-perfused mice hearts, with no effect on cardiac hemodynamic–contractile functions. These results are in line with ours in the sense that the protection afforded by calcium does not necessarily go through myocardial hemodynamic or metabolic changes.

Although there is a growing evidence for the participation of calcium in diverse kinds of preconditioning, assessed mainly by the administration of Ca^{2+} and the L-type Ca^{2+} channel blocker verapamil,[4–6,29,30] a detrimental effect of calcium during myocardial IR injury is also widely

TABLE 2. Metabolic and Hemodynamic Effects of Exercise

Maneuvre	HR, cycles per minute	MAP, mm Hg	CF, $mL \cdot min^{-1} \cdot g^{-1}$	MVO_2, mL/min per 100 g	AVO_2, mL per 100 mL	AV Lactate, mmol/L
Basal	107.8 ± 5.8	98.1 ± 3.9	0.44 ± 0.14	7.9 ± 2	12.2 ± 3.8	0.17 ± 0.16
E	158.2 ± 4*	113 ± 5.4*	0.71 ± 0.2*	12.8 ± 2.9*	13.1 ± 4.0	0.20 ± 0.11
VE	160 ± 6*	110 ± 8*	0.73 ± 0.15*	12.6 ± 3*	13.5 ± 3.5	0.18 ± 0.1

Values are presented as mean ± SEM.

*$P < 0.05$ versus basal.

AVO_2, coronary arteriovenous difference in oxygen content; AV lactato, coronary arteriovenous difference of lactate; CF, coronary flow; E, exercise; HR, heart rate; MAP, mean aortic pressure; MVO_2, myocardial oxygen consumption; VE, verapamil before exercise.

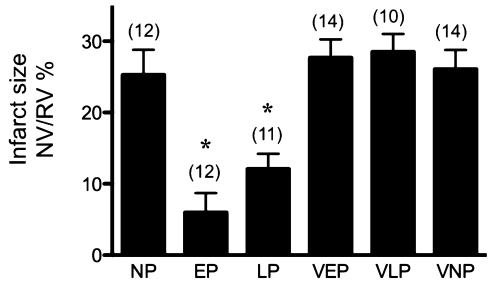

FIGURE 3. IS in the different groups: NP, nonpreconditioned dogs; EP, early preconditioning; LP, late preconditioning; VEP, verapamil before EP; VLP, verapamil before LP; VNP, verapamil in nonpreconditioned dogs; NV, necrotic volume; RV, risk volume. $*P < 0.05$ as compared with control.

recognized,[31–34] and calcium antagonists administered during the IR period have been used for years to limit the myocardial injury.[35–37] In this regard, we did not find a protective effect of verapamil in nonpreconditioned dogs, probably because we used a single low dose administered 1 hour before the indexed ischemia, suggesting that the cellular levels of verapamil during the IR period were not high enough to prevent the detrimental effects of calcium during this period.

We decided to use verapamil because of its cardioselectivity with negative chronotropic and inotropic effects, which were easily controlled by a small dose of the drug and our experimental protocol. This, together with a lower vasodilator effect of the drug and because it is the most studied Ca^{2+} channel blocker in preconditioning studies and the clinics as compared with others blockers.[5,6,36–39]

Exercise Preconditioning and NADPH Oxidase Activation

It is known that NADPH oxidase is a major source of reactive oxygen species (ROS) in cardiac tissue[40] and plays an important role in diverse forms of preconditioning,[41,42] including preconditioning by tachycardia and exercise.[2,43] In this regard, we previously found a loss of early exercise cardioprotection after NADPH oxidase inhibition, suggesting that ROS generated by this enzyme are important mediators of the preconditioning response and that early exercise preconditioning increased the NADPH oxidase activation with an increased membrane association of the regulatory subunits $p47^{phox}$ and rac1, with no change in the relative content of the membrane-bound catalytic subunit $gp91^{phox}$.[2] Frasier et al[43] reported recently that ROS generated by NADPH oxidase triggers the cardioprotection induced by exercise through the increase in glutathione reductase activity. In this study, we found that calcium is probably involved in NADPH oxidase activation because verapamil inhibited this activation simultaneously with the reversion of the EP effect of exercise.

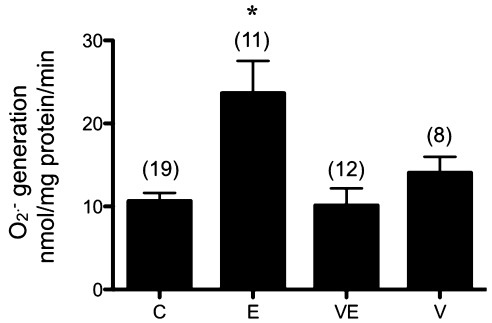

FIGURE 4. NADPH oxidase activity in different groups. C, control; E, only exercise; V, verapamil without exercise; VE, verapamil before exercise. $*P < 0.05$ as compared with control.

Interpretation of the Results

The protective effect afforded by exercise has been explained traditionally by hemodynamic and myocardial metabolic demand changes related to cytosolic calcium increase. We have found that this protective effect can be blocked by calcium inflow decrease despite the maintenance of the hemodynamic effects induced by exercise, suggesting that calcium dynamics mediates the protection in an independent way.

Based on our present and prior results[1–3] plus the antecedent that the calcium preconditioning is abolished by K^+ adenosine triphosphate channel blocker and by verapamil,[44] we believe that the transitory increase in cytosolic Ca^{2+} through the sarcolemma during exercise triggers the cascade for the preconditioning effect of exercise, which would be mediated by the activation of NADPH oxidase, mitochondrial adenosine triphosphate–sensitive K^+ channels, and the inhibition of the mitochondrial permeability transition pore,[45] which has been implicated as an end point in cardiomyocyte death.[46–48]

One of the limitations of our study is that we did not measure calcium entrance to the cell directly, which is needed to confirm our findings. Besides, we assessed the effect of exercise and verapamil on the NADPH oxidase activity in the early phase of preconditioning but not in the late phase, which is needed to a comprehensive understanding of the NADPH oxidase activity in exercise preconditioning. Further research is needed to confirm the precise mechanism by which verapamil blocks the exercise cardioprotection, including the effects of the drug on tissues besides the heart.

Clinical Implications

According to the final goal of myocardial ischemia research,[49–51] preconditioning may be one of the mechanisms by which exercise decreases the damage produced by the acute coronary syndrome.[52,53] The fact that verapamil prevents the exercise cardioprotection may have clinical implications because of the frequent use of L-type Ca^{2+} channel blockers in clinical practice. This presumptive detrimental effect should be balanced with the beneficial effect of Ca^{2+} blockers in patients with coronary artery disease. In summary, our results suggest that the preconditioning effect of exercise on the IS is triggered or mediated by calcium through NADPH oxidase.

ACKNOWLEDGMENTS

We thank Professor Julien I. E. Hoffman (University of California, San Francisco) for his advice and help to this study. The technical assistance of Juan Carlos Fuenzalida,

Guillermo Arce, Rodrigo Durán, and BQ Luis Montecinos is gratefully acknowledged.

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