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Beneficial Effects of Physical Training on the Cardio-Inflammatory Disorder Induced by Lung Ischemia/Reperfusion in Rats

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Abstract—Our laboratory demonstrated that training program attenuated the inflammatory responses in lung ischemia/reperfusion (IR). Considering the importance of the inflammatory responses on the cardiovascular system, we evaluate the effect of physical training on the vascular responsiveness and its underlying mechanism after lung IR. Male Wistar rats were submitted to run training and lung IR. Concentration–response curves for relaxing and contracting agents were obtained. Protein expressions of SOD-1 and $p47^{phox}$, plasma nitritre/nitrate (NO_x^-) and interleukin 6 (IL-6) were evaluated. A decreased in the potency for acetylcholine and phenylephrine associated with an upregulation of the $p47^{phox}$ expression were found after Lung IR as well as an increase in IL-6 and NO_x^- levels. Run training attenuated the vascular dysfunction that was accompanied by reduction of the $p47^{phox}$ protein expression and IL-6 levels. Our findings show the beneficial effect of training on the vascular function that was associated with reduction in inflammatory response in lung IR.

KEY WORDS: physical training; vascular dysfunction; inflammatory responses; lung ischemia/reperfusion.

INTRODUCTION

Surgical procedures such as cardiopulmonary bypass and organ transplantation provoke ischemia/reperfusion (IR) injury leading to an intense systemic inflammatory response and multiple organs failure that have been associated with high mortality rate [1–3]. The genesis of IR-induced deleterious effects has been linked to a massive reactive oxygen species (ROS) production by vascular endothelium and leukocytes [4–6]. In fact, a marked increase in neutrophil infiltration occurs in the first hours following IR process. Furthermore, a positive relationship between high ROS production and severe systemic hypotension in IR injury has been found. This hemodynamic complication can be controlled by pharmacological intervention with inotropic and vasopressive agents [7].

A healthy lifestyle has been strongly associated with regular physical activity, and evidences have shown that physically active subjects have more longevity with reduction of morbidity and mortality. Furthermore, physical exercise prevents or reduces the deleterious effects of pathological conditions such as arterial hypertension, atherosclerosis, diabetes mellitus, and metabolic syndrome [8]. Indeed, a significant reduction in systemic inflammation has been found in clinical and experimental models in response to physical exercise [9]. Recent study from our laboratory demonstrated that an 8-week run training program attenuated the pulmonary vascular permeability reducing lung edema, which was accompanied by decreasing in tumor necrosis factor-alpha (TNF-α) and interleukin 1β (IL-1β) serum levels and increased plasma superoxide dismutase (SOD) activity [10]. Considering that inflammatory mediators released after lung IR procedure have a great impact on the

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cardiovascular system affecting local and remote organ and the potential beneficial effects of the physical exercise on the remote organ after lung IR, we have designed experiments to evaluate whether regular run training prior to lung IR exerts beneficial effect on the vascular functional responses to relaxant as well as to vasoconstrictor agents. The protein expressions of SOD-1and $p47^{phox}$ subunit of NADPH oxidase in the mesenteric artery have also been performed to further elucidating the mechanisms of IR injury in remote tissue and a potential impact of physical exercise on the redox state.

MATERIALS AND METHODS

Animals

This study was approved by the Ethics Committee for Animal Research at the State University of Campinas (UNICAMP). Male Wistar rats, weighing 210–230 g, were obtained from the Animal Care Facility of UNICAMP and were maintained in a room at 20–21°C with normal 12 h light/dark cycle, house in groups of five animals and had free access to water and commercial chow (Purina Co., Campinas-SP, Brazil). Animals were divided into three groups, namely: sham-operated sedentary (SHAM/SD); ischemia/reperfusion sedentary (IR/SD); and ischemia/reperfusion trained (IR/TR). Body weight was measured before and weekly during the experimental protocol.

Training Program, Lung Ischemia/Reperfusion Procedure, and Concentration–Response Curves

Animals were trained in a treadmill for small animals with individual lanes; intensity of run training was determined according to the plasma lactate concentration curves, representing the maximal lactate steady state. The training program consisted in sessions of 60 min/day, 5 days/week for 8 weeks, 0% grade, in a speed of 1.2 km/h. In the beginning of the training program, the duration and speed started at 0.6 km/h for 30 min and were progressively increased until the total volume of training is reached [11]. Only animals adapted to the treadmill were used in the present study.

After 48 h of the last exercise training, rats were anesthetized with urethane (1.2 g/kg, i.p.). The left lung was immobilized atraumatically, and the left pulmonary artery, bronchus, and pulmonary vein were occluded with a noncrushing microvascular clamp for 90 min. Subsequently, the clamp was removed, and the lung was allowed to ventilate and reperfused for 120 min, as previously described [10].

Immediately after lung IR, the animals were killed and superior mesenteric artery was isolated carefully and placed in freshly prepared Krebs solution containing (mM): NaCl, 118: $NaHCO_3$, 25; glucose, 5.6; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$ $7H_2O$, 1.17; and $Cacl_2$ $2H_2O$, 2.5. In the sequence, mesenteric artery was cleaned of all adherent tissue and cut into rings of approximately 2 mm. Each ring was suspended between two wire hooks and mounted in 10 mL organ chambers with Krebs solution at 37°C, pH 7.4, and continuously gassed with 95% O_2 and 5% CO_2 under a resting tension of 10 mN. After 1 h of stabilization period, mesenteric rings with intact or denuded endothelium were precontracted with phenylephrine (PHE, 1 μM) and cumulative concentration–response curves to vasodilator agents: acetylcholine (ACh, 10 nM–10 μM), or sodium nitroprusside (SNP, 100 pM–1 μM) were obtained. The relaxations were plotted as percentages of the contraction induced by PHE. Concentration–response curves were also obtained for the α-adrenergic agonist, PHE (1 nM–10 μM), or thromboxane mimetic 9,11-dideoxy-11α,9α-epoxy methanoprostaglandin (U46619, 1 nM–1 μM) in rings with intact endothelium. The contractions were plotted as percentages of the contraction induced by KCl 80 mM.

All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{MAX}/((1 + (10^c/10^x)^n) + \Phi)$, where E is the effect of above basal; E_{MAX} is the maximum response produced by the agonist; c is the logarithm of the EC_{50}, the concentration of agonist that produces half-maximal response; x is the logarithm of the concentration of agonist; the exponential term, n is a curve-fitting parameter that defines the slope of the concentration response line, and Φ is the response observed in the absence of added agonist. Nonlinear regression analysis to determine the parameters E_{MAX}, log EC_{50}, and n were done using GraphPad Prism (GraphPad Software Inc., San Diego, CA) with the constraint that Φ=0. The responses for each agonist are shown as the mean ± standard error of the mean (SEM) of potency (pEC_{50}) and maximal response (E_{MAX}).

Western Blotting Analysis

In order to evaluate the contribution of SOD-1 and cytosolic NADPH oxidase subunit $p47^{phox}$ in response to lung IR and exercise training, expression of these proteins

were determined by Western blotting assays in mesenteric artery. Frozen segments of mesenteric arteries were homogenized in a lysing buffer containing 40 mM HEPES, 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4, and 1 mM phenylmethylsulphonyl fluoride. The tissue lysate was centrifuged at 10,000×*g* and the supernatant collected. The protein concentration was determined (Pierce BCA Protein Assay kit, Rockford-IL, USA).

Proteins from homogenized mesenteric arteries for $p47^{phox}$ (50 μg) and for SOD-1 (35 μg) were eletrophoretically separated by 7.5% or 12% SDS–PAGE, respectively. The proteins were subsequently transferred to polyvinylidene difluoride membranes overnight at 4°C by using a Mini Trans-Blot Cell System (Bio-Rad, Hercules, CA, USA) containing 25 mM Tris, 190 mM glycine, 20% methanol, and 0.05% SDS. After blockade of nonspecific sites in Tris-buffered solution (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) with 5% nonfat dry milk, membranes were incubated overnight at 4°C with the primary antibody with a mouse monoclonal antibody for SOD-1 (1:1000, Sigma–Aldrich CO, Saint Louis, MO, USA) or rabbit polyclonal antibody for $p47^{phox}$ (1:2000, Upstate, New York, Y, USA). After being washed (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20), membranes were incubated with anti-mouse 1:1500 dilution (BioRad, Hercules, CA, USA) or anti-rabbit 1:4,000 dilution (KPL, Gaithersburg, MD, USA) IgG antibody conjugated to horseradish peroxidase [12]. The membranes were thoroughly washed, and immunocomplexes were detected using an enhanced horseradish peroxidase-luminol chemiluminescent system (ECL Plus Amersham, Piscataway, NJ, USA) and subjected to autoradiography (Hyperfilm Amersham, Piscataway, NJ, USA). Scanning densitometry was used to quantify the immunoblot signals using Scion Image software. The same membrane was used to determine α-actin protein expression as an internal control using a monoclonal antibody anti-α-actin (1:30,000 dilution, Sigma–Aldrich, Saint Louis, MO, USA), and its content was used to normalize SOD-1 and $p47^{phox}$ protein expression in each sample.

NO_x^- and IL-6

Immediately after lung IR, arterial blood was collected, and the samples were centrifuged (3,500 rpm) for 15 min and the resulting plasma supernatant was stored at −80°C. For the concentration of NO_x^- (μM), plasma samples were ultra filtrated through microfilter cups (Microcon Centrifugal Filter Units, 10 kDa; Millipore, Bedford, MA, USA), and the resulting of filtrated solution was determined using ELISA commercially available kit (Cayman Chemical, Ann Arbor, MI, USA). Plasma IL-6 (pg/ml) levels were determined by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Since the artificial ventilation itself affects basal levels of plasma NO_x^- and IL-6, parallel experiments were also carried out using non-ventilated sedentary and trained rats, and this level was taken as the baseline and delta values were calculated.

Statistical Analysis

Data are expressed as mean ± SEM of *n* experiments. ANOVA followed by Tukey post-test was chosen for statistical analysis. A *p* value smaller than 0.05 was considered statistically significant.

RESULTS

The values of the body weight at initial time were similar in all groups (SHAM/SD: 221±7; IR/SD 222±6 and IR/TR: 218±6). After 8 weeks of run training, the values of the body weight were significantly lower in IR/TR (357±6, approximately 12% of reduction) as compared to sedentary groups (SHAM/SD: 412±5 and IR/SD: 410±8).

ACh (10 nM–10 μM) and SNP (100 pM–1 μM) produced a concentration-dependent relaxing response in isolated mesenteric rings (Fig. 1a, b, respectively). The pEC_{50} values for ACh were significantly decreased in IR/SD group as compared to SHAM/SD and IR/TR, approximately 1.6-fold, $p<0.05$ (Table 1). Removal of endothelium completely abolished the relaxation response for ACh in all groups (data not shown). No changes were seen in the E_{MAX} for ACh in all studied groups (Fig. 1a). Neither pEC_{50} nor E_{MAX} values for SNP were altered in mesenteric rings in all groups (Table 1 and Fig. 1b).

PHE (1 nM–10 μM) and U46619 (1 nM–1 μM) produced concentration-dependent contractile responses in isolated mesenteric rings in all groups (Fig. 1c, d, respectively). A rightward shift in the concentration–response curves were seen for PHE at the pEC_{50} level in isolated mesenteric rings from IR/SD as compared to SHAM/SD and IR/TR groups, approximately 2.0-fold,

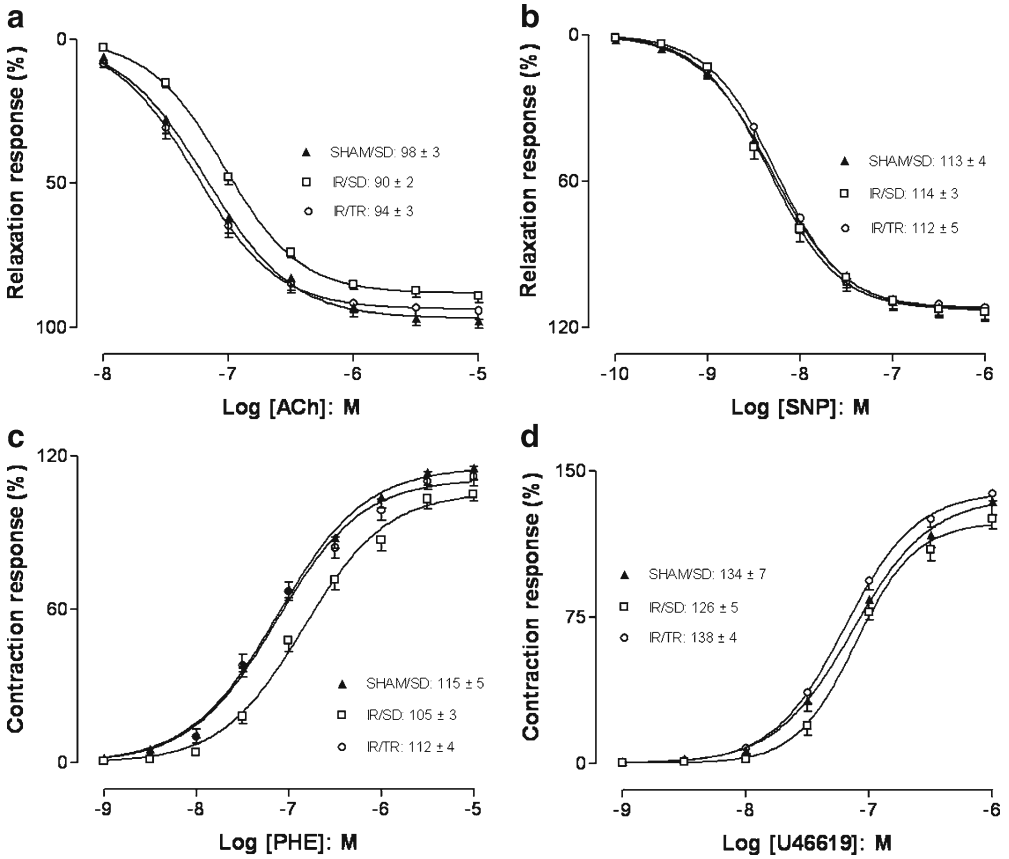

Fig. 1. Concentration-response curves to ACh (**a**), SNP (**b**), PHE (**c**), and U46619 (**d**) in mesenteric arteries with intact endothelium from: sham-operated sedentary (*filled triangles* SHAM/SD), ischemia/reperfusion sedentary (*unfilled squares* IR/SD) and ischemia/reperfusion trained (*unfilled circles* IR/TR). Maximal responses values were inserted in the figure. Data are mean ± SEM for n=5–7 animals per group.

$p<0.05$ (Table 1). No change was observed in the E_{MAX} values for this α-agonist (Fig. 1c). Neither the pEC_{50} nor the E_{MAX} values for U46619 were modified in all studied groups (Table 1 and Fig. 1d).

The quantification of SOD-1 protein expression in mesenteric artery showed that neither lung IR nor exercise training modified the expression of this antioxidant enzyme (Fig. 2a). On the other hand, the quantification of NADPH oxidase component $p47^{phox}$ was significantly increased in IR/SD group, approximately 70% (Fig. 2b). This increased expression of NADPH oxidase component $p47^{phox}$ seen in sedentary IR rats was attenuated by the exercise training (Fig. 2b).

The plasma concentration of the NO_x^- was increased in lung IR groups compared to SHAM/SD (Fig. 3a). Similar, plasma IL-6 levels were increased in

Table 1. Potency Values (pEC_{50}) Obtained from Concentration–Response Curves to ACh (10 nM–10 μM), SNP (100 pM–1 μM), PHE (1 nM–10 μM), and U46619 (1 nM–1 μM) in Mesenteric Arteries with Intact Endothelium from Sham-Operated Sedentary, Ischemia/Reperfusion Sedentary, and Ischemia/Reperfusion Trained Rats

GROUPS	ACh	SNP	PHE	U46619
SHAM/SD	7.22±0.05	8.29±0.02	7.12±0.04	7.14±0.07
IR/SD	7.03±0.04*	8.32±0.07	6.84±0.05*	7.11±0.03
IR/TR	7.27±0.05	8.27±0.06	7.14±0.06	7.20±0.04

Potency is represented as –log of the molar concentration to produce 50% of the maximal relaxation response. Data are mean ± SEM for 5–7 animals per group

*$p<0.05$ compared to SHAM/SD and IR/TR

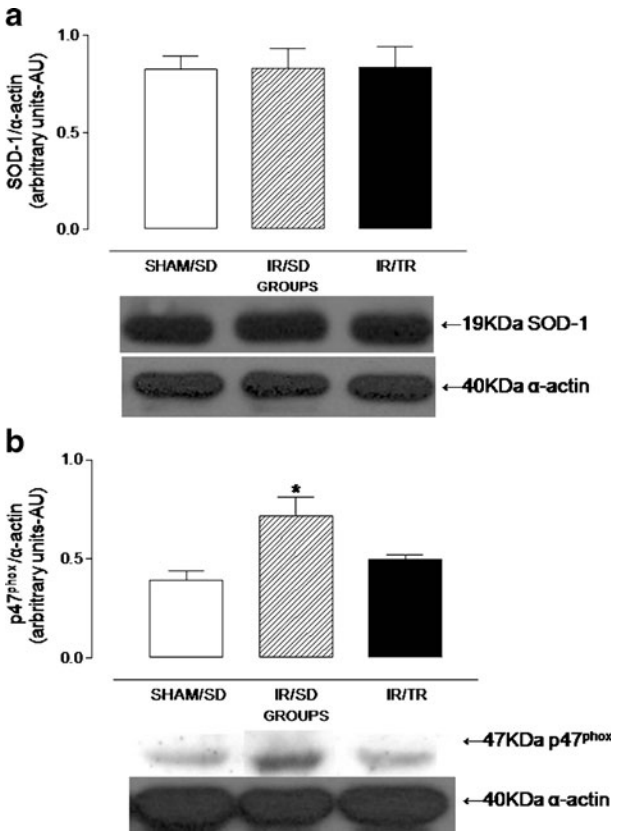

Fig. 2. Effects of lung ischemia/reperfusion and exercise training on the protein expressions of SOD-1 and $p47^{phox}$ subunit of NADPH oxidase from isolated rat mesenteric artery. *Bottom panel* representative Western Blot and *top panel* quantitative analysis for SOD-1 (**a**) and $p47^{phox}$ (**b**) protein expression. Data is normalized to the expression of α-actin. Data are means ± SEM of n=5–7 animals per group. *SHAM/SD* sham-operated sedentary, *IR/SD* ischemia/reperfusion sedentary, *IR/TR* ischemia/reperfusion trained. *p<0.05 compared to SHAM/SD and IR/TR.

IR sedentary animals as compared with SHAM/SD whereas exercise training attenuated this increment in IL-6 levels, approximately 35% (Fig. 3b).

DISCUSSION

We have previously demonstrated that IL-1β and TNF-α serum levels are markedly increased after lung IR model and exercise training attenuated this systemic inflammatory responses [10]. In the present work, we extended this study showing that lung IR provokes a vascular dysfunction that was positively associated with an upregulation of protein expression NADPH oxidase component $p47^{phox}$ in mesenteric tissue as well as increased IL-6 levels. On the other hand, exercise training performed previously to lung IR restores vascular responses and attenuated the inflammatory response in this experimental model.

A variety of surgical procedures including angioplasty, organ transplantation, aneurysm repair and cardiopulmonary bypass may result in ischemia/reperfusion leading to severe cellular damages and multiple failure organs. These complications following IR injury are attributed to a systemic inflammatory response with a massive production of ROS resulting in unbalance in redox state [13]. Additionally, the oxidant enzyme NADPH oxidase is the major sources of free radicals in pathological conditions, and it is believed that excessive production of superoxide (O_2^-) by NADPH oxidase leads to formation of peroxynitrite ($ONOO^-$) by the reaction with NO which in turn reduces NO bioavailability producing important functional alterations in the cardiovascular system [14, 15]. In our study, we clearly show that lung IR provokes a vascular dysfunction seen by

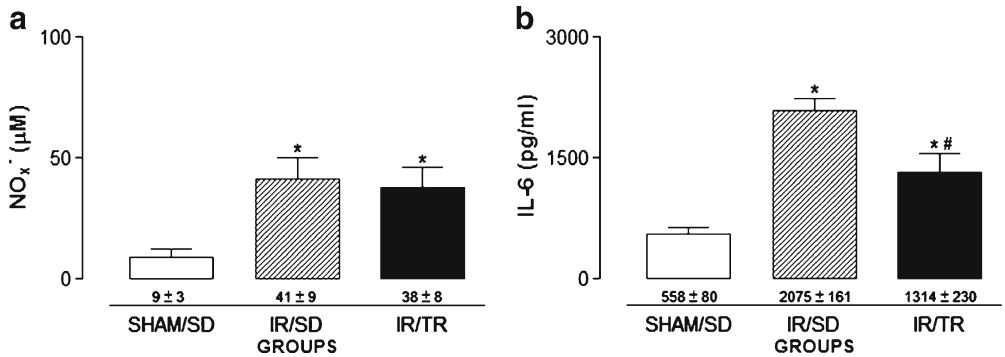

Fig. 3. NO_x^- (**a**) and IL-6 (**b**) concentrations in SHAM/SD, IR/SD, and IR/TR rats. Data are mean ± SEM for n=5–7 animals per group. *p<0.05 compared to SHAM/SD. $^{\#}p$<0.05 compared to IR/SD.

a rightward displacement of the concentration–response curves to endothelium-dependent agonist. Analyzing the mechanistic implications of this vascular alterations, we have found that protein expression of the NADPH oxidase component $p47^{phox}$ of the mesenteric artery was upregulated that could reduce NO bioavailability to the vascular smooth muscle. In fact, a previous study demonstrated that myocardial IR increased the activity of NADPH oxidase in coronary artery that was associated with endothelial dysfunction [16].

The lack of changing in the relaxing responses to SNP confirms our data indicating that lung IR does not alter the mesenteric smooth muscle relaxing property. Accordingly, previous studies have shown no alterations in the reactivity of vascular smooth muscle for SNP after IR process [17, 18].

The role of IL-6 in the cardiovascular system still not fully understood. Previous studies found increased levels of IL-6 expression in rejected lung transplantation patients [19] and in lung IR [20]. Recently, a study linked the high IL-6 plasma levels with cardiopulmonary damage following reperfusion [21]. Additionally, an elevated plasma IL-6 level was associated with the genesis of the peripheral arterial disease [22]. In our study, we found an increase of this cytokine level that was positively associated with upregulation of the protein expression of the NADPH oxidase component $p47^{phox}$ and vascular dysfunction. Recently, it was reported that high levels of plasma cytokines released in inflammatory states play an important role in the oxidant system by activation the enzyme NADPH oxidase [23]. Therefore, we hypothesized that increased plasma cytokines levels in our lung IR model might be the primary causes of the upregulation of NADPH oxidase component $p47^{phox}$ in mesenteric tissue provoking an impairment of the endothelium-dependent relaxing responses.

The health-promoting effect of regular physical activity has been linked to an increase of NO production and/or increase of the antioxidant defenses decreasing NO inactivation and consequently increasing NO bioavailability to the vascular smooth muscle [24–26]. Our study showed that physical training performed before lung IR was effective in preventing the rightward shift seen in the concentration-response curves to ACh that was not associated with increasing in NO production since we have not observed any differences in NO_x^- levels between IR/SD and IR/TR groups. The elevated concentration of NO_x^- likely is related to activation of inducible nitric oxide synthase in response to the systemic inflammation induced by lung IR. On the other hand, the reduction in the protein expression of NADPH oxidase component $p47^{phox}$ could decrease NO inactivation leading to improvement of the endothelium-dependent response by increasing NO bioavailability in mesenteric artery rings. Recently, we have demonstrated that regular physical training restores vascular dysfunction in isolated rat corpus cavernosum by reduction of NADPH oxidase component $gp91^{phox}$ in hypertensive rats showing the beneficial effect of physical exercise on the oxidative stress induced by certain pathological conditions [27]. Thus, our findings show that exercise training performed previously to lung IR is an important approach to prevent the deleterious effects of the cardiopulmonary surgeries on the cardiovascular system.

Regarding the contractile responses, our data show that the rightward shift to PHE seen in IR sedentary rats could be due to a desensitization of the contractile response mediated by α-adrenergic receptors as consequence of elevated sympathetic activity in response to lung IR. Our hypothesis of the α-adrenergic receptors desensitization in mesenteric rings is reinforced by the lack of alterations in the contractile response found for the thromboxane A_2 analog U46619 that is mediated by different receptor population. Additionally, it is well known that an over-stimulation of adrenergic receptors by catecholamines leads to a desensitization phenomenon [28, 29]. Accordingly, previous study has shown that catecholamine level is elevated after IR process [30, 31].

In conclusion, our present study showed the beneficial effect of aerobic physical exercise training on the cardio-inflammatory disease, preventing the upregulation of the NADPH oxidase component $p47^{phox}$ protein expression and the increased plasma IL-6 in lung IR. Therefore, physical training might be an additional approach in preoperative preparation to improve patient rehabilitation after cardiopulmonary surgeries which in turn could diminish the time of the hospitalization.

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