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Exercise training reduces pulmonary ischaemia–reperfusion-induced inflammatory responses

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ABSTRACT: Physical exercise reduces the deleterious effects of cardiovascular and inflammatory disorders. The purpose of the present study was to evaluate the beneficial effects of physical training on the inflammatory responses following lung ischaemia–reperfusion (IR) in rats.

Male Wistar rats were divided into sham-operated animals and sedentary and trained animals submitted to lung IR. The run training programme consisted of 5 sessions·week^{-1}, each lasting 60 min·day^{-1}, at 66% of maximal oxygen consumption for 8 weeks. The left pulmonary artery, bronchus and pulmonary vein were occluded for 90 min and reperfused for 2 h. Lung protein extravasation was measured as ^{125}I-human albumin accumulation, whereas lung neutrophil infiltration was measured as myeloperoxidase activity.

Lung IR in sedentary rats resulted in marked increases in protein extravasation and neutrophil influx, and in significant elevations of serum tumour necrosis factor (TNF)-α and interleukin (IL)-1β levels. Physical preconditioning attenuated the increased IR-induced protein leakage without affecting neutrophil influx. It also reduced serum TNF-α (and IL-1β) levels, but had no effect on IL-10 levels. Plasma superoxide dismutase activity was significantly increased in trained IR rats.

The present data show that physical preconditioning protects the rat lung from ischaemia–reperfusion injury by attenuating the pulmonary vascular permeability that may be a consequence of reduced levels of tumour necrosis factor-α and interleukin-1β and elevated superoxide dismutase activity.

KEYWORDS: Exercise training, inflammation, ischaemia–reperfusion, lung

A healthy lifestyle has been strongly associated with the practice of regular physical activity. Evidence has shown that physically active subjects exhibit more longevity, with a reduction in morbidity and mortality. Physical exercise prevents or reduces the deleterious effects of pathological conditions, such as arterial hypertension, coronary artery disease, atherosclerosis, diabetes mellitus, osteoporosis, and Parkinson's and Alzheimer's disease [1, 2]. Aerobic physical training of moderate intensity has also been recognised to improve cardiorespiratory function as a consequence of blood pressure and cardiac frequency reduction, improvement in lipid profile, increase in maximum oxygen consumption and upregulation of oxidative enzymes [3]. Furthermore, recent evidence shows that regular exercise exerts protective effects against diseases associated with systemic inflammation [4].

Acute respiratory distress syndrome (ARDS) is defined as a clinical disorder associated with a systemic inflammatory response and multiple organ failure, showing a high mortality rate (40–60%) strongly associated with lung injury [5, 6]. It is well known that ischaemia–reperfusion (IR) causes tissue damage and cellular dysfunction in local and remote regions, leading to ARDS and multiple organ failure [7]. Tissue injury by IR processes and other surgical conditions involving acute lung injury have been associated with microvessel endothelial injury, oedema formation and neutrophil infiltration, as a consequence of the release of systemic inflammatory mediators, including cytokines, lipid-derived mediators and reactive oxygen species (ROS) [8]. Therapeutic strategies for treating ARDS involve mechanical ventilation at low tidal volumes, which may be associated with the use of antioxidant agents and/or ischaemic preconditioning [7, 9]. Although the beneficial effects of exercise training on the cardiovascular system are well described, no studies exist investigating the influence of prior physical training on the inflammatory responses induced by lung IR.

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Therefore, the hypothesis that, besides improving chronic pathological conditions, exercise training also exerts protective effects against acute lung injury after lung IR in rats was tested. This was achieved by measuring plasma protein leakage and neutrophil influx into the pulmonary tissue, as well as circulating levels of tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-10, and plasma superoxide dismutase (SOD) activity.

METHODS

Animals

The present study was approved by the Animal Ethics Committee of the State University of Campinas (Campinas, Brazil), and followed the *Guide for the Care and Use of Laboratory Animals* [10]. Male Wistar rats were divided into three groups, namely false-operated (sham) animals and sedentary and trained animals submitted to lung IR.

Training programme

The trained animals underwent an 8-week run training programme, prior to lung IR injury, in a treadmill for small animals with individual lanes at a speed of 1.2 $km \cdot h^{-1}$, 0% grade, for 5 $sessions \cdot week^{-1}$, each lasting 60 $min \cdot day^{-1}$. This velocity was determined according to plasma lactate concentration curves, representing 66.6% of maximal oxygen consumption, in agreement with established training programme standards [11]. Only the animals adapted to the treadmill were used in the present study.

IR procedure

The rats were anaesthetised with pentobarbital (50 $mg \cdot kg$ body $weight^{-1}$) 48 h after the last exercise training session, and the femoral vein was cannulated in order to maintain the level of anaesthesia during the IR process. After tracheostomy, animals were artificially ventilated with a standardised inspired oxygen content of 66% at a frequency of 80 $breaths \cdot min^{-1}$ and positive end-expiratory pressure of 2 cmH_2O. Maximal peak pressures were maintained at <10 cmH_2O. Atropine (0.4 mg intramuscularly) and heparin (50 IU) were given to the animals in saline solution (total volume 500 μL). A heating blanket was used to keep the body temperature at 37 ± 0.8°C, monitored *via* a rectal thermometer. After thoracotomy, the left lung was immobilised atraumatically, and the left pulmonary artery, bronchus and pulmonary vein were occluded using a non-crushing microvascular clamp, maintaining the lung in a partially inflated state for 90 min. Lungs were kept moist with periodic applications of warm saline, and the incision was covered to minimise evaporative losses. Subsequently, the clamp was removed and the lung allowed to ventilate and reperfuse for 2 h. The heart–lung block was excised and the pulmonary circulation flushed through the main pulmonary artery with 20 mL saline, after which the lungs were separated from mediastinal tissues [12]. Lung IR-induced inflammation was evaluated by measurement of both pulmonary oedema formation (plasma protein extravasation) and neutrophil infiltration (myeloperoxidase (MPO) activity) into the right lung tissue, as well as serum levels of TNF-α, IL-1β and IL-10. Measurement of circulating cytokine levels and the contralateral (right) lung were made in order to exclude the direct mechanical effects of the left lungs.

Pulmonary plasma protein extravasation

Pulmonary plasma protein extravasation was measured by means of accumulation of intravenously injected ^{125}I-human serum albumin (92.5 $kBq \cdot rat^{-1}$). A blood sample was collected from the abdominal aorta and centrifuged for 10 min at $8,000 \times g$ to obtain a plasma sample. The right lung and plasma samples (0.1 mL) were monitored for radioactivity (gamma counter). Plasma extravasation was expressed as the volume (in microlitres) of plasma accumulated in the lung derived from the total count in 1 mL plasma.

Lung MPO activity

The right lung was removed and placed in a test tube in the presence of 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer (pH 6.0). The tissue sample was homogenised and centrifuged for 2 min at $12,000 \times g$ and the supernatant collected. An MPO assay was performed using a microplate spectrophotometer (Spectra Max 34; Molecular Devices, Sunnyvale, CA, USA). Briefly, the assay consisted of mixing a 5 μL sample with 200 μL *o*-dianisidine solution (0.167 $mg \cdot mL^{-1}$ *o*-dianisidine dihydrochoride; 0.0005% hydrogen peroxide) prior to reading the plate. The changes in absorbance were measured at 460 nm for 15 s over a total period of 5 min. MPO activity was expressed as units of enzyme activity per milligram of tissue. One unit of MPO was defined as that degrading one micromole of peroxide per minute at 25°C.

Determination of serum TNF-α and IL-1β and IL-10 levels

Serum levels of TNF-α, IL-1β and IL-10 were measured using commercially available ELISAs according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA).

Plasma SOD activity

Measurement of plasma SOD activity was carried out in sham and in sedentary and trained IR rats. Since the artificial ventilation (66% oxygen; 80 $breaths \cdot min^{-1}$) itself affects basal levels of plasma SOD activity, parallel experiments were also carried out using nonventilated sedentary and trained rats, and this was taken as the baseline. Briefly, arterial blood was collected and centrifuged (for 10 min at $8,000 \times g$), and the plasma supernatant was ultrafiltered through microfilter cups (Microcon Centrifugal Filter Units, 10 kDa; Millipore, Bedford, MA, USA). The white buffy layer was removed and discarded. Erythrocytes were lysed in four times their volume of ice-cold HPLC-grade water and centrifuged for 15 min at $10,000 \times g$ at 4°C. The supernatant was collected and kept on ice for assay. Samples, standards, radical detector and xanthine oxidase were prepared and the assay performed as described in commercial kits (Superoxide Dismutase Assay Kit; Cayman Chemical Co., Ann Arbor, MI, USA). The assays were performed in duplicate using various sample dilutions.

Statistical analysis

Data are presented as mean ± SEM, and were analysed by ANOVA followed by Bonferroni's modified t-test. A p-value of <0.05 was considered significant.

RESULTS

Body weight

Initial body weight was similar in all groups (200 ± 20 g). After 8 weeks of run training, body weight was 14% lower in the

trained groups (355±5 g; $p<0.05$) than in the sedentary group (415±17 g).

Peripheral leukocyte counts

The number of mononuclear and polymorphonuclear cells in peripheral blood was evaluated in both the trained and sedentary groups prior to performing lung IR protocols. The present data show that the number of mononuclear and polymorphonuclear cells in trained rats ($86\pm3\times10^6$ and $14\pm3\times10^6$ cells·mL^{-1}, respectively) did not change significantly in comparison with sedentary rats ($82\pm4\times10^6$ and $18\pm4\times10^6$ cells·mL^{-1}, respectively).

Effect of exercise training on IR-induced lung plasma protein leakage and neutrophil infiltration

The lung IR protocols caused a marked increase ($p<0.05$) in vascular permeability in the right lung of sedentary animals compared with the sham group. Physical preconditioning significantly attenuated ($p<0.05$) this increased vascular permeability (fig. 1a).

Measurement of right lung MPO activity was carried out in order to evaluate neutrophil infiltration into lung tissue. Figure 1b shows that lung IR caused a significant increase in MPO activity compared with the sham group that remained unaltered in trained rats.

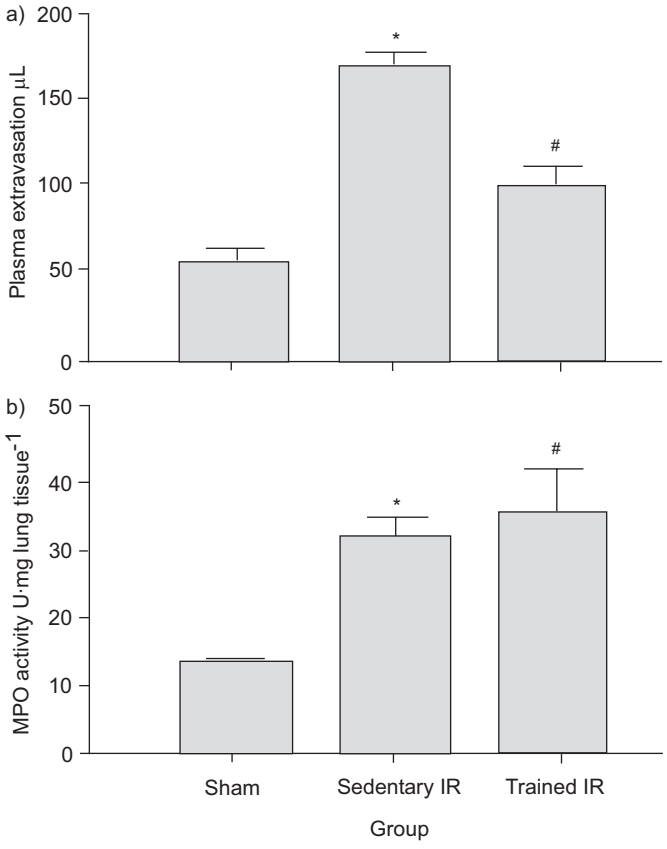

FIGURE 1. Effect of exercise training on induction by lung ischaemia–reperfusion (IR) of a) protein plasma extravasation and b) myeloperoxidase (MPO) activity. Protein plasma extravasation was measured *via* accumulation of ^{125}I-labelled human serum albumin in right lung tissue. Neutrophil content was estimated from MPO activity in right lung tissue. Data are presented as mean±SEM (n=5 for sham group; n=9 for IR groups). *: $p<0.05$ *versus* sham group; #: $p<0.05$ *versus* sedentary IR group.

Effect of exercise training on serum cytokine levels

The lung IR protocols resulted in increased serum TNF-α and IL-1β levels in the sedentary compared with the sham group, which were significantly attenuated by the run training programme (table 1). Conversely, serum levels of IL-10 were unaltered in all groups.

Effect of exercise training on plasma SOD activity

Figure 2 shows that plasma SOD activity in sedentary IR animals did not change significantly compared with the sham group (n=7). However, plasma SOD activity was significantly higher ($p<0.05$) in trained IR animals compared with both the sedentary IR and sham groups (n=6–8).

DISCUSSION

The present study was designed to evaluate the influence of physical preconditioning by run training on the pulmonary oedema formation and neutrophil influx caused by lung IR in rats, as well as on circulating levels of TNF-α, IL-1β and IL-10 and SOD activity. The present data show that prior physical training protects the rat lung from IR injury by attenuation of inflammatory oedema formation, accompanied by reductions in serum levels of TNF-α and IL-1β and increases in plasma SOD activity.

A variety of cellular and molecular events have been reported to mediate the inflammatory process in response to IR injury, including endothelial dysfunction, inflammatory oedema and leukocyte infiltration [5, 13]. Cytokine networks between alveolar–capillary cell membranes are important to the initiation and propagation of the inflammatory response that culminates in pulmonary injury. The cytokines TNF-α, IL-1β and IL-10, produced by alveolar macrophages, have shown to play critical roles in lung injury [8]. Accordingly, in the present study, using a model of normothermic lung IR, a marked increase was observed in plasma protein extravasation and neutrophil infiltration into right lung tissue, along with elevated levels of circulating TNF-α and IL-1β.

It is well established that regular physical exercise is effective in the prevention and treatment of cardiovascular diseases and other pathological conditions in both humans and animals [1, 4, 14, 15]. However, no studies have investigated the protective effects of physical exercise on lung IR injury. The present data

TABLE 1 Serum cytokine levels in rats submitted to pulmonary ischaemia–reperfusion (IR)

	IL-1β pg·mL^{-1}	TNF-α pg·mL^{-1}	IL-10 pg·mL^{-1}
Subjects n	5	9	9
Sham	26.9±0.8	3.8±1.6	568.0±51.5
Sedentary IR	38.2±2.0*	19.9±3.1*	567.3±123.3
Trained IR	29.6±2.0	9.1±2.8**	466.0±72.2

Data are presented as n or mean±SEM. IL: interleukin; TNF: tumour necrosis factor. *: $p<0.05$ *versus* sham group; **: $p<0.05$ *versus* sedentary IR group.

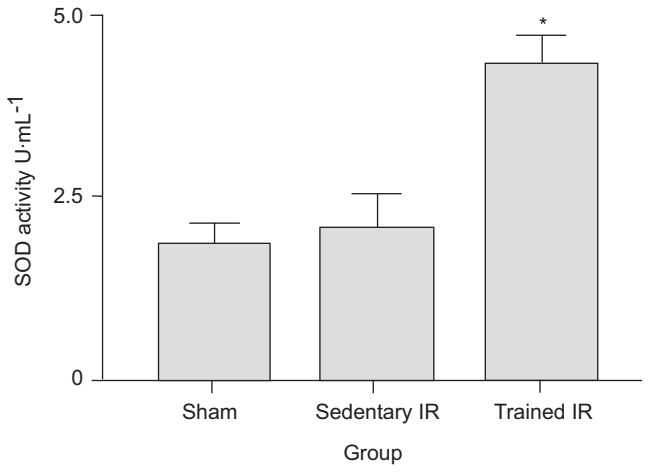

FIGURE 2. Effect of exercise training on induction by lung ischaemia–reperfusion (IR) of plasma superoxide dismutase (SOD) activity. Data are presented as mean±SEM (n=6–8). *: $p<0.05$ *versus* sham and sedentary IR groups.

clearly show that prior physical training exerted a beneficial effect in remote lung IR injury in terms of significant attenuation of the resulting inflammatory oedema (as assessed by measurement of pulmonary plasma protein leakage), accompanied by significant reductions in serum levels of TNF-α and IL-1β, without significantly affecting IL-10 levels. Both TNF-α and IL-1β are known to play important roles in the regulation of microvascular endothelial permeability [16]. In contrast, the cytokine IL-10 has been shown to reduce levels and expression of TNF-α, thus leading to clinical protection and reduction of lung pathologies, including lung IR injury [17]. In the present study, the elevations in TNF-α and IL-1β level were not accompanied by concomitant elevations in IL-10 levels, indicating that IR lung injury does not undergo downregulation by this cytokine under the present particular experimental conditions of lung IR.

It is known that ROS produced by vascular endothelium play important roles in IR injury, particularly in triggering endothelial damage and inflammatory oedema [18]. Additionally, signal transduction triggered by TNF-α involves increase in intracellular ROS in endothelial cells [19, 20]. Taking into consideration the fact that physical exercise at moderate intensity promotes upregulation of antioxidant enzyme expression in both humans and laboratory animals [21–27], it was hypothesised that beneficial effects of prior physical training in the present experimental model might reflect upregulation of antioxidant enzyme expression and hence lower ROS levels secondary to the reduced TNF-α production. Accordingly, the present data show that plasma SOD activity is significantly higher in trained compared with sedentary IR animals. It is likely, therefore, that increased SOD activity leads to lower superoxide anion levels and hence greater nitric oxide bioavailability in trained rats. An increase in SOD expression, of ~30%, has also been associated with improvement in the relaxation response to acetylcholine after a run training programme in isolated rat aorta [28]. It is of interest that recent findings showed an increase in the endothelium-dependent relaxation response to acetylcholine after physical preconditioning in mesenteric arteries from lung IR injury rats [29].

However, the failure of the prior physical training programme to affect lung IR-induced neutrophil influx (as assessed by measurement of MPO activity) is intriguing, especially since TNF-α plays critical roles in the regulation of neutrophil influx following lung IR injury [6, 30]. Polymorphonuclear neutrophils are know to release toxic oxygen metabolites, such as superoxide anion, hydroxyl radical and hydrogen peroxide, all of which can produce cellular oxidative damage in pulmonary endothelial and parenchymal cells [31]. Considering that the late (but not the early) phase of reperfusion is neutrophil-dependent [12], one possible explanation might be that the prior physical training at moderate intensity was able to overcome the increased ROS production required to generate the inflammatory oedema during early phases, but not the neutrophil influx during later phases. Thus additional studies investigating the influence of preconditioning on lung injury after varying time periods of IR are required.

In conclusion, the present study is the first to show that physical preconditioning reduces lung oedema formation after pulmonary ischaemia–reperfusion in the rat. Thus it is likely that subjects undertaking regular aerobic physical exercise have a better prognosis when submitted to nonsurgical or surgical procedures involving acute lung inflammation.

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