Exercise preconditioning alleviates cardiopulmonary damage during endotoxemia

Dong-Zi Shao[a], Che-Ning Chang[b], Yu-Chung Chen[c], Yu-Wen Chen[d,1], Ching-Hsia Hung[b,*,1]

[a]Department of Cosmetics Application and Management, Chung Hwa University of Medical Technology, Tainan, Taiwan;

[b]Department of Physical Therapy, National Cheng Kung University, Tainan, Taiwan;

[c]Division of Physical Therapy, Department of Physical Medicine and Rehabilitation, Cheng Hsin Rehabilitation Medical Center, Taipei, Taiwan;

[d]Department of Physical Therapy & Graduate Institute of Rehabilitation Science, China Medical University, Taichung, Taiwan

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Address reprint requests to: C. H. Hung, Department of Physical Therapy, National Cheng Kung University, No.1 Ta-Hsueh Road, Tainan 701, Taiwan. Tel.: +886-6-2353535 ext 5939. Fax: +886-6-2370411

E-mail Address: chhung@mail.ncku.edu.tw

[1] Drs. Ching-Hsia Hung and Yu-Wen Chen contributed equally to this work.

Abstract

AIM: During endotoxemia, inflammatory responses were induced and results in cardiopulmonary dysfunction. However, heat shock protein 72 (HSP72) which is found after exercise can protect the cardiopulmonary system from lethal damage. Therefore, our hypothesis was that exercise-trained rats could increase the expression of HSP72 in heart and lung to attenuate cardiopulmonary damage during endotoxemia.

METHODS: Male Wistar rats were separated into sedentary and exercise groups. The exercise group was trained to run on the treadmill 30-60 minutes/day at 1.0 mile/hr, 5 days/week for 3 weeks. Twenty-four hours after the last training session, we compared the temporal profiles of hemodynamic parameters in urethane-anesthetized rats receiving an injection of lipopolysaccharide (LPS). We also determined arterial blood gas, lung injury, tumor necrosis factor-alpha (TNF-α) level in serum and bronchoalveolar lavage. In addition, HSP72 expression in the heart and lung were determined.

RESULTS: We found a significant increase in all hemodynamic data, decreased serum and lavage TNF-α level, and reduced lung edema in the exercise group compared to the non-exercise group during endotoxemia. HSP72 expression in heart and lung was significantly increased in exercised rats.

CONCLUSION: Exercise training can attenuate the occurrence of inflammatory responses and alleviate cardiopulmonary damage to increase survival rate during endotoxemia.

KEYWORDS: lipopolysaccharide, hemodynamic, lung injury, HSP72, tumor necrosis factor-alpha

Abbreviations:

CI = cardiac index

CO = cardiac output

HR = heart rate

HSP72 = heat shock protein 72

LPS = lipopolysaccharide

MAP = mean arterial pressure

PVDF = polyvinylidene fluoride

SV = stroke volume

SVI = stroke volume index

TNF-α = tumor necrosis factor-alpha

Introduction

Endotoxemia is a systemic infection mainly caused by endotoxin (lipopolysaccharide; LPS) of gram-negative bacteria.[1] Endotoxin stimulates monocytes, macrophages, and endothelial cells to release proinflammatory mediators, such as Tumor necrosis factor-alpha (TNF-α).[2] As a result, endotoxemia frequently leads to overwhelming septic shock and multiple organ failure, including cardiac depression and lung injury,[3] and cause high mortality.[4] The serum level of TNF-α was raised during lethal endotoxemia[5] and previous finding showing that TNF-α in the lung and plasma were significantly increased at 240 min after administration of LPS.[6] Diffuse pulmonary inflammation and irreversible cardiovascular collapse are induced in animals that have received recombinant human TNF injection.[7] On the contrary, monoclonal TNF-α antibodies or agents which antagonize LPS-induced production of TNF-α are effective in preventing LPS-induced lethal toxicity.[5,8] Thus, TNF-α is believed to play an important role in the pathogenesis of endotoxin-induced shock and lung injury.

It is well known that cardiopulmonary function can be improved by regular exercise.[9] We observed that heat shock protein 72 (HSP72) was detected in various organs of rats with exercise training.[10,11] It has also been demonstrated that HSP72 prevents cardiac mitochondrial dysfunction, decreases the LPS-induced acute lung

injury,[12] and reduces the mortality rate.[13] This raises the possibility that exercise training may protect against the LPS-induced septic shock and lung injury.

Hence, the present study attempts to determine whether preconditioning exercise can alleviate cardiopulmonary damage of rats during endotoxemia. The mortality rate, lung injury, HSP72 expression in heart and lung, and the changes of TNF-α in serum and bronchoalveolar lavage fluid were determined in sedentary and exercised rats after administration of LPS.

Materials and methods

Experimental animals

Adult male Wistar rats (320±20 g) were purchased from the Animal Resource Center of the National Cheng Kung University in Taiwan. All protocols were approved by the Institutional Animal Care and Use Committee of National Cheng Kung University, Tainan, Taiwan, and all experimental procedures were conducted in compliance with the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals." The animals were housed in groups of four at an ambient temperature of 24 ± 1 ˚C. Pelleted rat chow and tap water were allowed ad libitum.

Experimental groups

We separated all animals randomly into exercise and sedentary groups. Each group

was arranged into the following subgroups: endotoxemia group and saline group. In other words, we separated the rats into four groups: sedentary rats received normal saline administration (S), sedentary rats received LPS administration (L), exercised rats received normal saline administration (ES), and exercised rats received LPS administration (EL). The exercise training protocol was performed according to the previously described method with some modification.[11] The trained rats ran on a treadmill (treadmill exerciser T510, Diagnostic & Research Instruments, Singa) 5 days/week with intensity 1.0 mile/hr for 3 weeks at room temperature. Before the exercise training, the rats were acclimated to run 15 min with intensity 1.0 mile/hr for 3 days. Some electrical shocking (1.0 mA) was needed in the beginning to force animals to run forward. Subsequently, they ran without electrical stimulation. The duration of the exercise bouts were increased progressively so that the rats were running for 30 min at the first 2 weeks, and 60 min at the last week of training. Twenty-four hours after the last training session, adequate anesthesia was maintained to abolish the corneal reflexes and pain reflexes induced by tail pinch throughout the course of experiments after a single dose (1.4 g/kg, i.p.) of urethane. After rats received an intravenous injection of lipopolysaccharide (LPS, 15 mg/Kg) or saline, the survival time and cardiovascular parameters were continuously monitored. LPS (from Escherichia coli 0111:B4, Sigma, St. Louis, MO) was used as a fresh solution

in phosphate buffered saline (pH 7.40) at a concentration of 10 mg/mL.

Different groups of animals were used for three experiments: **(I)** determination of survival rate in sedentary and exercised rats after receiving an injection of LPS; **(II)** determination the change of hemodynamic parameters in urethane-anesthetized rats during endotoxemia; and, **(III)** determination of arterial blood gas, lung injury, tumor necrosis factor-alpha (TNF-α) level in serum and bronchoalveolar lavage, HSP72 expression in heart and lung at 240 min after LPS injection.

Measurement of hemodynamic parameters

Rats were anesthetized with urethane and cannulated in the right femoral artery with polyethylene catheters (PE-50). Mean arterial pressure (MAP) and heart rate (HR) were recorded using a polygraph (MP35, BIOPAC, Goleta, CA, USA) every 20 min from the arterial tube. The rat's trachea was intubated for artificial ventilation (Small Animal Ventilator Model 683, Harvard Apparatus, Holliston, MA, USA) at 50 breaths/min with a tidal volume of 8 ml/kg room air and a positive end expiratory pressure of 5 cmH_2O. After incision into the rat's chest, a Transonic Flowprobe (2.5PSB923, Transonic System Inc, Ithaca, NY, USA) was implanted around the root of the ascending aorta and connected to a Transonic transit-time blood flowmeter (T403, Transonic System Inc, Ithaca, NY, USA). The cardiac output (CO) was

calculated from the aortic blood flow, and stroke volume (SV) was expressed as CO divided by HR. The body weights of the rats were normalized in order to obtain a cardiac index (CI) and stroke volume index (SVI), which was computed by dividing CO and SV by body weight.

Determination of TNF-α in serum and lavage

To determine the TNF-α level, venous blood and bronchoalveolar lavage samples were taken from rats at 240 min after the administration of LPS or saline. Blood samples were allowed to clot for 30 min at room temperature and then were centrifuged for 20 min (2000g, 4 ^{o}C). The supernatants were harvested and stored at -70 ^{o}C until measurement. Lavage samples were collected by perfusing saline (15 ml) from the endotracheal tube. The concentration of TNF-α in serum and lavage were assayed using double-antibody sandwich ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and the optical density of each well was determined by a microplate photometer (Multiskan EX, Thermo Fisher Scientific Inc, Waltham, MA, USA).

Detection of lung vascular/epithelial permeability

Albumin content in lavage was determined to assess the damage of endothelial cell of the capillaries in lung. The lavage samples were centrifuged for 20 min (1000 rpm, 4 ^{o}C) to remove cells. The supernatant of lavage was analyzed by Bio-Rad protein assay

system (Bio-Rad Hercules, CA) with bovine serum albumin as the standard. The albumin content in lavage was calculated by dividing albumin by dried weight of lung to evaluate pulmonary capillary/endothelial cell permeability.

Measurement of lung edema

At 240 min after administration of LPS, the entire lungs were taken, and weighed as the wet weight. Dry weight was measured after the lungs were dried at 63 ℃ for 48 hrs. The wet/dry weight ratio of lung was calculated by dividing the wet weight by dry weight to evaluate lung edema.

Analysis of arterial blood gas

In order to determine the arterial pH, arterial partial pressure of O_2 (PaO_2), CO_2 ($PaCO_2$), and O_2 saturation (SO_2) of rats, 0.4 ml of arterial blood was sampled anaerobically from the femoral artery by a syringe rinsed with heparin to prevent exchange of gases in the atmosphere with those in the blood sample at 240 min after administration of LPS and analyzed by blood gas analyzer (Synthesis1725, Diamond Diagnostics Inc, Hollinton, MA, USA).

Western blotting analysis of HSP72

For determination of HSP72 expression in heart and lung of rats with or without exercise training, rats were sacrificed by decapitation at 240 min after LPS injection.

The heart and lung were removed and stored at -70 ^{o}C until analysis. The detection of HSP72 was carried out following the previous method [10]. Briefly, the tissue was homogenized and denatured in SDS sample buffer (0.5 M Tris-HCl [pH 6.8], 10% SDS, 0.1% bromphenol blue, 2-mercaptoethanol, and glycerol). Protein contents were assayed using the Bio-Rad kit (Bio-Rad, Hercules, CA, USA) and an ELISA reader (Multskan EX, Thermo, MA, USA) at 630 nm. After equal amounts of protein extract were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was incubated at 4 ˚C overnight with mouse monoclonal anti-HSP72 primary antibody (SPA 810; StressGen Biotechonologies, Victoria, BC, Canada), diluted 1:2000 in 5% skim milk. Immunodetection for HSP72 was performed using the enhanced chemiluminescence protocol with Renaissance reagent (NEN Life Science Products, Boston, MA, USA) and X-ray film (Fuji, Tokyo, Japan). Mouse anti-actin monoclonal antibody was used as the internal control. Quantification of blot band was performed using an optical scanner and ImageMaster TotalLab 1D Elite software (version 2.01; Amersham Pharmacia, Piscataway, NJ, USA).

Statistical analysis

Data from experiments were expressed as mean ± S.E.M. for each point. Statistical

analysis was conducted using the analysis of variance (ANOVA) for factorial experiments, and repeated measures ANOVA was performed for comparing the hemodynamic parameters (followed by the Duncan post hoc test). The Wilcoxon rank sum test was used for the analysis of survival time, while the Kaplan-Meier test was used for the survival rate. Pearson correlation coefficient (r) was used as a measure of linear association between 2 variables. Significant differences between groups were assumed to be present at values of $P<0.05$.

Results

After exercise training for 3 weeks, the body weight in exercised rats was significantly lower than that in sedentary rats (354.1 ± 5.8 gm vs. 385.4 ± 9.3 gm; $P<0.05$). After administration of LPS, exercise training significantly ameliorated the survival rate (Fig 1). As shown in Figure 2, the level of TNF-α in serum and larvage was markedly elevated after LPS challenge. However, the increase of TNF-α was ameliorated significantly in rats with exercise training. The time course for change of cardiovascular parameters during endotoxemia in rats with or without exercise training is indicated in Figure 3. It shows that the values of heart rate increased slightly and then dropped dramatically while the values of all other hemodynamic parameters decreased progressively throughout endotoxemia. However, the change of

heart rate, mean arterial pressure, cardiac index, and stroke volume index were found to be alleviated significantly in the exercised rats as compared to sedentary ones after LPS challenge.

The albumin content in lung and the lung wet-dry weight ratios increased significantly after administration of LPS, but the values were diminished significantly in rats with exercise training (Fig 4). Even though the albumin content in EL group was significantly lower than L group, the value was still significantly higher than that in S or ES groups. After LPS challenge, the value of $PaCO_2$ decreased significantly, whereas the value of PaO_2 and SaO_2 were significantly greater indicating that hyperventilation occurred, but these values were indistinguishable between exercised and non-exercised rats (Table 1). The protective effect of exercise on blood gas changes is considered minor.

The levels of HSP72 expression in heart and lung were found to be increased in rats with exercise training as compared to those without exercise training (Fig 5) and the expression of HSP72 in ES and EL groups were indistinguishable (data not shown). A negative correlation between lung HSP72 level and lavage TNF-α level was demonstrated ($r=-0.742$, $P<0.05$).

Discussion

In the present study, we found that exercise training attenuated cardiopulmonary damage in rats during endotoxemia. The increase levels of TNF-α in serum and lavage by LPS were markedly attenuated in exercised rats as compared to those in sedentary rats. We also demonstrated that prior exercise training could increase the HSP72 expression in heart and lung.

Previous studies showed that HSP72 overexpression was induced in multiple organs after exercise training.[10] HSP72 is introduced to play a critical role in the protection of cellular damage from stresses.[14] In fact, exercise activates heat shock factors to bind the heat shock elements in nucleus to produce new HSP72. It has been documented that HSP72 protects the heart from ischemia-reperfusion injury in myocardial ischemia by reducing proinflammatory cytokines[15] and shields the heart against cardiac dysfunction in exercised rats.[16] HSP72 induced by heat was also able to protect cardiomyocytes from systemic inflammation.[17] Additionally, some articles make an association between the HSP72 and protection against endotoxemia. HSP72 induced by heat was reported to reduce lung damage, pulmonary fibrosis, attenuated neutrophil recruitment and acute lung leak induced by endotoxin.[18,19] The LPS-induced secretion of TNF-α of mononuclear cells was dampened by purified HSP70 in order to limit further tissue damage.[20]

High expression of TNF-α increased the mortality rate due to septic shock and

multiple organ failure caused by endotoxin.[21] The present results are in good agreement with those of previous finding showing that TNF-α in the lung and plasma were significantly increased at 240 min after administration of LPS in non-exercised rats.[22] However, HSP72 inhibited the LPS-induced TNF-α production and organ damage in the endotoxemia model of rats.[23] HSP72 can prevent the generation of nuclear factor-κB and diminish TNF-α level.[24] In fact, HSP72 minimized the phosphorylation of inhibitor-κB and maintained the nuclear factor-κB/inhibitor-κB complex to interfere the synthesis of TNF-α.[25] Moreover, HSP72 is mentioned to prevent cardiac mitochondrial dysfunction and attenuate the hyporesponsiveness of platelets and other hemodynamic alterations induced by endotoxemia.[13,26] Therefore, the induction of HSP72 may play an important role in endotoxemic rats. In the present study, exercise training increased the expression of HSP72. Also, the increase TNF-α level in serum and lavage induced by LPS challenge became lower in exercised rats. We found that lavage TNF-α levels were negatively correlated with HSP72 levels in the lung. Hence, these results suggested an implication that the attenuation of TNF-α during endotoxemia may be related to the higher expression of HSP72 induced by exercise. However, exercise may also increase a number of other factors (e.g., interleukin-6, glucocorticoids, etc) which are immunomodulators. Further studies should be conducted to explore whether these factors contribute to the protection

against lethal endotoxemia.

The arterial pressure and total peripheral resistance are decreased from the dilation of peripheral vessels caused by LPS-induced endotoxemia.[27] Previous study has mentioned that heart rate was increased in order to compensate the low blood pressure[28,29] which is consistent with our results during the early stage of endotoxemia. However, the compensating mechanism then was broken down and heart rate dropped dramatically.[30] Cardiac output and stroke volume, representing the left ventricular function, also explicitly diminished. Moreover, coronary flow was reduced to result in myocardial dysfunction during endotoxemia.[31] The decreased ventricular function is believed to result in the inefficient discharge of blood and contribute to pulmonary hypertension.[32] In the present study, we found that exercise training increases the survival rate and alleviates the reduction of blood pressure. However, it has been manifested that HSP72 prevents impaired myocardial contractile function and maintains coronary flow in endotoxemia.[33] As our results showed that prior exercise training increased the expression of HSP72 in heart, the HSP72 may be used to confer the partial protection against hemodynamic alterations after LPS administration in exercised rats.

Numerous polymorphonuclear cells, which releasing TNF-α to damage pulmonary vessels and increase lung vascular/epithelial permeability, were found in thickened

alveolar interstitial tissue during endotoxemia.[34] Furthermore, endotoxin-induced kidney failure makes albumin releasing from urine due to poor filtering function. The altered albumin levels in vessels and tissues change hydrostatic pressure and retard liquid recycling. The overflowing liquid in tissue causes acute pulmonary edema.[35] However, HSP72 was able to protect respiratory systems from systemic inflammation[36] and decreased the LPS-induced acute lung injury.[37] HSP72 also facilitated the action of an anti-inflammatory cytokine, IL-10, resulting in a decrease of TNF-α induced lung damage.[38] In this study, we have already demonstrated that exercised rats had significantly lower albumin content, TNF-α level in lavage, and lung edema than sedentary rats during endotoxemia. Moreover, lavage TNF-α level had a negative correlation to the HSP72 level in lung. Hence, the attenuation of TNF-α during endotoxemia may be, in part, related to the higher expression of HSP72 in lung induced by exercise. Furthermore, our results are similar to previous finding[39] showed hyperventilation occurred during endotoxemia since $PaCO_2$ decreased and PaO_2 increased significantly, but inconsistent with the other study.[40] The controversial findings may be due to the different dosage of LPS injection.

Meanwhile, there are some limitations to this study. It remains unclear whether HSP72 plays the critical role in protection against endotoxin-induced lethal damage. Additional studies using HSP72 antisense or siRNA in the exercised rats will be

helpful. Through exercise training, HSP72 could be induced in other organs, such as liver and kidney. Role of HSP72 expressed in these organs after exercise training for protection against endotoxemia-induced damage shall also be elucidated in addition. Although the increase of TNF-α level were alleviated after LPS challenge in exercised rats, further studies are necessary to determine the responses of other cytokines or inflammatory mediators (e.g. nitric oxide, interleukin-1β, Macrophage inflammatory protein-1α, etc.) during endotoxemia. Since these data are derived from a rapidly lethal model of endotoxemia, apparently all rats had succumbed to LPS by approximately 6 hr. The present results may not be extrapolatable to other more chronic or less severe models.

Conclusion

Exercise preconditioning could attenuate cardiopulmonary damage during endotoxemia, confer significant protection against the high mortality rate and TNF-α overproduction. This protective effect may be related with the increase of HSP72 expression. These findings provide a concept that exercise may play an important role in preventing endotoxin-induced septic shock and acute lung injury.

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Table 1. Effects of LPS administration on arterial blood gas in rats with or without exercise training

Figure captions

Fig 1. The survival rate after LPS administration (15mg/kg, i.v.) in different groups. L, rats injected with LPS; EL, rats with exercise injected with LPS. (n=10 for each group) † P <0.05, compared with the L group (Kaplan-Meier test).

Fig 2. The TNF-α level in serum and brochoalveolar lavage fluid. S, rats injected with saline; L, rats injected with LPS; ES, exercised rats injected with saline; EL, exercised rats injected with LPS. U.D., underdetective. Data are expressed as means ± S.E.M. of eight rats per group. * P <0.05, compared with the S group; † P <0.05, compared with the L group (one-way ANOVA)

Fig 3. Effects of LPS administration on HR (heart rate), MAP (mean arterial pressure), CO(cardiac output), CI (cardiac index), SV(stroke volume) and SVI (stroke volume index) in different groups. S, rats injected with saline (◯); L, rats injected with LPS (●); ES, exercised rats injected with saline (▽); EL, exercised rats injected with LPS (▼). Data are expressed as means ± S.E.M. of eight rats per group. * P <0.05, compared with the S group; † P <0.05, compared with the L group; $^{\#}$ P <0.05,

compared with the ES group (repeated measures ANOVA)

Fig 4. The albumin content in lung and the lung wet-dry weight ratios in different groups. Albumin content in the brochoalveolar lavage fluid was calculated as [(mg of albumin)/(ml of lavage fluid)]/ (g of dried lung weight). S, rats injected with saline; L, rats injected with LPS; ES, exercised rats injected with saline; EL, exercised rats injected with LPS. Data are expressed as means ± S.E.M. of eight rats per group. * P <0.05, compared with the S group; † P <0.05, compared with the L group; $^{\#}$ P <0.05, compared with the ES group (one-way ANOVA)

Fig 5. The expression of HSP72 in heart and lung of sedentary and exercised rats. Protein levels are expressed as the ratio to sedentary group. Below each column is a representation Western blot of HSP72. Data are expressed as means ± S.E.M. of eight rats per group. * P <0.05, compared with sedentary group.