Anti-inflammatory and metabolic effects of remote ischemic preconditioning and postconditioning on the contractile performance of the diaphragm in endotoxemic rats

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Background
Respiratory failure is a major cause of mortality during septic shock and is due in part to the decreased ventilatory muscles contraction. These muscles depend mainly on fatty acid oxidation as an important source of ATP. We investigated the effects of remote ischemic preconditioning and postconditioning (RpostC) on the contractile functions of the diaphragm in relation to metabolic functions during systemic inflammation in lipopolysaccharide (LPS)-induced endotoxemia model.

Materials and methods
A total of 24 adult male albino rats were divided equally into four groups: (i) the control group (group 1); (ii) LPS group (group 2), in which rats were treated with a single intraperitoneal injection of LPS (0.8 mg/kg intraperitoneal single dose); (iii) preconditioned group (group 3), in which remote ischemic preconditioning was induced with three ischemia/reperfusion cycles of the right hind limbs just before LPS injection; and (iv) postconditioned group (group 4), in which remote ischemic postconditioning was induced as in group 3 just after LPS injection. The animals were killed 5 days after the treatment. Among all groups, the contractility of the diaphragm was examined and the gene expressions of the carnitine palmitoyl transferase 1β (*CPT-1*β), the nuclear receptor peroxisome proliferator-activated receptor-α (*PPAR*α), peroxisome proliferator-activated receptor γ coactivator-1α (*PGC-1*α), and the nuclear factor erythroid 2-related factor 2 (*Nrf2*) – the master of antioxidant response element – by real-time reverse transcription-PCR in the rat diaphragm tissue were determined; in addition, the serum levels of inflammatory markers, tumor necrosis factor α (TNFα) and interleukin-6, and lipids were measured.

Results
Both types of remote ischemic, preconditioning and postconditioning, significantly improved the contractile performance of the diaphragm ($P < 0.001$) compared with rats in the LPS group. These improvements were accompanied with significant elevation in the diaphragmatic expression of *PPAR*α, *PGC-1*α, and *CPT-1*β, together with decreased serum lipids. In addition, there was a significant improvement in *Nrf2* with a reduction in serum TNFα and interleukin-6.

Conclusion
Our results showed that the improvement of contractile performance of the diaphragm during sepsis was related to the improvement in the expression of nuclear hormone receptors involved in fat oxidation and modulation in oxidative stress and its consequences on inflammatory response. On the basis of these findings, we can suggest that early rational postconditioning interventions may be one of the promising strategies in the management of sepsis.

Keywords:
carnitine palmitoyl transferase 1β, interleukin-6, Nrf2, PGC-1, PPAR-alpha, TNF alpha

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Introduction

Respiratory failure in patients with septic shock is a major cause of morbidity and mortality [1]. This respiratory insufficiency is usually attributed to lung injury, but there is increasing evidence that decreased ventilatory muscle contraction, particularly of the diaphragm, contributes significantly to this respiratory failure [2,3].

The diaphragm is the principal muscle of respiration, and is primarily composed of fatigue-resistant slow-twitch type I and fast-twitch type IIa myofibers.

Disease processes that interfere with diaphragmatic innervations, contractile properties, or mechanical coupling to the chest wall can result in diaphragmatic dysfunction. Such dysfunction, in turn, can lead to dyspnea, decreased exercise performance, sleep-disordered breathing, and respiratory failure [4].

The prognostic significance of impaired respiratory muscle strength has not been established yet. However, it emerges as a novel, independent predictor of prognosis in patients with congestive heart failure [5] and acute respiratory distress syndrome [6].

The mechanisms accounting for the ventilatory muscle failure during inflammation are likely to be multifactorial. Inflammation compromises blood perfusion of, and nutrient uptake in, skeletal muscles. It interferes with its structural integrity and contractile function. Inflammatory cytokines mediate these effects by affecting gene expression and adaptive responses [7].

Moreover, a systemic inflammatory state could impact negatively on skeletal muscle performance because of the alterations in the muscle energetic. Muscle depends on both glucose and free fatty acids (FA) as an energy source required for muscle contraction and, because of its high energy demands, the diaphragm is particularly dependent on FA oxidation, which, compared with the oxidation of glucose, can produce greater amounts of ATP [8].

FA oxidation in muscle and other tissues is regulated by the activation of nuclear hormone receptors, particularly peroxisome proliferator-activated receptors (PPARs), thyroid hormone receptors, and estrogen-related receptor α [9,10]. These nuclear hormone receptors form obligate heterodimers with retinoid X receptor, allowing for the activation of gene transcription. Many studies have shown that the expression of retinoid X receptor, PPARα, thyroid hormone receptor α and β, and estrogen-related receptor α decrease in liver, kidney, heart, and diaphragm following the administration of lipopolysaccharide (LPS) [11–14].

Other studies have also indicated that in models of sepsis there is a reduction in the expression of crucial nuclear hormone receptor coactivators, such as peroxisome proliferator-activated receptor γ coactivator-1α and β (PGC-1α and PGC-1β), which are required for the nuclear hormone-mediated increases in gene transcription [12,14].

These data suggest that the decrease in these nuclear hormone receptors and their coactivators plays an important role in regulating FA metabolism during infection. Many studies have demonstrated a decrease in FA oxidation in the liver, kidney, heart, and diaphragm during infections [14–16].

A clear association between oxidative stress and depressed skeletal muscle performance has been described in several acute and chronic conditions, such as systemic inflammation and chronic obstructive lung diseases. This can be attributed to the elevated levels of oxidant-derived post-translational protein modifications, including protein carbonylation inside skeletal muscle fibers when oxidative stress develops. This is supported by recent studies that showed that several myofilament (myosin heavy chain and actin), mitochondrial (aconitase, creatine kinase), and cytosolic [enolase, aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and carbonic anhydrase III] proteins are carbonylated inside the skeletal muscle fibers in many animal models of muscle dysfunction, and in humans with impaired skeletal muscle contractility [17].

In the last decade, the understanding on oxidative stress has broadened considerably, and it is now often seen as an imbalance, with origins in our genes, and the ways in which gene expression is regulated. At the center of this new focus is the transcription factor called nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 is referred to as the 'master regulator' of the antioxidant response, modulating the expression of hundreds of genes, including not only the familiar antioxidant enzymes but also a large number of genes that control seemingly disparate processes such as immune and inflammatory responses, tissue remodeling and fibrosis, carcinogenesis and metastasis, and even cognitive dysfunction and addictive behavior [18]. Thus, the dysregulation of Nrf2-regulated genes provides a logical explanation for the relation between observable oxidative stress and perhaps 200 human diseases involving these various physiological processes, each reflecting a network involving many gene products. The evolutionary self-association of these many genes under the common control of Nrf2 suggests that the immune and inflammatory systems may present the largest demand for increased antioxidant protection, apart from constitutive oxidative stress resulting from the mitochondrial oxygen consumption for metabolic purposes [18].

Remote preconditioning has a greater potential for clinical application than does conventional preconditioning, as it can be performed in a nonvital organ, avoiding the high risk of inducing ischemia by preconditioning in the vital organ such as the brain or the heart [19].

Remote ischemic preconditioning (RIPC) or postconditioning (RpostC), achieved with repeated

brief periods of ischemia and reperfusion of one organ before or after a prolonged ischemic period, has been reported to protect distant organs against ischemic injury [20]. The effects are associated with the downregulation of key steps leading to cell death and systemic inflammatory responses [20,21].

Indeed, systemic effects of RIPC and RpostC stimuli exist, which might be used to harness their protection not only in acute ischemic syndromes but also in many other types of tissue injuries. Recent studies also suggest RIPC and RpostC-mediated protective effects in systemic inflammatory situations [22]. More recently, a study from our group has shown that RIPC carried out in the lower limbs attenuates the cisplatin-induced hepatotoxicity in rats [23].

However, little research has been conducted examining the effects of RpostC in the setting of LPS-induced sepsis model. We investigated whether RpostC could improve contractile muscle performance of the diaphragm compared with RIPC and suppress LPS-induced proinflammatory cytokines. In addition, we assessed whether RpostC might upregulate PPARα, PGC-1α, and carnitine palmitoyl transferase 1β (CPT-1β) leading to the improvement of LPS-induced energetic alterations. An assessment of the potential antioxidant role through the effect on the expression on Nrf2, the master of antioxidant response, was also carried out.

Materials and methods

Experimental animal

The study was conducted on 24 inbred strains of adult male albino rats matched for age and weight (6–9 months and 150–200 g). Rats were inbred in the Experimental Animal Unit, Faculty of Medicine, Cairo University. They were kept at room temperature and normal dark–light cycles, in groups of four per cage, and were maintained according to the standard guidelines of Institutional Animal Care and Use Committee, and after obtaining the approval from the Institutional Review Board. Animals had free access to standard rat chow and had unrestricted access to drinking water.

Rats were divided equally into four groups, and were treated as follows: group 1 (control group), which was treated with saline solution (1 ml/100 g body weight, intraperitoneal); group 2 (LPS group), which was treated with a single intraperitoneal injection of LPS (0.8 mg/kg intraperitoneal single dose) [24]; group 3 (preconditioned group), in which RIPC was induced just before the LPS injection according to the protocol described below; and group 4 (postconditioned group), in which RpostC was induced as in group 3 just after the LPS injection.

Five days after saline or LPS administration, blood samples were collected from all rats before killing and the diaphragms were dissected for the demonstration of contractile performance in the right hemidiaphragm. The left hemidiaphragms were blotted dry, quickly frozen in liquid nitrogen, and stored at -80°C for quantitative real-time PCR gene expressions of *CPT-1β*, *PPAR-α*, *PGC-1α*, and *Nrf2*. The serum level of lipids and proinflammatory cytokines, tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), were measured for all groups.

Induction of remote ischemia preconditioning/postconditioning

Anesthesia

Before starting, the experimental animals were anesthetized with thiopental (50–60 mg/kg) administered intraperitoneally together with heparin (500 IU) [25]. Then, an area of the right hind limb was shaved.

Remote ischemic preconditioning/postconditioning

Remote ischemic conditioning was induced by a brief occlusion either before or after LPS. Occlusion was induced by using a tourniquet (a rubber band) tied to the right hind limb. The blood circulation of the limb was stopped by increasing the pressure on the trochanter with the rubber band. Cessation of the blood flow in descending branches of the femoral artery was indicated by the appearance of clear signs of ischemia (cyanosis and cold skin).

The protocol for ischemic conditioning consisted of three cycles of 10 min limb ischemia followed by 10 min of reperfusion [25].

In-vitro assessment of rat diaphragmatic contractile function

The rats were anesthetized with pentobarbital sodium (70 mg/kg body weight, intraperitoneal). The diaphragm and the adherent lower ribs were quickly excised after a combined thoracotomy and laparotomy, and were immediately submersed in cooled and oxygenated (95% O_2 and 5% CO_2) Krebs' solution at pH 7.4. This Krebs' solution consisted of 137 mmol/l NaCl, 4 mmol/l KCl, 2 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 1 mmol/l KH_2PO_4, 24 mmol/l $NaHCO_3$, and 7 mmol/l glucose. The right hemidiaphragm was dissected and then hung by a string to a wide-range force transducer (MLT1030/D-310; AD Instruments, Sydney, New

South Wales, Australia). The diaphragm was mounted vertically in tissue baths containing Krebs' solution bubbling with 95% O_2 and 5% CO_2 with a pH of 7.4. Temperature of the solution was maintained at 37°C. The muscle was stimulated directly by using platinum plate electrodes (electronic square wave stimulator; AD Instruments, Castle Hill, Australia) placed in close apposition of the bundle. A direct isometric twitch response was elicited by stimulating the muscle supramaximally with 0.2-Hz rectangular pulses of 0.5 ms duration and recorded by using an isometric transducer [26]. Muscle preload force was adjusted until the optimal fiber length for maximal twitch tension was achieved. After 10 min of thermoequilibration, contractile activity was continuously monitored and recorded using a Power Lab Data Acquisition System (4/30-ML866-AD Instruments) with an ML110 Bridge Bioelectric Physiographic Amplifier (Castle Hill, Australia).

In the averaged twitch record we measured the following:

(1) The tension developed (from the baseline to the peak).
(2) Contraction time (from the onset of tension record to the peak).
(3) Half-relaxation time (from the peak to fall of tension to the half of the peak value).
(4) Peak rate of muscle contractions (dp/dt_{max}) and peak rate of relaxations (dp/dt_{min}), which were obtained through electronic differentiation of the twitch curve.

Data analysis was carried out offline by using peak analysis. The peak analysis is a specific software for the detection and analysis of all recorded signal peaks in acquired waveforms. All the calculated parameters except for half-relaxation time (HRT) are displayed in a table form, showing the values of all peaks with an average value for all. Then we selected any of these peaks to be displayed separately for measuring HRT using manually controlled cursors according to its above-mentioned definition.

Serum lipid assay

The levels of high-density lipoprotein (HDL), triglycerides (TGs), and cholesterol were determined in the serum of rats in all groups using conventional laboratory methods.

Assessment of serum proinflammatory cytokines

Measurement of serum tumor necrosis factor α

TNFα was measured by using the ELISA (Quantikine; R&D Systems, Minneapolis, USA) according to the manufacturer's instructions [27].

Measurement of serum interleukin-6

IL-6 was measured by using the ELISA (Quantikine; R&D Systems) according to the manufacturer's instructions [28].

Detection of carnitine palmitoyl transferase β, peroxisome proliferator-activated receptor-α, peroxisome proliferator-activated receptor γ coactivator-1α, and erythroid 2-related factor 2 gene expression by quantitative real-time polymerase chain reaction

RNA isolation and reverse transcription

Total RNA was isolated from the tissue homogenate using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), and then was quantified on a Nano Drop 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). The OD 260 nm/280 nm ratio was among 1.9–2.0. RNA samples were further assessed through electrophoresis on 1.5% agarose gels, and then visualized under UV light after ethidium bromide staining. RNA samples were stored at -80°C in aliquots until use. cDNA was synthesized with 1 μg total RNA and 1 μl d(T)18 oligo using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Thermo Scientific) according to the manufacturer's instruction.

Quantitative real-time polymerase chain reaction

Quantitative PCR was carried out in an optical 96-well plate with an ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems, Carlsbad, California, USA) and universal cycling conditions at a minimum temperature of 95°C (40 cycles of 15 s at 95°C and 60 s at 60°C). Each 10 μl reaction contained 5 μl SYBR Green Master Mix (Applied Biosystems), 0.3 μl gene-specific forward and reverse primers (10 μmol/l), 2.5 μl cDNA, and 1.9 μl nuclease-free water. The sequences of PCR primer pairs used for each gene are shown in Table 1. Data were analyzed by using the

Table 1 Primer sequences used for real-time polymerase chain reaction

Primers	Sequence
CPT-1β	Forward primer: 5′-CGGTTCAAGAATGGCATCATC-3′
	Reverse primer: 5′-ATCACACCCACCACCACGATA-3′
PPAR-α	Forward primer: 5′-ACTTATCCTGTGGTCCCCGG-3′
	Reverse primer: 5′-CCGACAGAAAGGCACTTGTGA-3′
PGC-1α	Forward primer: 5′-TGTGCAACTCTCTGGAACTG-3′
	Reverse primer: 5′-TGAGGACTTGCTGAGTGGTG-3′
Nrf2	Forward primer: 5′-CCTCAACTATAGCGATGCTGAATCT-3′
	Reverse primer: 5′-AGGAGTTGGGCATGAGTGAGTAG-3′
GAPDH	Forward primer: 5′-CTCCCATTCTTCCACCTTTG-3′
	Reverse primer: 5′-CTTGCTCTCAGTATCCTTGC-3′

CPT-1β, carnitine palmitoyl transferase 1β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nrf2, nuclear factor erythroid 2-related factor 2; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PPAR-α, peroxisome proliferator-activated receptor-α.

ABI Prism Sequence Detection System software and quantified using the v1.7 Sequence Detection software (PE Biosystems, Foster City, California, USA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH genes [29].

Statistical analysis

The data were coded and entered using the statistical package SPSS (version 15; SPSS Inc., Chicago, Illinois, USA). The data were summarized using descriptive statistics: mean ± SD for all variables. Statistical differences between groups were tested using analysis of variance for quantitative normally distributed variables. When a significant *F* was obtained, multiple comparison after tests were used to determine which groups were significantly different. *P*-values less than or equal to 0.05 were considered statistically significant.

Results

The results of contractile performance of the diaphragm: contraction time (t_c), developed tension (D_t), peak rate of contraction (dp/dt_{max}), peak rate of relaxation (dp/dt_{min}), and half-relaxation time

These results are summarized in Table 2. There was a statistically significant elevation in t_c ($P < 0.001$) in rats of the LPS group compared with the control group. In contrast, the D_t together with dp/dt_{max} were statistically markedly reduced ($P < 0.001$ for both) in the same group compared with the control group. Clearly, there was an extreme slowing of relaxation in LPS rats as indicated by significant ($P < 0.001$) decrease in dp/dt_{min} and HRT compared with controls.

Both types of remote ischemic conditioning significantly improved all parameters of contractile performance ($P < 0.001$, <0.001, <0.001, 0.001, <0.001 for t_c, D_t, dp/dt_{max}, dp/dt_{min}, and HRT, respectively) compared with the LPS group. Interestingly, except for dp/dt_{max} and dp/dt_{min}, all other contractile parameters in both preconditioned and postconditioned groups showed significant difference compared with control rats.

There was no significant difference between the two types of remote ischemic conditioning on the contractile performance of the diaphragm, except for developed tension, which was significantly lower with RpostC intervention compared with the preconditioned group.

The results of serum lipids: triglyceride, cholesterol, and high-density lipoprotein

There was a statistically significant elevation of TG and cholesterol ($P < 0.001$ for both), and a depression in HDL ($P < 0.001$) in LPS-treated rats compared with the control rats.

All measured serum lipids were significantly improved in RIPC and RpostC ($P < 0.001$ for all) compared with the LPS group. Both types of remote ischemic conditioning reduced TG to the degree to be insignificant compared with the level for the control group, whereas both interventions significantly partially improved cholesterol and HDL. There was no significant difference between the two types of remote ischemic conditioning on serum lipids except for HDL, which was significantly slightly higher in the RpostC group ($P = 0.032$) compared with the preconditioned group (Table 3).

The changes in the serum level of proinflammatory cytokines: tumor necrosis factor α and interleukin-6

Both cytokines exhibited notable statistically significant rise ($P < 0.001$ for both) in rats of the LPS group. They were significantly reduced in RIPC and RpostC rats ($P < 0.001$ in both) compared with LPS rats, but they were still significantly ($P < 0.001$ for both) higher compared with the control animals (Table 4).

The changes in the levels of diaphragm-relative expression of carnitine palmitoyl transferase 1β, peroxisome proliferator-activated receptor-α, peroxisome proliferator-activated receptor γ coactivator-1α, and nuclear factor erythroid 2-related factor 2

The changes are summarized in Fig. 1. There was a statistically significant depression of gene expression of

Table 2 The contractile performance of the diaphragm showing the values of contraction time (t_c) (ms), developed tension (D_t) (g), peak rate of contraction (dp/dt_{max}), peak rate of relaxation (dp/dt_{min}), and half-relaxation time (s) in the studied groups ($n = 6$ in each group)

Groups	Parameters (mean ± SD)				
	Contraction time (ms)	Developed tension (g)	dp/dt_{max} (g/s)	dp/dt_{min} (g/s)	Half-relaxation time (s)
Control	0.15 ± 0.006	4.523 ± 0.45	15.623 ± 1.831	12.869 ± 4.083	0.11 ± 0.005
LPS	0.26 ± 0.019*	1.291 ± 0.42*	8.0493 ± 2.05*	7.174 ± 2.889*	0.150 ± 0.006*
Preconditioned	0.20 ± 0.016*,#	2.98 ± 0.508*,#	17.36 ± 1.439#	14.317 ± 1.553#	0.129 ± 0.005*,#
Postconditioned	0.195 ± 54.63*,#	2.18 ± 0.409*,#,@	15.028 ± 3.193#	13.088 ± 3.175#	0.127 ± 0.009*,#

dp/dt_{max}, peak rate of contraction; dp/dt_{min}, peak rate of relaxation; LPS, lipopolysaccharide; *Statistically significant as compared with controls at $P \leq 0.05$; #Statistically significant as compared with the LPS group at $P \leq 0.05$; @Statistically significant as compared with the preconditioned group at $P \leq 0.05$.

Table 3 The level of serum lipids: triglycerides (mg/dl), cholesterol (mg/dl), and high-density lipoprotein (mg/dl) in the studied groups (*n* = 6 in each group)

Groups	Parameters (mean ± SD)		
	TG	Cholesterol	HDL
Control	88.383 ± 8.787	144.4000 ± 9.168	58.467 ± 3.26
LPS	124.383 ± 15.877*	196.9833 ± 10.457*	36.70 ± 2.791*
Preconditioned	99.833 ± 7.635#	172.65 ± 10.172*,#	45.083 ± 3.4*,#
Postconditioned	95.367 ± 7.063#	165.267 ± 14.291*,#	49.867 ± 4.629*,#,@

HDL, high-density lipoprotein; LPS, lipopolysaccharide; TG, triglyceride; *Statistically significant as compared with control at $P \leq 0.05$; #Statistically significant as compared with the LPS group at $P \leq 0.05$; @Statistically significant as compared with preconditioned at $P \leq 0.05$.

Table 4 Serum level of proinflammatory cytokines: tumor necrosis factor α (pg/ml) and interleukin-6 (pg/ml) in the studied groups (*n* = 6 in each group)

Groups	Parameters (mean ± SD)	
	TNFα	IL-6
Control	37.05 ± 5.352	33.73 ± 2.1
LPS	156.63 ± 31.19*	123.62 ± 15.19*
Preconditioned	85.7 ± 12.877*,#	77.67 ± 14.74*,#
Postconditioned	73.68 ± 11.804*,#	67.4 ± 13.06*,#

IL-6, interleukin-6; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; *Statistically significant as compared with controls at $P \leq 0.05$; #Statistically significant as compared with the LPS group at $P \leq 0.05$.

*CPT-1*β, *PPAR*α, *PGC-1*α, and *Nrf2* in LPS-treated rats (P = 0.03, <0.001, <0.001, <0.001, respectively) compared with controls.

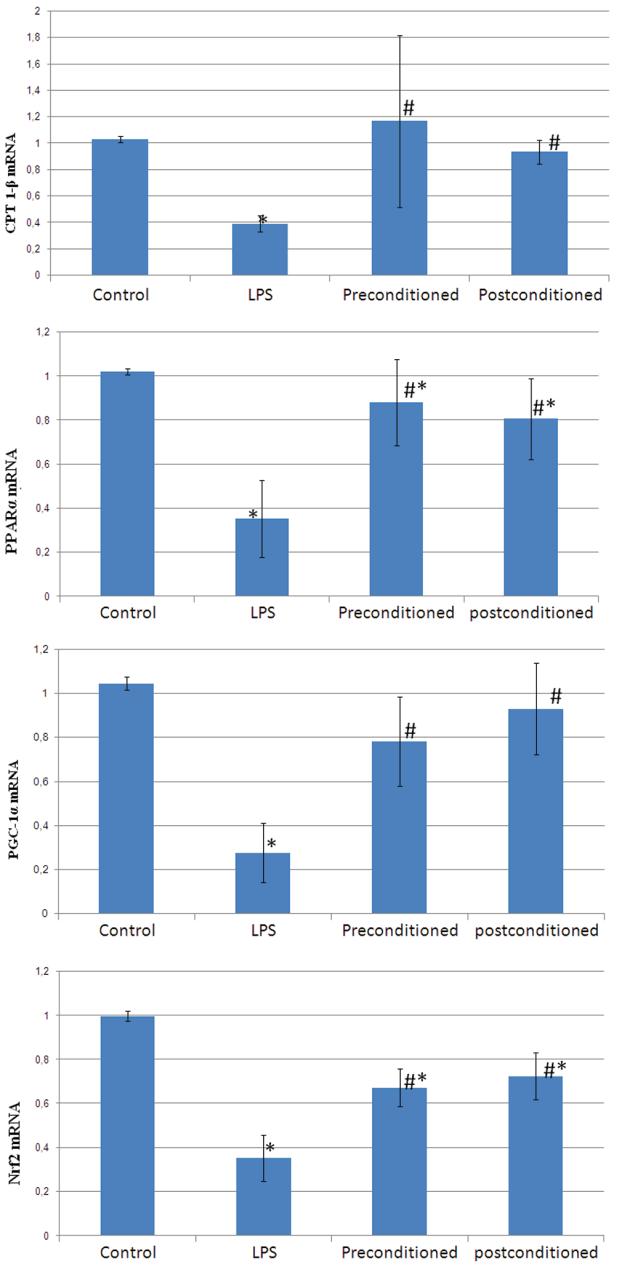
All expressed genes were significantly improved in the RIPC and RpostC groups (P = 0.01, <0.001, <0.001, 0.001, <0.001, respectively) compared with the LPS group. Both types of remote ischemic conditioning elevated all expressed genes to be comparable to the control level ($P > 0.05$) except for *Nrf2*, which was significantly ($P < 0.001$) still less than the control level. There were no significant differences between the two types of remote ischemic conditioning on the previously expressed genes.

Discussion

We investigated the effect of RIPC and RpostC in relation to their anti-inflammatory, antioxidant, and energetic response on the diaphragmatic contractile performance, during the early phase of muscle dysfunction [6] in LPS-induced sepsis in a rat model.

In the current work, RIPC and RpostC significantly produced comparable effects in improving the contractile performance of the diaphragm and suppressing serum proinflammatory cytokines. These effects were accompanied with significant elevation in diaphragmatic expression of PPARα, PGC-1α, and CPT-1β – the key enzyme that transports FA moieties across the mitochondrial membranes together with decreased serum lipids. Moreover, there was an

Figure 1



The levels of diaphragm-relative expression of carnitine palmitoyl transferase 1β (*CPT-1*β) (a); peroxisome proliferator-activated receptor-α (*PPAR*α) (b); peroxisome proliferator-activated receptor γ coactivator-1α (*PGC-1*α) (c); and nuclear factor erythroid 2-related factor 2 (*Nrf2*) (d). Mean ± SD in the studied groups (n = 6 in each). *Statistically significant as compared with controls at $P \leq 0.05$. #Statistically significant as compared with the lipopolysaccharide (LPS) group at $P \leq 0.05$.

improvement in the expression of Nrf2: the master of antioxidant response element (ARE).

In the present study, the developed tension of the diaphragm in the LPS group was significantly lower than that in the control group. This characteristic suggests a functional impairment of the diaphragm, which may impede adequate ventilation. In contrast, as weak muscles need to work closer to maximum contractile capacity [30], LPS rats may be predisposed to respiratory muscle fatigue, thus contributing to respiratory failure.

Although the developed tension was significantly higher in preconditioned rats compared with the postconditioned rats, it was significantly improved by both types of interventions compared with LPS rats. In addition, both interventions resulted in comparable improvement of all other measured contractile parameters, t_c, dp/dt_{max}, dp/dt_{min}, and HRT, compared with the LPS results.

Inflammatory reaction plays a critical role in skeletal muscle dysfunction [31]. The release of inflammatory cytokines and the aggregation and infiltration of inflammatory cells are the key steps in inflammation [32]. TNFα is primarily produced by macrophages, and it acts to promote inflammatory cascade by increasing the release of other proinflammatory cytokines and by influencing neutrophil recruitment [33]. TNFα, as an important cytokine in inflammation, plays a pivotal role in the inflammation induced by LPS [33]. Indeed, TNFα can induce the release of other inflammatory mediators, increase the expression of cell adhesion factors, and promote the adhesion of neutrophils to endothelial cells. Previous studies suggest that the level of TNFα increases significantly after LPS [34]. Interestingly, treatment with TNFα has been shown to also decrease diaphragmatic pressure and contraction in a way similar to endotoxin administration [34]. The administration of TNFα antibodies partially blocked the deleterious effects of LPS on diaphragmatic contractility, suggesting that the effects of sepsis and LPS are mediated by cytokines [33].

Measuring the levels of TNFα and IL-6 were relevant in the context of this study because these cytokines have the potential to induce skeletal muscle proteolysis via the ubiquitin–proteasome pathway [35]. In addition, TNFα activates the proteolytic enzymes caspase-8 and caspase-3 in the diaphragm of mice exposed to LPS [36].

Data observed in the current work were in agreement with the previous studies, as they indicated the presence of an association between systemic proinflammatory markers and strength in the diaphragm during endotoxemia. We found that serum TNFα and IL-6 protein levels in the LPS group were significantly higher compared with the control group.

In contrast, RIPC and RpostC interventions before and after LPS injection, respectively, significantly decreased these proinflammatory cytokines. This is in agreement with the findings of a recent study by Gyurkovics *et al*. [37], who also showed lower TNFα levels and reduced distant organ (lung and renal) impairment with RpostC treatment.

The major biological role of the PPAR/PGC-1α complex in the muscles appears to be the transcriptional control of enzymes involved in fatty acid uptake and oxidation [38]. PGC-1α activates mitochondrial biogenesis [37] and induces the expression of reactive oxygen species scavengers [39].

In addition, PPARα also governs inflammation by downregulating gene expression through a mechanism called transrepression. Specifically, it has been shown that activated PPARα binds to c-Jun and to the p65 subunit of nuclear factor κB (NF-κB), thereby inhibiting NF-κB-mediated signaling. Moreover, PPARα induces the inhibitory protein IκBα, which normally retains NF-κB in a nonactive form [40]. NF-κB is considered as an amplifying mechanism that can exaggerate the disease-specific inflammatory process through the coordinated activation of several inflammatory genes [41].

We found that LPS injection led to a downregulation of gene expression of the PGC-1α and PPARα. Thus, the decrease in coactivator, coupled with a decrease in the levels of the receptors that regulate FA metabolism, would be expected to lead to reductions in the enzymes and transport proteins. Then, one can speculate that the inability to generate energy through FA oxidation might contribute to the development of diaphragm weakness. In agreement with our findings were the results of a study by Feingold *et al*. [11,12,14]. Although a decrease in the expression of any one of these genes could decrease FA oxidation, it is likely that the LPS-induced reduction of FA oxidation activity is the result of a coordinated downregulation of several genes that mediate multiple steps along the FA oxidation pathway [14].

In the current work, the LPS-mediated increase in serum lipids were reduced significantly by both RIPC and RpostC interventions. The effect of RIPC and RpostC on LPS-induced changes in the serum lipid may be due to their effects on lipid delivery to the peripheral organs, primarily muscles that result in lowering their

serum levels, or it may be related to the improvement in the mitochondrial function that affects fatty acid oxidation. This hypothesis is supported by our finding of improved expression of CPT-1β together with the induction of both PPARα and PGC-1α. Furthermore, our assumption was supported by Schilling *et al*. [42], who demonstrated that the reduction in myocardial TG is an effect of increased PGC-1.

Interestingly, Feingold *et al*. [12] assumed that, whereas FA oxidation was decreased, the incorporation of FAs into TGs was somewhat increased in the diaphragm. This increase appears to be substrate driven as the expression of the enzymes that catalyze the incorporation of FAs into TGs is decreased rather than increased. Thus, in the diaphragm, similar to the liver, heart, and kidney, infection induces a decrease in FA oxidation with an increase in FA incorporation into TGs [12] accompanied with a reduction in the nuclear hormone receptor and coactivator expression [14].

Most of the changes in protein synthesis and lipid metabolism that occur during infection are beneficial to the organism [14]. One can only speculate on the potential benefits of decreasing FA oxidation in these organs. Infection stimulates an increase in TG-rich lipoproteins, which have been shown to directly bind toxic bacterial products, such as endotoxin and lipoteichoic acid, and thereby reduce their harmful effects [43]. Moreover, TG-rich lipoproteins could provide FAs for metabolism by cells of the immune system that play a crucial role in host defense or tissue repair. For example, studies have shown that LPS stimulates the uptake of TG by macrophages [44].

However, the decrease in FA oxidation in the heart, kidney, and diaphragm could also have detrimental effects. As compared with the oxidation of glucose or lactate, the oxidation of FA produces the most energy per molecule. ATP generated from FA oxidation is an important energy source for metabolically active tissues such as the heart, kidney, and diaphragm. During severe sepsis, multiorgan failure, including renal and heart failure, often occur and one can speculate that the inability to generate energy via FA oxidation might contribute to the development of these abnormalities [44].

Since mitochondrial dysfunction is evident in animal models of sepsis [45,46] and contributes to respiratory muscle weakness [47] and organ damage [48], one can speculate the PGC-1α/PPARα pathway system as a potential mechanism that links between the improvement in energetic metabolism and RIPC or RpostC.

As the pathogenesis of sepsis-induced mitochondrial injury is associated with free radical generation and resulting oxidative stress, one should assess this pathway in mediating organ dysfunction.

Given the regulatory role of the transcription factor Nrf2, the 'master regulator' of the ARE, in modulating the expression of hundreds of genes, including antioxidant enzymes, inflammation response, and others [18], we assessed the alteration in its expression after the LPS injection and its response to preischemic and postischemic conditioning. We found that the LPS injection led to a downregulation of Nrf2, which was significantly improved with both preconditioning and postconditioning.

This finding was in agreement with that of Song *et al.* [49], who found that nuclear expression of Nrf2 decreased in preterm lamb after LPS exposure *in utero*, and the significant association between Nrf2 protein level.

Under nonstressed conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex and constitutively degraded through the ubiquitin–proteasome system by binding to kelch-like ECH-associated protein 1 (Keap1). Oxidative or covalent modification of thiols in some cysteine residues of Keap1 lead to dissociation of Nrf2 from Keap1 and subsequently nuclear accumulation of Nrf2. Within the nucleus, Nrf2 binds to the ARE, a regulatory enhancer region within gene promoters. This binding triggers the production of many phase II detoxifying and antioxidant enzyme genes such as hemeoxygenase 1 (HO-1) and NAD(P)H: quinoneoxidoreductase 1 (NQO1), both of which protect cells against oxidative stress and a wide range of other toxins [50].

Thus, the fall in nuclear Nrf2 with inflammation would be responsible for the downregulation of antioxidant gene expression during infection.

In the current work, it is noted that RIPC has a protective effect similar to RpostC. However, a number of advantages favor the latter in clinical practice, as the most frequent clinical situation is that of establishing the treatment when the process of inflammation has already occurred and not otherwise. However, further carefully controlled human studies are needed to determine whether clinical differences exist in the muscle injury and recovery trajectories of sepsis in patients with and without concomitant acute respiratory distress syndrome.

Conclusion

This research presents evidence that endogenous protective mechanism triggered by RIPC and

RpostC lead to improvement in anti-inflammatory, antioxidants and energetic of the diaphragm during sepsis. These findings suggest that early rational postconditioning interventions may be one of the most promising strategies in the management of sepsis to prevent or retard diaphragmatic dysfunction and related respiratory failure.

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Conflicts of interest

There are no conflicts of interest.

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