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The oxidative stress and the mitochondrial dysfunction caused by endotoxemia are prevented by α-lipoic acid

VIRGINIA VANASCO, MARIA CECILIA CIMOLAI, PABLO EVELSON, & SILVIA ALVAREZ

Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

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Abstract
The aims of this work were to study the mitochondrial function and to evaluate (a) the oxidative stress in real time in an acute model of endotoxemia and (b) the effect of α-lipoic acid (LA, 100 mg/kg) as a therapeutic strategy to be considered. In rats treated with lipopolisaccharide (LPS, 10 mg/kg), a 1.4-fold increase was observed in *in situ* skeletal muscle chemiluminescence. Experimental sepsis increased oxygen consumption in tissue cubes (1 mm^3) by 30% for heart and diaphragm and impaired state 3 mitochondrial respiration rate in the three organs (liver, diaphragm and heart) studied. Only complex I activity in heart and diaphragm and complex IV activity in diaphragm were found impaired in this septic model. The production of NO by submitochondrial membranes was found increased by 80% in the diaphragm and by 35% in the heart of septic rats. The treatment with LA prevented the oxidative stress and mitochondrial dysfunction observed in this model.

Keywords: *Endotoxemia, α-lipoic acid, oxidative stress, mitochondrial function*

Introduction

Sepsis constitutes a major cause of death following trauma and a persistent problem in surgical patients. It is a paradigm of acute whole body inflammation, with massive increases of nitric oxide (NO) and inflammatory cytokines in biological fluids, systemic damage to vascular endothelium and impaired tissue and whole body respiration despite adequate oxygen supply [1,2].

Although many studies have reported the occurrence of oxidative stress in different models of sepsis [3], no measurements in real time and in non-invasive manner in an acute model of endotoxemia were done. In this present work, we have used *in situ* chemiluminescence (CL) to evaluate the reactive oxygen species steady-state concentrations. *In situ* CL is a low-intensity emission in the visible range mainly due to the decay of excited states of molecular oxygen (singlet oxygen and excited carbonyls) [4,5], which are formed during the termination steps of the chain reaction of lipid peroxidation. The spontaneous CL of the organs *in situ* correlates with the development of oxidative stress and has been used in several models of toxicity to the lung, heart and liver [6,7].

Several authors addressed the importance of mitochondrial function during experimental sepsis, as it may play a role in the genesis of the tissue injury described in this syndrome [8,9]. Moreover, different organ sensitivity to this inflammatory condition has been described. There are several mechanisms by which mitochondria may lead to tissue dysfunction: (a) reduction in cellular high-energy

Correspondence: Silvia Alvarez, Fisicoquímica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, C1113AAD, Buenos Aires, Argentina. Tel/Fax: (54-11) 4508-3646. Email: salvarez@ffyb.uba.ar

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(understood as adenosine triphosphate (ATP)) levels due to impairment of mitochondrial metabolic pathways [10], (b) generation of active species, that can damage cell organelles directly (through the reaction with cellular components) or indirectly (by the activation of signaling pathways) [11] and (c) involvement in the intrinsic pathway of cellular apoptosis [12].

Previous work in rodent septic models has shown that mitochondria isolated from vascular smooth muscle and skeletal and heart muscle exhibit impaired respiration due to inhibition of electron transfer and oxidative phosphorylation [9]. These observations may be explained by an excessive production of NO by mitochondrial nitric oxide synthase (mtNOS) and inducible nitric oxide synthase (iNOS) [9]. NO may inhibit mitochondrial respiration [13] via (a) an acute and reversible inhibition of cytochrome oxidase in competition with oxygen and (b) irreversible inhibition of multiple sites by reactive nitrogen species (such as peroxynitrite). The main findings with respect to mitochondrial NO metabolism and oxidative stress in sepsis are (a) a marked increase in mtNOS activity and NO production [14,15], (b) an increased production of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) [15], (c) elevated steady-state concentrations of O_2^-, H_2O_2 and $ONOO^-$ secondary to an increase in the NO steady-state level [16] and (d) a parallel and related increase in mtNOS and Mn-SOD activity [17]. Also, contractile failure following mitochondrial impairment was described [17] and translocation of cytosolic iNOS to mitochondria was suggested [13]. The molecular mechanism that leads to mtNOS activation and elevated NO production is a complex event that involves the transcription of NFkB [14]. This transcription factor induces the expression of mRNA of a variety of pro-inflammatory mediators including TNF-α, interleukins, adhesion molecules and enzymes (as cyclo-oxygenase 2 and iNOS).

Treatment of sepsis usually includes respiratory support to optimize tissue oxygenation, intravenous fluid administration, broad-spectrum antimicrobial therapy, anti-inflammatory agents and blood pressure support. Competitive inhibitors of NOS activity were considered as potentially useful, but failed to show beneficial effects [18].

α-Lipoic acid (LA) is a disulphide derivative of octanoic acid. Two LA enantiomers exist: the *R* form is biologically active as prosthetic group of various cellular enzymatic complexes and both *R* and *S* forms have antioxidant activity. It is taken up and reduced by cells (in mitochondria) to dihydrolipoate, a more powerful antioxidant than the parent compound. LA inhibits nuclear translocation of NFkB by preventing the activation of IkK and phosphorylation/degradation of IkB-α [19]. This compound has been proposed to be a therapeutic agent in the prevention or treatment of pathological conditions mediated *via* oxidative stress [20,21]. Pathologies and situations involving inflammation present abundant evidence of oxidative damage, raising the prospect of antioxidant therapies [22].

The aim of this work was to evaluate, in real time, the oxidative stress present in this acute model of endotoxemia, using CL as a non-invasive technique. Also, the oxygen consumption in whole organ was determined. These results are analysed in relation with the mitochondrial function during experimental endotoxemia assessed by mitochondrial oxygen consumption, respiratory chain complexes activities and NO production. This study was mainly conducted in muscle organs: heart, diaphragm and hind limb adductor, as muscle is the tissue mostly affected in this condition due to its high oxygen utilization. Liver was used for comparative purposes. Finally, the administration of α-lipoic acid as a therapeutic strategy is considered.

Materials and methods

Drugs and chemicals

D,L-α-Lipoic acid (racemic mixture) and lipopolisaccharide (LPS, serotype 026:B6 from *Escherichia coli*) were from Sigma-Aldrich (St. Louis, MO, USA). Other reagents, enzymes and enzyme substrates were reagent grade and were also purchased from Sigma-Aldrich.

Experimental design

Rats (Sprague-Dawley, female, 150 g), from the animal facility of the University of Buenos Aires, were used. The animals were housed under specific conditions in a temperature and humidity controlled environment and unlimited access to water and food (pelleted rodent non-purified diet). LPS was injected in a single dose of 10 mg/kg body weight. α-Lipoic acid was injected i.p. in a single dose of 100 mg/kg body weight. The treatments were performed 6 h before sacrifice. The four groups studied were: (a) control group: animals were injected i.p. with saline solution, (b) LA group: animals were injected i.p. only with α-lipoic acid (100 mg/kg body weight), (c) LPS group: animals were only injected i.p. with LPS (10 mg/kg) and (d) LPS + LA group: animal were co-injected (at the same time in a single injection) i.p. with LPS and α-lipoic acid (10 mg/kg and 100 mg/kg, respectively). Animal treatment was carried out in accordance with the guidelines of the 6344/96 regulation of the Argentinian National Drug, Food and Medical Technology Administration (ANMAT).

Isolation of mitochondria and preparation of submitochondrial membranes

Rats were anaesthetized (ketamine (10 mg/kg) plus xylazine (0.2 mg/kg)) and liver, heart and diaphragm were immediately excised. The tissues were homogenized in a glass-Teflon homogeinizer in a medium consisting of 0.23 M mannitol, 0.07 sucrose, 10 mM Tris-HCl and 1 mM EDTA, pH 7.4 at a ratio of 1 g/9 ml of medium. The homogenates were centrifuged at 700 g for 10 min to discard nuclei and cell debris, the sediment was discarded and the supernatant was centrifuged at 7000 g for 10 min to obtain mitochondria. The mitochondrial pellet was washed [23] twice and resuspended in the same buffer; it consisted of mitochondria able to carry out oxidative phosphorilation. Purity of isolated mitochondria was assesed by determining lactate dehydrogenase activity; only mitochondria with less than 5% impurity were used. Submitochondrial membranes (SMM) were obtained by freezing and thawing mitochondria three times, homogenizing by passage through a 29G hypodermic needle and centrifuging at 100 000 g for 30 min [23]. Protein content was assayed with the Folin reagent using bovine serum albumin as standard.

Oxidative stress evaluation by in situ chemiluminescence (CL)

The whole animal was covered with aluminium foil, in which a window was cut allowing exposure of the organ (liver or leg muscle) only. For leg muscle CL, the hind limb adductor was exposed. Determinations were performed with a Johnson Research Foundation photon counter. An EMI 9658 photomultiplier (responsive in the range 300–900 nm) cooled at -20°C with an applied potential of -1.4 kW was used. The phototube output was connected to an amplifier-discriminator adjusted to a single photon counting which was in turn connected to both a frequency counter and a recorder. Efficient light collection and isolation from the sample were established by using a lucite rod as optical coupler placed in front of the exposed organ *in situ*. Results were expressed as counts/second per unit of organ surface (cps/cm^2) [24].

Oxygen consumption by tissue cubes

A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria) was used. Briefly, organs were cutted into cubes of 1 mm^3 and oxygen consumption rates were measured in a reaction medium containing 118 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 5.5 mM glucose at 30°C. Results were expressed as ng-at O/minute per gram of tissue [25].

Mitochondrial function evaluation

Mitochondrial respiration. A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria) was used. Mitochondrial respiratory rates were measured in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 5 mM phosphate and 0.2% bovine serum albumin at 30°C. Malate 6 mM and glutamate 6 mM were used as substrates to measure state 4 respiration; 1 mM ADP was added to measure state 3 respiration [26]. Respiratory control (RC) was calculated as the relation between state 3 respiration and state 4 respiration.

Respiratory complexes activity. For the determination of NADH-cytochrome *c* reductase (complex I + III), submitochondrial membranes were added to 100 mM phosphate buffer (pH 7.4), 0.2 mM cytochrome *c* and 0.5 mM KCN and followed spectrophotometrically at 550 nm ($\varepsilon = 19.6$ mM^{-1} cm^{-1}) and 30°C. Enzyme activity was expressed as nmol reduced cytochrome c/minute per mg protein. Succinate cytochrome *c* reductase activity (complex II + III) was similarly determined and expressed, except that NADH was substituted by 20 mM succinate. Cytochrome oxidase activity (complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of 50 μM ferrocytochrome *c* [27]. The activity was expressed as nmol oxidized cytochrome c/minute per mg protein.

Nitric oxide synthase activity. Nitric oxide production was determined by the oxidation of oxyhemoglobin to methemoglobin, followed spectrophotometrically at two wavelengths 577–591 nm ($\varepsilon = 11.2$ mM^{-1} cm^{-1}) in a Beckman DU 7400 diode array spectrophotometer at 30°C [28], in a reaction medium containing 50 mm phosphate buffer (pH 7.2), 0.1 mM $CaCl_2$, 0.2 mM L-arginine, 100 μM NADPH, 10 μM dithiothreitol, 4 μM Cu,Zn-SOD, 0.1 μM catalase, 0.5–2.0 mg protein/ml of SMM and 20 μM oxyhaemoglobin. Control measurements in the presence of 2 mM N^G-methyl-L-arginine (L-NMMA) were performed to consider only L-NMMA-sensitive haemoglobin oxidation, usually 90–95% and due to NO formation. Results were expressed as nmol NO/minute per mg protein.

Statistics

Results were expressed as mean values ± SEM and represent the mean of six independent experiments. ANOVA followed by Dunnett test was used to analyse differences between mean values of more than two groups. Statistical significance was considered at $p < 0.05$.

Results

Oxidative stress evaluation by in situ chemiluminescence (CL)

The level of the steady-state concentration of oxidants was assessed by measuring the spontaneous *in situ* chemiluminescence of leg muscle and liver. The results of spontaneous and *in situ* liver and leg muscle CL are shown in Figure 1. We selected leg muscle (hind limb adductor) as an example of muscle organ and liver was included for comparative purposes.

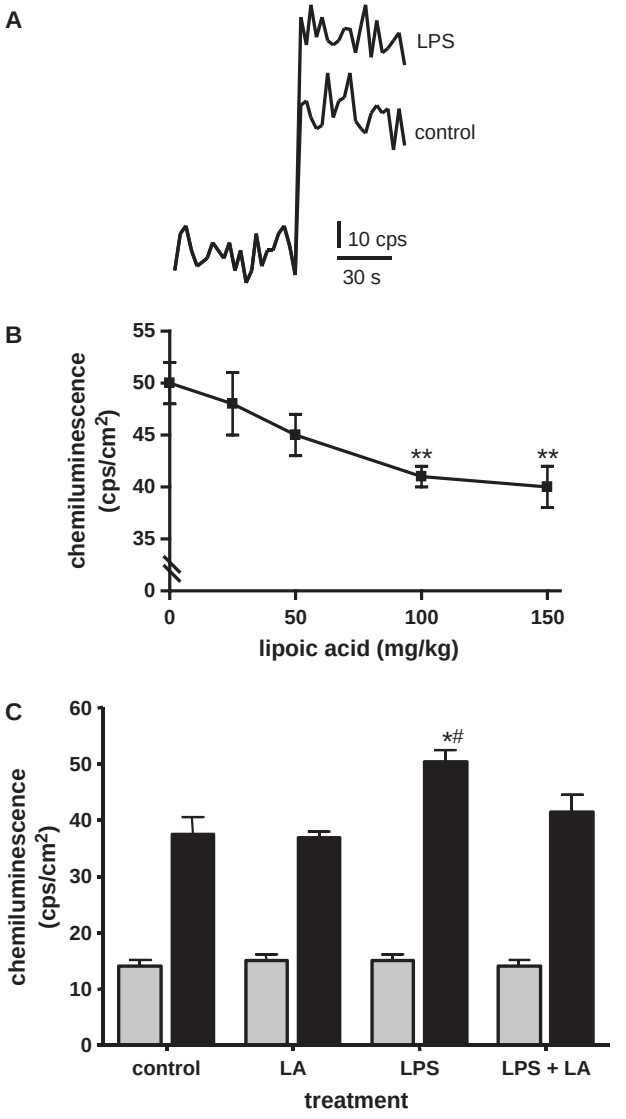

Figure 1. (A) Trace obtained during the determination of the *in situ* CL of leg muscle of control and LPS-treated animals. (B) LA dose–response curve analysed by *in situ* CL of leg muscle. Animals were co-injected with LPS and different LA doses (control value, without treatments: 38 ± 3 cps/cm^2). (C) Liver (grey bars) and skeletal muscle (black bars) chemiluminiscence from septic rats pre-treated with lipoic acid. The animal was anaesthetized with ketamine (10 mg/kg) plus xylazine (0.2 mg/kg) and covered with aluminium foil, in which a window was cut allowing exposure of the organ (liver or muscle) only. $^{\star}p < 0.01$ with respect to control group; $^{\star\star}p < 0.05$ with respect to LPS group; $^{\#}p < 0.05$ with respect to LPS + LA group.

Figure 1A shows an overlay trace of two independent and representative measurements of the leg muscle CL of control and LPS-treated animals. The lower trace corresponds to the background emission and the upper traces correspond to the measurements in control and treated muscle. A dose–response analysis of LA was performed to choose the dose (100 mg/kg) used in our studies. Animals were co-injected with LPS (10 mg/kg) and different LA doses (0; 25; 50; 100 and 150 mg/kg) and after 6 h the hind limb adductor was exposed for CL measurements. Figure 1B shows that only the two higher doses are significatly different from the LPS value; for that reason 100 mg/kg was the dose chosen for our studies. For rats treated with LPS, a 1.4-fold increase (when compared to the control value: 38 ± 3 cps/cm^2) was observed for skeletal muscle while liver CL remained unchanged (control value: 14 ± 1 cps/cm^2) (Figure 1C). The co-treatment with α-lipoic acid prevented the increase observed in muscle CL and the value obtained showed no signifficative difference with respect to the control value.

Oxygen consumption by tissue cubes

To evaluate the oxygen consumption by whole tissue, we used tissue cubes (1 mm^3) as organ sample. Experimental endotoxemia increased oxygen consumption by tissue cubes by $\sim$ 30–35% in heart (control value: 1073 ± 66 ng-at O/min mg tissue) and diaphragm (control value: 546 ± 41 ng-at O/min mg tissue) and only 25% in liver (control value: 926 ± 56 ng-at O/min mg tissue) (Figure 2). The *in vivo* co-treatment with α-lipoic acid reverted the increment observed in the heart and diaphragm; no significant differences were found when compared to the control value in heart and diaphragm tissue cubes.

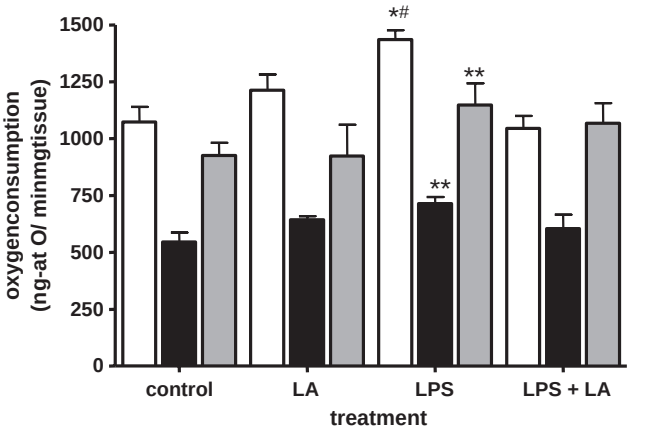

Figure 2. Oxygen consumption in heart (white bars), diaphragm (black bars) and liver (grey bars) tissue cubes from septic rats pre-treated with LA. Oxygen consumption was assessed in tissue cubes of 1 mm^3 at 30°C. $^{\star}p < 0.01$ with respect to control group; $^{\star\star}p < 0.05$ with respect to control group; $^{\#}p < 0.01$ with respect to LPS + LA group.

Mitochondrial function evaluation

Mitochondrial respiration and respiratory complexes activities. To evaluate the occurrence of dysfunction at the mitochondrial level, two different approaches were used. We measured the oxygen consumption in state 4 (resting or controlled respiration) and in state 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis) and the respiratory controls were calculated. Also, the activity of the respiratory chain complexes I, II and IV were measured.

LPS treatment significantly decreased mitochondrial oxygen consumption in state 3 by 20% for heart (control value: 234 ± 10 ng-at O/min mg prot), 40% for diaphragm (control value: 247 ± 10 ng-at O/min mg prot) and 17% for liver (control value: 220 ± 10 ng-at O/min mg prot), as shown in Table I. No significant changes were observed in state 4. Respiratory controls from treated mitochondria were found decreased, as a reflection of the decreased state 3 respiration. The respiratory impairement may be due to an inhibition of the electron transfer, as it is inferred from the decreased state 3 respiratory rate, simultaneous with an unchanged state 4 respiration. The co-treatment with α-lipoic acid improved the oxygen consumption rate of the corresponding LPS-treated values. Regarding state 3 respiration, no significant difference was observed for the three organs when the LPS + LA value was compared to the control value; significant difference was obtained for heart and diaphragm when the LPS + LA value was compared to the corresponding LPS value.

Table I. Mitochondrial respiration (in state 4 and in state 3) and respiratory control in heart, diaphragm and liver from control, LA, LPS and LPS+LA treated animals.

	Oxygen consumption (ng-at O/min mg protein)		
Organ/treatment	State 4	State 3	Respiratory control
Heart			
Control	53 ± 4	234 ± 10	4.4
LA	54 ± 5	240 ± 10	4.4
LPS	59 ± 5	$182 \pm 8^{*\#}$	3.1
LPS+LA	57 ± 5	224 ± 14	3.9
Diaphragm	52 ± 4	247 ± 10	4.8
Control			
LA	50 ± 4	246 ± 10	4.9
LPS	48 ± 4	$156 \pm 8^{*\|\|}$	3.3
LPS+LA	55 ± 5	217 ± 8	3.9
Liver	53 ± 5	220 ± 10	4.1
Control			
LA	55 ± 5	198 ± 9	3.6
LPS	54 ± 4	$183 \pm 7^{**}$	3.4
LPS+LA	55 ± 6	204 ± 10	3.7

$^{*}p < 0.01$ with respect to control group; $^{**}p < 0.05$ with respect to control group; $^{\|\|}p < 0.01$ with respect to LPS+LA group; $^{\#}p < 0.05$ with respect to LPS+LA group.
Oxygen consumption was assayed in mitochondria at 30°C, using malate 6 mm and glutamate 6 mm as substrates to measure state 4 respiration; and 1 mm ADP was added to measure state 3 respiration.

Complex I–III activity, shown in Table II, was found to be decreased in heart (30% with respect to the control group, control value: 328 ± 21 nmol/min mg prot) and diaphragm (40% when compared to the control group, control value: 256 ± 24 nmol/min mg prot) of septic rats, while this activity remained unchanged in liver (control value: 327 ± 21 nmol/min mg prot). These decreased values returned to the corresponding control values when the animals were also treated with α-lipoic acid.

Complex II–III activity showed no significant changes in any of the assessed organs, neither with LPS treatment nor with the co-treatment with α-lipoic acid. Complex IV activity was observed to be significantly diminished only in septic diaphragm (50% with respect to the control, control value: 100 ± 5 nmol/min mg prot), but the co-treatment with α-lipoic acid reverted this effect (Table II).

Mitochondrial nitric oxide production. As NO plays an important regulatory role on mitochondrial respiration, we decided to determine if the mitochondrial dysfunction previously observed depended on the mitochondrial NO production. For that reason we measured the mtNOS activity. The production of NO by submitochondrial membranes was significantly increased, by 80%, in diaphragm (control value: 0.84 ± 0.09 nmol NO/min mg prot) and by 35% in heart (control value: 0.66 ± 0.03 nmol NO/min mg prot) of septic rats (when compared to the control values). These results are shown in Figure 3.

NO production by liver submitochondrial membranes (control value: 0.51 ± 0.04 nmol NO/min mg prot) remained unchanged for all the treatments performed. When the animals where co-treated with α-lipoic acid, NO production by submitochondrial membranes isolated from diaphragm and heart significantly decreased with respect to the LPS value ($p < 0.01$ and $p < 0.05$, respectively) and returned to the corresponding control value.

Discussion

The present study shows that acute endotoxemia increases the steady-state concentration of oxidants in muscle, as measured *in vivo* and in real time by *in situ* CL. This result may be related to the increased oxygen consumption by the tissue. Mitochondrial dysfunction is also present, mainly in muscle organs (heart and diaphragm), assayed by mitochondrial oxygen consumption, respiratory complexes activities and NO production. α-Lipoic acid seems to contribute to ameliorate the mitochondrial dysfunction

Table II. Activity of the mitochondrial respiratory chain complexes (I, II and IV) in heart, diaphragm and liver from control, LA, LPS and LPS+LA treated animals.

Organ/treatment	Complex I–III (nmol/min mg prot)	Complex II–III (nmol/min mg prot)	Complex IV (nmol/min mg prot)
Heart			
Control	328±21	120±5	75±10
LA	360±25	115±5	80±10
LPS	234±9$^{*\#}$	110±5	75±10
LPS+LA	396±22	110±10	80±10
Diaphragm			
Control	256±24	110±15	100±5
LA	228±20	120±18	95±10
LPS	155±31**	105±18	55±5$^{*\#}$
LPS+LA	215±10	110±10	110±10
Liver			
Control	327±21	90±8	80±7
LA	433±43	85±8	85±5
LPS	369±18	85±8	80±6
LPS+LA	386±45	95±7	80±6

$^{*}p<0.05$ with respect to control; $^{**}p<0.01$ with respect to control; $^{\#}p<0.01$ with respect to LPS+LA group.
Complexes activity was assayed in submitochondrial membranes at 30°C.

and the oxidative stress observed in this septic condition.

Oxidative stress is a common event in the septic condition and it is accepted that muscle is one of the first organs to be affected. Organ CL is a non-invasive and non-destructive assay that allows the study of the ocurrence of oxidative stress. It directly determines the *in vivo* steady-state level of excited species. These species (mostly singlet oxygen and excited carbonyls) are mainly formed during the termination steps of the chain reaction of lipid peroxidation. Thus, CL values may be associated to the extent of lipid peroxidation and tissue oxygen consumption rate. In this way, organ CL provides an accurate measurement of the redox status of the tissue. Muscle CL was found to be increased in LPS-treated animals, but liver CL was not modified. This result is in agreement with a previous observation [29] in an animal model of sepsis produced by cecal ligation and double perforation. Muscle is described as the target organ in sepsis, probably due to the high O_2 utilization; meanwhile the liver may develop oxidative stress as a late phenomenon. Organ CL seems to be a useful tool for studying the occurrence of oxidative stress and to evaluate the redox status of the tissues in real time in several pathologies [30], including sepsis.

Tissue oxygen uptake (determined in cubes of 1 mm^3) was found increased in diaphragm and heart during the septic condition. Several mechanisms could explain the raise observed in tissue oxygen consumption. One mechanism is related to the mitochondrial oxygen consumption and production of reactive oxygen species. In physiological conditions, mitochondria utilize 90% of the oxygen consumed by the tissue, being of importance the disturbances in mitochondrial function during sepsis. These rates of oxygen uptake may be interpreted as the result of a shift of mitochondria from state 4 to state 3, driven by local energy demands and ADP availability [31]. It must be considered that, under physiological conditions, a mitochondrial sub-population is exposed to high ATP levels and another sub-population is exposed to ADP levels that stimulate respiration [31]. The high tissue O_2 consumption may be originated by an elevated mitochondrial sub-population in state 3 in order to respond to the high ATP demand, despite the inhibition of mitochondrial respiration due to the increased mitochondrial NO production. This observation has also been described for the rat ovary under hormone stimulation [32]. Also, it is worth noting that, due to the isolation method employed to obtain mitochondria from muscle organs (heart and diaphragm), the preparation would consist mainly of subsarcolemmal mitochondria and not interfibrillary mitochondria. These two sub-populations may play different metabolic roles in the cell and previous work has shown that one of the two populations of muscle mitochondria may increase in number in certain physiological or pathological situations [33]. Subsarcolemmal mitochondria have been found increased in gastrocnemius muscle after thyroidectomy [34] and have been described as the main mitochondria population for the supply of energy for active transport through the sarcolemma in soleus muscle during endurance training [35]. Another mechanism that may explain the raise in tissue oxygen consumption is the activation of NADPH oxidase and non-mitochondrial production of reactive oxygen species. Inflammatory processes (as endotoxemia and sepsis) include leukocyte recruitment and activation (respiratory burst) in tissues, thus of importance in this pathological processes as a mechanism of tissue injury [36]. It has been shown

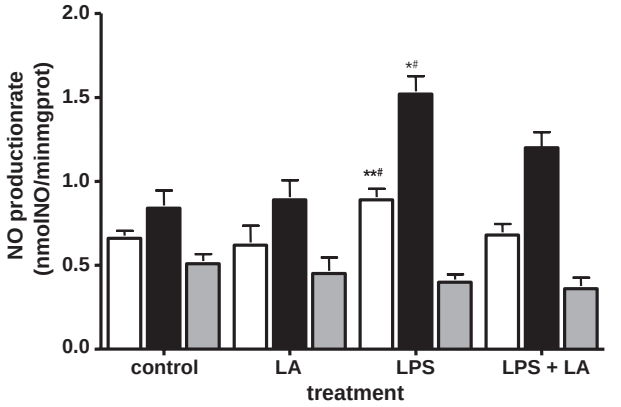

Figure 3. Mitochondrial NO production in heart (white bars), diaphragm (black bars) and liver (grey bars) from septic rats pretrated with lipoic acid. Nitric oxide production was determined by the oxidation of oxyhaemoglobin to methaemoglobin at 30°C. Controls whith the mtNOS inhibitor L-NMMA were performed (heart: 0.06±0.01 nmol NO/min. mg prot.; diaphragm: 0.07± 0.01 nmol NO/min mg prot; liver: 0.04±0.01 nmol NO/min mg prot) and the results are expressed as NO production rate sensitive to L-NMMA. $^{*}p<0.01$ with respect to control group; $^{**}p<0.05$ with respect to control group; $^{\#}p<0.05$ with respect to LPS+LA group.

that NADPH oxidases are important sources of reactive oxygen species in endothelial cells under inflammatory conditions [37] and that iNOS expression requires NADPH oxidase-dependent redox signalling in microvascular endothelial cells [38]. NADPH-oxidase inhibitors (as diphenyleneiodonium chloride (DPI) and apocynin) have been extensively used as an attempt to distinguish this last mechanism from others, but the specificity of this approach is under debate [37,39].

Nitric oxide produced by mitochondria (through the mtNOS) has important implications for the metabolism of cellular energy. Mitochondrial respiration is regulated not only by O_2 and ADP, but also by NO [40]. In the septic syndrome, state 3 respiration was found highly decreased in diaphragm and heart mitochondria, while in liver mitochondria the decrease in state 3 respiration was less significant ($p<0.05$). This result agrees with the increased NO production in mitochondria, observed for septic heart and diaphragm; liver production showed no significant variation. This concerted regulation has been observed in other experimental models [41]. NO has been recognized to be an inhibitor of cytochrome oxidase (complex IV) [42] and complex I [43]. The inhibition of cytochrome oxidase, under physiological conditions, is largely competitive and has regulatory importance in mitochondrial respiration. In our model, LPS-treatment decreased complex I and complex IV activity in diaphragm, complex I activity in heart, whereas in liver no changes were observed. In a previous work, we calculated the mitochondrial steady-state NO concentration in the heart and diaphragm of LPS-treated animals which were 28 and 27 pM, respectively, and allows a reversible inhibition of cytochrome oxidase [17]. This last observation is in accordance with the inhibition of complex IV by NO through the binding of a NO molecule to the reduced cytochrome a_3 to render cytochrome a_3^{2+}-NO [42]. Higher NO concentrations (>1 μM) can also induce irreversible inhibition. Complex I is less potently but more persistently inhibited by NO and other reactive nitrogen species by different mechanisms, such as S-nitrosilation and nitration [43]. Conversely, complex II may only be damaged by high levels of reactive nitrogen species. These associated events give evidence for the proposed functional association between complex IV and mtNOS and complex I and mtNOS [43].

α-Lipoic acid (LA) has been extensively used as a therapeutic agent in the prevention or treatment of pathological conditions involving oxidative stress [44–46]. LA is rapidly absorbed from the diet and reduced intracellularly to dihydrolipoic acid, having an important role in mitochondrial energy metabolism [21]. Addittionally, it can scavenge reactive oxygen species, regenerate antioxidants, chelate metal ions and has been used to attenuate inflammatory responses [47]. The administration of α-lipoic acid (100 mg/kg body weight) prevented the mitochondrial dysfunction and oxidative stress observed in LPS-treated rats. The significant findings, concerning LA treatment, were: (a) a marked decreased in the oxidative stress generated during the septic condition (assessed by *in vivo* CL), (b) mitochondrial NO production not significantly different from control values and (c) improvement of mitochondrial function (as determined by oxygen consumption in tissues and in mitochondria and by assessing mitochondrial complex I, II and IV activities). Although this work was designed to study LA effects on mitochondria, it cannot rule out the preventive effects of this compound by other mechanisms, the inhibition of NADPH oxidase being one of the most important. The inhibition of NF-kB signalling pathway activation was recently described by LPS in human monocytic THP-1 cells [47] and the inhibition of LPS-induced activation of iNOS in macrophages independently of the heat shock response [48].

This work provides new evidence that the *in vivo* treatment with α-lipoic acid is a therapeutic strategy to be considered for the septic syndrome, based on the improvement of the mitochondrial function, decreased production of mitochondrial NO and whole tissue oxidative stress. Although this work is mainly focused on the effect of this compound on the mitochondrial function, it is worth noting that the mechanism of action of α-lipoic acid in cells is complex. Modulation of glutathione and other thiol

levels, regeneration of antioxidants and regulation of various signalling pathways must be taken into account when discussing the overall effect of α-lipoic acid in pathology.

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