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Endotoxemia impairs heart mitochondrial function by decreasing electron transfer, ATP synthesis and ATP content without affecting membrane potential

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Abstract Acute endotoxemia (LPS, 10 mg/kg ip, Sprague Dawley rats, 45 days old, 180 g) decreased the O_2 consumption of rat heart (1 mm^3 tissue cubes) by 33% (from 4.69 to 3.11 μmol O_2/min. g tissue). Mitochondrial O_2 consumption and complex I activity were also decreased by 27% and 29%, respectively. Impaired respiration was associated to decreased ATP synthesis (from 417 to 168 nmol/min. mg protein) and ATP content (from 5.40 to 4.18 nmol ATP/mg protein), without affecting mitochondrial membrane potential. This scenario is accompanied by an increased production of $O_2^{\bullet-}$ and H_2O_2 due to complex I inhibition. The increased NO production, as shown by 38% increased mtNOS biochemical activity and 31% increased mtNOS functional activity, is expected to fuel an increased $ONOO^-$ generation that is considered relevant in terms of the biochemical mechanism. Heart mitochondrial bioenergetic dysfunction with decreased O_2 uptake, ATP production and contents may indicate that preservation of mitochondrial function will prevent heart failure in endotoxemia.

Keywords ATP · Bioenergetics · Endotoxemia · LPS · Mitochondrial function · Rat heart

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
m-CCCP	carbonyl cyanide m-chlorophenyl hydrazone
HRP	horseradish peroxidase
iNOS	inducible nitric oxide synthase
LPS	lipopolisaccharide
MOF	multiple organ failure
mtNOS	mitochondrial nitric oxide synthase
L-NMMA	L-N^G-mono-methyl-L-arginine
Rh123	Rhodamine 123
SOD	superoxide dismutase

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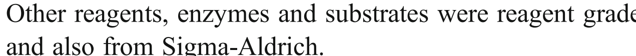
Introduction

Sepsis and endotoxemia are described as a paradigm of acute whole body inflammation: with massive increases of nitric oxide (NO) and inflammatory cytokines in biological fluids, with systemic damage in the vascular endothelium, and with impaired tissue and whole body respiration despite adequate O_2 supply (Szabó 2000). Without timely and effective therapeutic intervention, this scenario evolves to multiple organ failure (MOF) and ultimately to death mainly by heart failure. Heart requires large amounts of energy to sustain contractile function, and is the major consumer of energy in the body on a weight basis. It has been shown that during heart failure, the myocardium has a low ATP content due to a decreased ability to generate ATP by oxidative metabolism, and thus unable to effectively transfer chemical energy to contractile work (Maack and O'Rourke 2007).

Although the precise mechanisms by which sepsis leads to organ failure are not clear, there is a current awareness about a central role of mitochondrial dysfunction in the genesis of organ failure in this syndrome (Boveris et al. 2002a; Callahan and Supinski 2005; Escames et al. 2007). Although microvascular flow redistribution occurs in endotoxemia and sepsis, we and others have shown that



mitochondrial dysfunction is important in this condition indicating that an inadequate use of cellular O_2 coexists with impaired O_2 delivery (Fink 2002; Vanasco et al. 2008). The idea that mitochondrial dysfunction implies decreased oxidative phosphorylation despite normal pO_2 is a valid hypothesis for the pathogenesis of MOF (Fink 2002). In this context, inhibition of the electron transfer chain (Vanasco et al. 2008; Zapelini et al. 2008), decreased ATP production (Brealey et al. 2002; Escames et al. 2007), and defects in the inner and outer mitochondrial membrane (Crouser et al. 2004), were reported. It is worth to note that these findings correspond to different experimental models and to various organs.

Mitochondria provide energy to the cell through the synthesis of ATP by F_o-F_1 ATP synthase, that is located in the inner mitochondrial membrane. Therefore, in endotoxemia, any alteration in the respiratory chain, in the electrochemical proton gradient, or in the F_o-F_1 ATP synthase activity would lead to a deficiency in ATP production resulting in bioenergetic dysfunction and organ failure. Moreover, mitochondrial ATP content has been suggested to be related to patient outcome (Brealey et al. 2002; Carre et al. 2010). If the development of organ dysfunction is related to a cellular energetic failure, then strategies aimed at preventing the impairment of mitochondrial energy production may be potentially beneficial.

In the septic condition, cellular NO production and NO steady-state levels rise significantly, as a consequence of increased expression of inducible nitric oxide synthase (iNOS) and of mitochondrial nitric oxide synthase (mtNOS), as previously shown in our laboratory and by others (Alvarez and Boveris 2004; Protti and Singer 2007; Reynolds et al. 2009), contributing to heart mitochondrial dysfunction. Besides the well-known NO inhibitory effect at cytochrome oxidase, NO-mediated inhibition of complex III (ubiquinol-cytochrome c reductase) increases the generation of superoxide anion ($O_2^{\bullet-}$) (Cleeter et al. 2001; Poderoso et al. 1996). Intramitochondrial NO and $O_2^{\bullet-}$ react together to generate peroxynitrite ($ONOO^-$) (Beckman et al. 1993).

To our knowledge, no systematic analysis through a bioenergetic approach has been carried out for this specific model of endotoxemia, thus lacking precise information about mechanisms impaired during cellular energy metabolism. This work is focused on the mechanism of mitochondrial bioenergetic dysfunction, the relevance of decreased O_2 uptake, and decreased ATP production and content in an acute model of endotoxemia.

Materials and methods

Drugs and chemicals

Lipopolisaccharide (LPS, serotype 026:B6 from *Escherichia coli*) was from Sigma-Aldrich (St. Louis, MO, USA). Other reagents, enzymes and substrates were reagent grade and also from Sigma-Aldrich.

Experimental design

Rats (Sprague–Dawley, female, 180±10 g, 45±5 days old), from the Animal Facility of the School of Pharmacy and Biochemistry of the University of Buenos Aires, were used. The animals were housed under standard conditions of light, temperature and humidity with unlimited access to water and food (pelleted rodent food). LPS was injected in a single dose of 10 mg/kg body weight. Treatments were performed 6 h before sacrifice. The two groups studied were: a) control: animals injected i.p. with saline solution (vehicle) and b) LPS: animals injected i.p. with LPS (10 mg/kg). Animal treatments were carried out in accordance with the guidelines of the 6344/96 regulation of the Argentine National Drug, Food and Medical Technology Administration (ANMAT).

Sample preparation

Tissue cubes preparation

Rats were anesthetized with ketamine (50 mg/kg) plus xylazine (0.5 mg/kg) and the heart was rapidly excised, immediately placed in 118 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 5.5 mM glucose at 0–1 °C and subsequently cut into cubes of 1 mm^3.

Heart mitochondria isolation

Heart was homogenized in a glass-Teflon homogenizer in a medium consisting of 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl and 1 mM EDTA, pH 7.4 at a ratio of 1 g tissue/9 ml medium. The homogenate was centrifuged at 700 g for 10 min to remove nuclei and cell debris, the sediment was discarded and the supernatant was centrifuged at 7,000 g for 10 min to precipitate mitochondria. The supernatant was used to obtain the heart microsomal fraction, and the mitochondrial pellet was washed twice and resuspended in the same buffer (Cadenas and Boveris 1980). This suspension consisted of mitochondria able to carry out oxidative phosphorylation. Purity of isolated mitochondria was assessed by determining lactate dehydrogenase activity; only mitochondria with less than 3% of cytosolic lactate dehydrogenase activity were used.

Mitochondrial membranes preparation

Fragments of mitochondrial membranes were obtained by freezing and thawing three times mitochondrial suspension

and homogenizing them by passage through a 29G hypodermic needle (Cadenas and Boveris 1980). Protein content was determined by the Lowry assay using bovine serum albumin as standard.

Heart microsomes preparation

Heart microsomes were obtained by differential centrifugation. Briefly, the 7,000 g supernatant was ultracentrifuged at 105,000 g during 1 h at 4 °C. The pellet was resuspended in 100 mM KH_2PO_4/K_2HPO_4, pH 7.4. This subcellular fraction consists of plasma membrane and t-tubules fragments and sarcoplasmic reticulum (Webster and Williams 1964). The yield of the preparation was 12–14 mg of protein/g heart.

Oxygen uptake by tissue cubes and mitochondria

A two-channel respirometer for high-resolution respirometry (Hansatech Oxygraph, Hansatech Instruments Ltd, Norfolk, England) was used. The oxygen consumption rates of 1 mm^3 heart cubes were measured in a reaction medium containing 118 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 5.5 mM glucose and 2–4 tissue cubes, at 30 °C (Poderoso et al. 1994). In order to quantify the O_2 uptake by non-mitochondrial sources, 4 mM KCN was added to the measurement medium to inhibit mitochondrial cytochrome oxidase (Villani and Attardi 2007). Results were expressed as μmol O_2/min. g tissue.

Heart mitochondrial respiration was measured in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM KH_2PO_4/K_2HPO_4 , pH 7.4 and 0.1–0.2 mg of fresh heart mitochondria, at 30 °C. Malate and glutamate 6 mM or 7 mM succinate were used as substrates; 1 mM ADP was added to establish state 3 respiration (Boveris et al. 1999). Results were expressed as ng-at O/min. mg protein. Respiratory control was calculated as the ratio state 3/state 4 respirations. Oligomycin (2 μM) was used as ATP synthase inhibitor and m-CCCP (2 μM) as a protonophore uncoupler.

Mitochondrial superoxide anion production

Superoxide anion production was determined spectrophotometrically, at 30 °C, following the reduction of partially acetylated cytochrome c^{3+} to cytochrome c^{2+} at 550–546 nm (ε: 19 mM^{-1} cm^{-1}). Before the determination the mitochondrial fragments were washed twice in 140 mM KCl, 20 mM Tris–HCl buffer, pH 7.4, to eliminate endogenous SOD. Production of $O_2^{\bullet-}$ was measured in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl pH 7.4, 0.1 μM catalase, 50 μM NADH as substrate, mitochondrial membranes 0.05–01 mg/ml and 15 μM partially acetylated cytochrome c^{3+}. Cu,Zn-SOD 2 μM was added as control for the specificity of the $O_2^{\bullet-}$ assay, being the observed inhibition greater than 95%. The SOD-sensitive rate of reduced cytochrome formation was expressed as nmol $O_2^{\bullet-}$/min. mg protein (Azzi et al. 1975).

Mitochondrial hydrogen peroxide production

Hydrogen peroxide generation was determined in mitochondria (0.1–0.3 mg protein/ml) in metabolic state 4 by the scopoletin-horseradish peroxidase (HRP) method (Boveris 1984), following the decrease in fluorescence intensity in a Hitachi F-3010 fluorescence spectrophotometer at 365 nm (excitation) and 450 nm (emission) at 30 °C (Boveris 1984). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, pH 7.4, 1 μM HRP, 1 μM scopoletin, 0.3 μM Cu,Zn-SOD, 6 mM malate and glutamate, and 0.1–0.3 mg/ml of heart mitochondrial suspension. The fluorescence decrease that was sensitive to 0.2 μM catalase, usually 95–98%, was expressed as nmol H_2O_2/min. mg protein.

Mitochondrial membrane potential (ΔΨ)

Mitochondrial membrane potential was determined by measuring rhodamine 123 (Rh123) fluorescence (Emaus et al. 1986; Scaduto and Grotyohann 1999) in a Hitachi F-3010 fluorescence spectrophotometer at 503 nm (excitation) and 527 nm (emission) at 30 °C. Rat heart mitochondria (0.05 mg/ml) were suspended in 250 mM sucrose, 10 mM HEPES, 100 μM EGTA, 4 mM KH_2PO_4/K_2HPO_4, pH 7.4, and supplemented with 0.1 μM Rh123, 6 mM malate and 6 mM glutamate or 7 mM succinate, 1 mM ADP, 2 μM m-CCCP, and 2 μM oligomycin. Rh123 fluorescence was recorded before and after addition of heart mitochondrial suspension. Measurements were made in state 4, in state 3 and after the additions of oligomycin and m-CCCP. After stabilization had been reached, the fluorescence of the suspension was recorded and the content of the cuvette was centrifuged 3 min at 15,000 g to pellet mitochondria. The amount of dye taken up by mitochondria was calculated by subtraction from the initial fluorescence and the supernatant fluorescence. Since Rh123 distribution depends on the partitioning coefficient in lipid membranes, the concentration of free Rh123 in the matrix ($[Rh123]_{matrix}$) was calculated as follows: $[Rh123]_{mit} = Ki\,[Rh123]_{matrix} + Ko\,[Rh123]_{outside}$ where Ki = 37 and Ko 109 at 30 °C (Scaduto and Grotyohann 1999). Inner mitochondrial membrane potential was calculated using the Nernst-Guggenheim equation: $\Delta\Psi_{mit} = 59 \log ([Rh123]_{matrix}/[Rh123]_{outside})$ and results were expressed as mV (Valdez et al. 2006).

Mitochondrial ATP content and production rate

The assay is based in the chemiluminescent detection using the luciferine-luciferase reaction; the ATP content was

measured in a reaction medium containing 120 mM KCl, 20 mM Tris–HCl, 1.6 mM EDTA, 0.08% BSA, 8 mM K_2HPO_4/KH_2PO_4, 0.08 mM $MgCl_2$, pH 7.4, 40 μM luciferine, 1 μg/ml luciferase and 30–50 μg of heart mitochondria at 30 °C. To determine the ATP production rate, 6 mM malate, 6 mM glutamate, 0.1 mM ADP, and 0.15 mM di (adenosine)pentaphosphate were added to the reaction medium (Vivez-Bauza et al. 2007). The measurement was made in a LKB Wallack 1209 Rackbeta liquid scintillation counter. The production of ATP in the presence of 2 μg/ml oligomycin was determined and a calibration curve using ATP as standard (0–20 nmoles) was used (Vivez-Bauza et al. 2007). ATP content was expressed as nmol ATP/mg protein and ATP production rate as nmol ATP/min. mg protein.

Nitric oxide synthase activity in mitochondria

Nitric oxide synthase *biochemical activity* was determined as NO production analyzing the oxidation of oxyhemoglobin to methemoglobin, followed spectrophotometrically at 577–591 nm (ε=11.2 mM^{-1} cm^{-1}) in a Beckman DU 7400 diode array spectrophotometer at 37 °C (Boveris et al. 2002b), in a reaction medium containing 50 mM K_2HPO_4/ KH_2PO_4, pH 7.4, 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, 0.2–0.5 mg protein/ml of mitochondrial membranes, and 20 μM oxyhemoglobin. Control measurements in the presence of 2 mM N^G-methyl-l-arginine (L-NMMA) were performed to consider only L-NMMA-sensitive hemoglobin oxidation, usually 90–95%, as due to NO formation. Results were expressed as nmol NO/min. mg protein.

The mtNOS *functional activity* was calculated as the difference between the rates of O_2 uptake at maximal levels of NO, by addition of 0.10 mM L-arginine and 1 μM Cu,Zn-SOD, and the minimal levels of NO, by addition of 1 mM L-NMMA and 20 μM HbO_2 (Valdez et al. 2005).

NADPH oxidase activity in heart tissue cubes and microsomes

NADPH oxidase activity was measured as NADPH-dependent $O_2^{\bullet-}$ production by (a) tissue cubes and (b) heart microsomes, using the lucigenin chemiluminescent assay (Li et al. 1998). (a) Briefly, $O_2^{\bullet-}$ production rate by 1 mm^3 heart cubes was measured in a reaction medium containing 99 mM NaCl, 4.7 mM KCl, 1 mM K_2HPO_4, 1.2 mM $MgSO_4$, 1.9 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES, pH 7.4, 5 μM lucigenin, 10 μM NADPH and 2–4 tissue cubes, at 30 °C (Wang et al. 2009). (b) An aliquot of the microsomal suspension was diluted at 0.5 mg/ml in 150 mM sucrose, 50 mM KH_2PO_4/K_2HPO_4 containing 1 mM EGTA, pH 7.4, 2.5 μM lucigenin and 100 μM NADPH as substrate. Chemiluminiscence was measured at 15 s intervals for 3 min in a LKB Wallack 1209 Rackbeta liquid scintillation counter in the out of coincidence mode. Control measurements were performed in the presence of 1 μM SOD. Data were corrected for background emission and only the SOD-sensitive signal was considered as NADPH-dependent $O_2^{\bullet-}$ production. Results were expressed as cpm/g tissue and as cpm/mg microsomal protein.

Statistics

Results were expressed as mean values ± SEM and represent the mean of six independent experiments. Student's *t*-test for unpaired data was used to analyze differences between mean values of two groups. ANOVA followed by Tukey test was used to analyze differences between mean values of more than two groups (Tables 2 and 6). Statistical significance was considered at $p<0.05$.

Results

Oxygen metabolism pathways in tissue cubes and mitochondria

Heart O_2 consumption was determined in 1 mm^3 tissue cubes. Figure 1a shows a representative measurement of heart O_2 consumption in control conditions; the trace also shows the effect of 4 mM KCN. A 30% decrease in tissue O_2 consumption was observed in endotoxemic animals (control value: 4.69±0.21 μmol O_2/min. g tissue; LPS value: 3.11±0.22 μmol O_2/min. g tissue) (Table 1). With the aim of evaluating the existence of other relevant pathways of cellular O_2 utilization, tissue O_2 consumption was determined in the presence of 4 mM KCN (cytochrome oxidase inhibitor). In control conditions, 88% of heart cubes O_2 uptake is due to mitochondrial pathways, being the rest (12%) due to non-mitochondrial sources. Interestingly, LPS treatment increased up to 27% the heart cubes O_2 uptake due to non-mitochondrial pathways. This observation agrees with the observation that NADPH oxidase is a non-mitochondrial source of O_2 consumption and an active source of reactive O_2 species in inflammatory conditions.

Analysis of mitochondrial O_2 consumption is the classical approach to characterize mitochondrial function by the determination of the respiratory rates in the mitochondrial respiratory states: in state 4 (resting or controlled respiration) and in state 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis). Also, respiratory control ratios were taken into account. Figure 1b shows a representative measurement of mitochondrial O_2 consumption in control conditions; the trace also

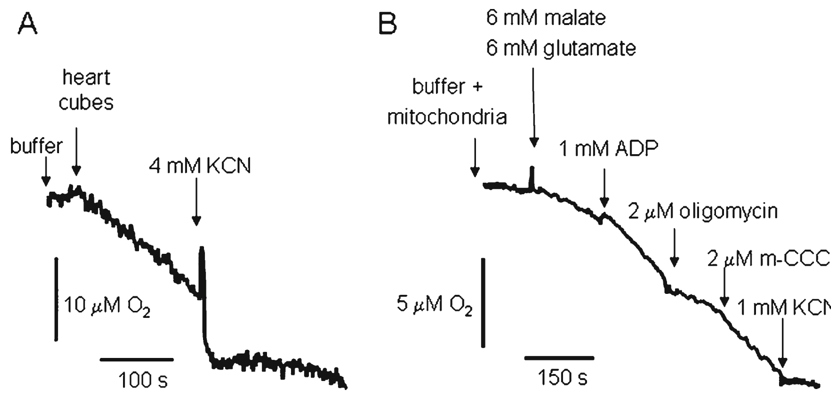


Fig. 1 Representative traces obtained during the measurement of O_2 consumption in control conditions. (**a**) Oxygen consumption of heart tissue cubes before and after the addition of 4 mM KCN. (**b**) Oxygen consumption of heart mitochondria in state 4 and in state 3, and after the addition of 2 μM oligomycin and 2 μM m-CCCP

shows the effect of 2 μM oligomycin and 2 μM m-CCCP on O_2 uptake. As shown in Table 2, LPS treatment significantly decreased, by 30%, the O_2 consumption in state 3 with malate-glutamate, being the effect lower, by 20%, with succinate. Respiratory control ratios were decreased in mitochondria from LPS-treated animals, due to decreased state 3 respiration. Information about mitochondrial membrane integrity can be obtained analyzing oligomicyn and m-CCCP effects on respiratory rates. The state 3 O_2 consumption switched to a slower state 4o respiration by the addition of the F_oF_1 ATP synthase inhibitor oligomicyn; the subsequent addition of the protonophore m-CCCP restores the O_2 consumption rate to an active respiration state known as state 3 uncoupled (state 3u). Values corresponding to the relationship between state 3 + olygomycin and m-CCCP (state 3u) and state 3 respiration + oligomicyn (state 4o) show no difference with respiratory control values corresponding to control and LPS-treated animals. This result indicates maintenance of mitochondrial membrane integrity in endotoxemia. State 3u is controlled exclusively by substrate oxidation and electron transfer rates and detects dysfunction in the respiratory chain complexes. The closeness of the respiratory rates of state 3 and state 3u values observed with malate-glutamate as substrates, agrees with the idea of a respiratory complex I dysfunction and limiting respiratory rate, as reported (Crouser et al. 2004).

Table 1 Oxygen consumption in heart cubes from control and LPS-treated rats, in the absence or in the presence of 4 mM KCN

	O_2 consumption (μmol O_2/min. g tissue)	
	− KCN	+ KCN
Control	4.69±0.21	0.58±0.02*
LPS	3.11±0.22*	0.85±0.06**#

*$p<0.001$ (Unpaired *t* test) with respect to control group, $n=6$

**$p<0.001$ (Unpaired *t* test) with respect to LPS group, $n=6$

#$p<0.005$ (Unpaired *t* test) with respect to control + KCN group, $n=6$

Mitochondrial dysfunction in endotoxemia was associated with increased $O_2^{\bullet-}$ and H_2O_2 production. Figure 2 shows representative traces corresponding to the production of $O_2^{\bullet-}$ and H_2O_2 in heart mitochondrial fragments and mitochondria, respectively, from control rats. The observed results (Table 3) show that mitochondrial $O_2^{\bullet-}$ production was increased by 66% in LPS-treated animals (control: 2.34±0.10 nmol $O_2^{\bullet-}$/min. mg protein, $p<0.0001$). Mitochondrial H_2O_2 production was also significantly increased, by 68% in this case, in endotoxemic rats (control value: 1.20±0.06 nmol H_2O_2/min. mg protein, $p<0.0001$).

Mitochondrial membrane potential, ATP production rates and ATP content

Mitochondrial inner membrane potential is another indicator parameter of mitochondrial bioenergetic function that determines the electrical compound (Nernst) of the electrochemical potential created by the H^+ pumping to the intermembrane space. No significant differences were observed between control and LPS-treated rats (determined either in state 3 or state 4 respiration) indicating mitochondrial integrity in endotoxemia (Table 4).

Mitochondrial ATP content or ATP steady-state level, are classical indicators and purpose of mitochondrial function. LPS treatment significantly decreased, by 60%, ATP production in heart mitochondria (control value: 417±34 nmol ATP/min mg protein, $p<0.005$) (Table 5). ATP content was also reduced in endotoxemic animals by about 23% (control: 5.40±0.33 nmol ATP/mg protein, $p<0.05$). The P/O ratio, calculated as ATP production rate/state 3 O_2 uptake using malate-glutamate as substrate, were 2.59 for control animals and 1.43 for endotoxemic rats.

Mitochondrial NO production

The *biochemical activity* of mtNOS, i.e. the NO production by heart mitochondrial fragments determined by the oxyhemoglobin assay, was markedly and significantly increased, by 35 %, in endotoxemia (control value: 0.66±0.03 nmol

Table 2 Respiration of heart mitochondria from control and LPS-treated rats[a]

Substrate	Mitochondrial states	O_2 consumption	
		Control (ng-at O/min. mg prot.)	LPS-treated
Malate + glutamate	State 4	32±4	27±4
	State 3	161±9	117±9*
	Respiratory control	5.03	4.33
	State 3 + oligomycin (state 4o)	34±4	32±4
	State 3 + oligomycin + m-CCCP (state 3u)	170±11	118±7*
	State 3u/state 4o	5.06	3.68
Succinate	State 4	43±3	41±3
	State 3	157±8	136±6**
	Respiratory control	3.65	3.32
	State 3 + oligomycin (state 4o)	44±5	46±4
	State 3 + oligomycin + m-CCCP (state 3u)	166±7	135±7*
	State 3u/state 4o	3.77	2.93

[a]State 4o is state 3 in the presence of the inhibitor of F_oF_1-ATP synthase oligomycin. State 3u is state 3 in the presence of oligomycin and the uncoupler m-CCCP

*$p<0.01$ with respect to control group by ANOVA-Tukey test, $n=6$

**$p<0.05$ with respect to control group by ANOVA-Tukey test, $n=6$

NO/min. mg protein, $p<0.005$) (Table 6). The *functional activity* of mtNOS, defined as the difference between O_2 consumption in situations of maximal and minimal steady-state levels of NO, constitutes an useful analysis that quantifies the fine regulation exerted by NO in the respiratory chain (Valdez et al. 2005) and was 31 % higher in the LPS-treated rats. The analysis was performed using malate-glutamate as substrate because the difference between control and LPS values for O_2 consumption is more pronounced with malate-glutamate than with succinate as substrate. Therefore, there is a quantitative agreement in the increases in mtNOS biochemical and functional activities (35 and 31 %). The values of mtNOS functional activities obtained are in full agreement with the computational model of NO effects on cytochrome oxidase (Antunes et al. 2007).

NADPH oxidase activity in heart cubes and microsomes

Plasma membrane NADPH oxidase seems to have a relevant role under inflammatory conditions as a source of $O_2^{\bullet-}$, as indicated by the KCN-insensitive respiration in heart tissue cubes. The activity of this enzyme was assayed as NADPH-dependent $O_2^{\bullet-}$ production in tissue cubes and in the microsomal fraction (that contains fragments of the plasma membrane, sarcoplasmic reticulum and mitochondria). The activity was found increased by 109 % in tissue cubes (from 22.1±2 to 46.0±3 10^5 cpm/g tissue) and by 70 % in the microsomal fraction (from 0.054±0.005 to 0.092±0.008 cps/mg protein) (Table 7).

Discussion

Mitochondrial dysfunction and organ failure are key features in endotoxemia and the associated MOF syndrome including heart failure (Callahan and Supinski 2005; Svistunenko et al. 2006; Vanasco et al. 2008). Mitochondrial dysfunction in endotoxic shock has been observed including inhibition of electron transfer and ATP synthesis using a series of experimental designs. However, no systematic analysis with a bioenergetic approach has been carried out using a unique experimental model, thus lacking precise information about impaired cellular energy metabolism. This study is focused on heart mitochondrial function, uses an acute model of

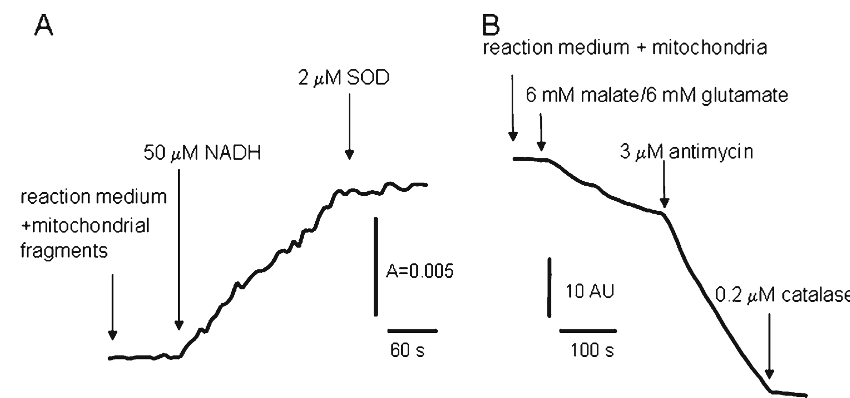


Fig. 2 Representative traces obtained during the measurement of $O_2^{\bullet-}$ and H_2O_2 production in heart mitochondria from control rats. (**a**) Production of $O_2^{\bullet-}$ by heart mitochondrial fragments before and after the addition of 2 µM SOD. (**b**) Production of H_2O_2 by heart mitochondria before and after the addition of 3 µM antimycin and 0.2 µM catalase

Table 3 Mitochondrial production of $O_2^{\bullet -}$ and H_2O_2 in heart from control and LPS-treated rats

	$O_2^{\bullet -}$ production (nmol/min. mg protein)	H_2O_2 production (nmol/min. mg protein)
Control	2.34±0.10	1.20±0.06
LPS-treated	3.89±0.17*	2.02±0.09*

*$p<0.0001$ (Unpaired t test) with respect to control group, $n=6$

endotoxemia (LPS, 10 mg/kg, 6 h), and shows that complex I (as previously reported in (Vanasco et al. 2008)) and mitochondrial O_2 consumption impairment lead to decreased ATP content without affecting mitochondrial membrane potential. This scenario is accompanied by an increased mitochondrial production of $O_2^{\bullet -}$, H_2O_2, NO and $ONOO^-$.

Heart tissue oxygen consumption was found decreased in the LPS-treated rats, but the corresponding KCN-insensitive respiration was observed increased. An increased NADPH oxidase activity accounts for an increased production of $O_2^{\bullet -}$ at the extracellular space (Brandes et al. 1999; Wu et al. 2008). In addition, increased generations of $O_2^{\bullet -}$ and H_2O_2 by the respiratory chain were determined. Interesting to note, $O_2^{\bullet -}$ and H_2O_2 production were found increased in heart mitochondria and the relationship between these two production rates indicates a stoichiometry of 2 and defines $O_2^{\bullet -}$ as the precursor of mitochondrial H_2O_2 in agreement with previous work (Chance et al. 1979; Boveris and Cadenas 1975; Dionisi et al. 1975).

Mitochondrial O_2 consumption in state 3 was decreased both with malate-glutamate and with succinate as respiratory substrates, although the decrease was higher when complex I substrates were used. This observation agrees with

Table 4 Inner membrane potential in heart mitochondria from control and LPS-treated rats[a]

Substrate	Mitochrondrial states	Membrane potential (mV)	
		Control	LPS
Malate plus glutamate	State 1	111±7	110±11
	State 4	158±9	152±8
	State 3	145±10	141±14
	State 3 plus oligomycin	157±8	148±9
	State 3 plus CCCP	96±7	85±8
Succinate	State 4	144±17	143±18
	State 3	132±8	135±14
	State 3 plus oligomycin	143±4	139±9
	State 3 plus CCCP	88±3	81±9

[a] Mitochondrial state 1 was achieved using heart mitochondrial suspension in the absence of substrates and ADP

Table 5 ATP synthesis, ATP content, and P/O ratios in heart mitochondria from control and LPS-treated rats[a]

	ATP production (nmol ATP/ min. mg protein)	ATP content (nmol ATP/ mg protein)	P/O
Control	417±34	5.40±0.33	2.59
LPS-treated	168±12*	4.18±0.38 **	1.43

[a] P/O ratio was calculated as the ratio between ATP production and state 3 O_2 consumption using malate-glutamate as substrates

*$p<0.005$ (Unpaired t test) with respect to control group, $n=5$

**$p<0.05$ (Unpaired t test) with respect to control group, $n=5$

previous data showing that heart mitochondrial complex I activity was found inhibited in endotoxemia (Vanasco et al. 2008). The analysis of the data presented in Table 2 indicates a decreased mitochondrial function with maintenance of the inner membrane permeability barrier to H^+ and of membrane integrity.

Several reports have indicated that mitochondrial complexes I, III, and IV interact to form supercomplexes with a defined stoichiometry (Schafer et al. 2006; Bornhovd et al. 2006; Vonck and Schafer 2009). Moreover, it has been reported that mtNOS is structurally adjacent to complex I (Franco et al. 2006; Parihar et al. 2008) . The functional association of mtNOS with complex I has implications for the molecular mechanisms involved in endotoxemia. The so-called "complex I syndrome" describes complex I dysfunction as an inhibition in complex I activity, accompanied by an increase in $O_2^{\bullet -}$ and H_2O_2 production, and an increase in oxidation and nitration products (Galkin et al. 2009; Navarro et al. 2010; Navarro et al. 2009). The lower rates of electron transfer through complex I observed in endotoxemia, critically restrict ATP synthesis and significantly increase $O_2^{\bullet -}$ and H_2O_2 production a situation that leads to further organ damage through a self-sustaining process. Although endotoxemia provoked an inhibition of complex I activity accompanied by decreased mitochondrial O_2 consumption in state 3, the magnitude of these events was not enough to affect the inner membrane potential but allowed a decreased ATP production rate and ATP steady-state levels. It has been claimed that mitochondrial ATP content are critical in endotoxic shock to determine patients outcome (Galkin et al. 2009; Navarro et al. 2010; Navarro et al. 2009). In this scenario, there are marked increases in the primary mitochondrial production of $O_2^{\bullet -}$ (66 %) and of NO (35 %); both free radicals react in the matrix space at diffusion-controlled rates, that will be 120 % increased in the LPS-treated rats. Then, protein nitration by $ONOO^-$ and protein and complex I inactivation may account for the molecular mechanism of mitochondrial dysfunction in endotoxemia. Nitration of ATP-synthase at Tyr269 was observed in aging, affecting the ADP binding to the active site,

Table 6 Biochemical and functional mtNOS activity in heart from control and LPS-treated rats[a]

	Assay	Control	LPS-treated
Biochemical activity	Mitochondrial NO production (mtNOS) (nmol NO/min. mg prot)	0.66±0.03	0.89±0.04*
Functional activity	O_2 consumption (ng-at O/min. mg prot.)		
	State 3	161±9	117±9**
	(a) State 3 plus L-arginine and SOD	145±7	98±5**
	(b) State 3 plus L-NMMA and HbO_2	192±10[#]	143±8**[#]
	Functional Activity (%) [(b-a)/st 3]×100	29	38

[a] *Biochemical activity*: NO production was determined spectrophotometrically following the oxidation of oxyhemoglobin to methemoglobin at 577–591 nm. *Functional activity*: O_2 consumption was determined using a Clark electrode, using 6 mM malate-6 mM glutamate as substrates. Data are means ± SEM of six rats for each group

*$p<0.005$ with respect to control group by unpaired-*t* test

**$p<0.01$ with respect to control group by ANOVA-Tukey test

[#] $p<0.01$ with respect to (a) by ANOVA-Tukey test

without nitration of cytosolic proteins, an observation that indicates a closed mitochondrial compartment for $ONOO^-$ (Lam et al. 2009).

In the heart, mitochondrial metabolism of fatty acids (as palmitate) accounts for 60–90% of the total energy production (energetic value: 20.1 kJ /L O_2) in the form of ATP, with carbohydrates (as glucose) contributing the remaining 10–40% (energetic value: 21.7 kJ /L O_2). Although palmitate does contain more energy per gram of substrate (40.5 kJ/g) as compared to glucose (20.1 kJ/g), both substrates produce about the same ATP yield with similar amounts (Ventura-Clapier et al. 2011).

In the inflammatory condition that is central in endotoxemia, NO and $ONOO^-$ acquire a prominent role in the development of mitochondrial dysfunction. Mitochondrial NO production was increased significantly, mainly as a consequence of a significantly higher mtNOS activity. The production of NO by mtNOS is important by the diffusional vicinity of the NO source with complex I as target. It is worth to note that in others inflammatory conditions iNOS induction has a prominent role in cellular NO production (Ganster and Geller 2000). NO and $O_2^{\bullet-}$ rapidly react to form the powerful oxidant peroxynitrite ($ONOO^-$), that exhibits multiple inhibitory actions in the mitochondrial respiratory chain, specially in complexes I and III (Beckman et al. 1990; Stadler et al. 2008). The biological situations in which the regulation by NO of mitochondrial respiration is considered relevant include hypoxia, ischemia-reperfusion, inflammation, apoptosis and aging (Valdez et al. 2005). Nevertheless, respiratory chain dysfunction by mitochondrial permeability transition opening cannot be ruled out (Crouser et al. 2004).

Table 7 NADPH oxidase activity in heart cubes and heart microsomes from control and LPS-treated rats

	Heart cubes (10^5 cpm/g tissue)	Heart microsomes (cps/mg protein)
Control	22.1±2	0.054±0.005
LPS-treated	46.0±3*	0.092±0.008*

*$p<0.0001$ (Unpaired *t* test) with respect to control group, $n=6$

The regulation of mitochondrial O_2 uptake, i. e. the mtNOS functional activity is considered as one of the major pathways by which NO exerts its role as an intracellular regulator in physiological, pathological and pharmacological conditions, in addition to its function as activator of guanilate cyclase activity (Valdez et al. 2005). In this model of endotoxemia, mtNOS functional activity (determined by its effect on mitochondrial O_2 consumption) was observed increased in the heart in full agreement with increased mitochondrial NO production. This positive relationship was also observed in other pathological situations and pharmacological treatments (Boveris et al. 2003; Lores-Arnaiz et al. 2004; Navarro et al. 2010).

The key implication of our study is that strategies to preserve mitochondrial O_2 consumption (as the beneficial effects of α-lipoic acid, previously shown by our laboratory (Vanasco et al. 2008)) and ATP content could limit heart mitochondrial impairment and bioenergetic failure as well as help to prevent organ dysfunction in endotoxemia.

To our knowledge this is the first study in which “Complex I syndrome” is described in endotoxemia. Overall, the data support the hypothesis that heart complex I activity and mitochondrial O_2 consumption impairment in endotoxemia, contribute to a decreased ATP production by F_oF_1-ATP synthase and to decreased mitochondrial ATP content without affecting inner membrane potential. This scenario is accompanied by an increased production of $O_2^{\bullet-}$ by auto-oxidation of reduced complex I and to an increased NO production by mtNOS that may lead to an increased production of $ONOO^-$.

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