Melatonin restores the mitochondrial production of ATP in septic mice

Luis C. López [1], Germaine Escames [1], Francisco Ortiz [1], Eduardo Ros [2] & Darío Acuña-Castroviejo [1,3]

[1] Instituto de Biotecnología, Departamento de Fisiología, Universidad de Granada, Granada, Spain.
[2] Servicio de Angiología y Cirugía Vascular, Hospital Universitario San Cecilio, Granada,
[3] Servicio de Análisis Clínicos, Hospital Universitario San Cecilio, Granada, Spain.

Correspondence to: Darío Acuña-Castroviejo, MD, PhD
Departamento de Fisiología, Facultad de Medicina,
Avenida. de Madrid 11,
E-18012, Granada, SPAIN
PHONE: +34 958 246631; FAX: +34 958 246295;
EMAIL: dacuna@ugr.es

Submitted: May 26, 2006 *Accepted: July 28, 2006*

Key words: **sepsis; melatonin; ATP; ETC; knockout iNOS; i-mtNOS; ATPase;**

Neuroendocrinol Lett 2006; **27**(5):623–630 **PMID:** 17159820 NEL270506A09 © Neuroendocrinology Letters **www.nel.edu**

Abstract

OBJETIVES: To evaluate the changes in the mitochondrial ATP production during sepsis and the participation of iNOS in these changes. We also assessed the effect of melatonin administration in this experimental paradigm.

METHODS: The activity of ATPase, the level of adenine nucleotides, and the ATP production were measured in mitochondria of diaphragm and hind leg skeletal muscle of wild type ($iNOS^{+/+}$) and knockout iNOS ($iNOS^{-/-}$) mice. Three experimental groups were done: control group; group of septic mice induced by cecal ligation and puncture (CLP), and group of septic mice treated with melatonin. Mice were killed 24 hours after CLP. Melatonin was administrated in four doses (30 mg/kg b.w.) as follows: 30 min before CLP (i.p.) and 30 min, 4 h and 8 h after CLP (s.c.).

RESULTS: Mitochondrial production of ATP decreased in $iNOS^{+/+}$ but not in $iNOS^{-/-}$ mice after sepsis. No changes in the ATPase activity were found in any group. Melatonin treatment normalized the production of ATP in $iNOS^{+/+}$ mice, without affecting $iNOS^{-/-}$ animals.

CONCLUSIONS: The reduction of the ATP production in $iNOS^{+/+}$ but not in $iNOS^{-/-}$ mice suggest the participation of iNOS in the impairment of mitochondrial function in the former. Because ATPase was unaffected by sepsis, it is suggested the ATP deficit dependend on the sepsis-induced respiratory chain damage. The normalization of the production of ATP with melatonin may explain the reduction of the mortality reported elsewhere in experimental and clinical sepsis after treatment with the indoleamine.

ORIGINAL ARTICLE

Abbreviations:

ATPase – ATP synthase
CLP – cecal ligation and puncture
ETC – electron transport chain
i-mtNOS – inducible mitochondrial nitric oxide synthase
iNOS – inducible nitric oxide synthase
LPO – lipid peroxidation
aMT – melatonin
NO• – nitric oxide
$ONOO^-$ – peroxynitrite
OXPHOS – Oxidative Phosphorylation
RNS – Reactive Nitrogen Species
ROS – Reactive Oxygen Species
SOD – superoxide dismutase

Introduction

Mitochondria are essential organelle to sustain the life because they provide the energy required for almost all cellular processes, including muscle contraction. This energy is generated by the oxidative phosphorylation (OXPHOS) system, through the production of ATP. In this process, mitochondria consume 98% of the oxygen reaching the cell. Mitochondrial failure to produce ATP is involved in many pathologies [38]. Sepsis courses with mitochondrial dysfunction and energy failure, which was related to multiorgan failure and death. Mitochondria are the primary targets for injury in several organs, including skeletal muscle and diaphragm, in sepsis. The cause for mitochondrial dysfunction in sepsis was related to an increase of reactive oxygen (ROS) and nitrogen (RNS) species [14]. Among RNS, nitric oxide (NO•) plays several functions in the mitochondria. Under physiological conditions, NO• regulates the activity of mitochondrial complex IV competing with oxygen by the same site of the enzyme [8]. At high concentrations, however, NO• and peroxynitrite ($ONOO^-$), a product of the reaction between NO• and superoxide anion ($O_2^{\bullet -}$), irreversibly impairs the respiratory chain complexes. Among other mitochondrial enzymes, ATP synthase may be also inactivated during sepsis leading to energy depletion [4, 5, 8, 11, 36]. This situation occurs during sepsis, where the induction of iNOS increases the NO• levels [15, 16, 24, 37]. The recently identified mitochondrial iNOS (i-mtNOS) supports the relationship between an excess of *in situ* production of NO• and mitochondrial dysfunction [17, 18, 25, 46]. Recent data showed that the sepsis-induced i-mtNOS was accompanied by an inhibition of the respiratory complexes [18, 25], an increase in ROS/RNS production and lipid peroxidation (LPO), and a reduction in the antioxidative defense in muscle mitochondria [2, 18, 25]. These alterations did not occur in iNOS deficient ($iNOS^{-/-}$) mice. Others authors have found modifications in mitochondrial respiration [4, 10, 20, 33] and adenine nucleotide levels [6, 7] in different models of sepsis.

The neurohormone melatonin (aMT) is an efficient antioxidant and free radical scavenger, showing antiseptic properties in different experimental and clinical conditions [19]. aMT inhibited the expression and activity of iNOS and the iNOS-induced mutiorgan failure in the LPS model of sepsis [15]. The neurohormone also counteracted i-mtNOS-dependent respiratory chain damage in the cecal ligation and puncture (CLP) model of sepsis in diaphragmatic and hind leg skeletal muscle mitochondria [17, 18, 25]. In different models of mitochondrial dysfunction, aMT also improved the ability of the mitochondria to produce ATP both *in vitro* and *in vivo* [12, 29, 31, 41].

Mitochondrial respiratory chain damage can be responsible for the bioenergetic failure and ventilatory muscle impairment in sepsis [6, 14, 17, 23, 25]. However, there are not studies regarding the role of aMT on the ATP synthase activity and ATP production and its relation to the i-mtNOS induction in sepsis. Thus, we evaluated the mitochondrial complex V activity, adenine nucleotide levels and ATP production in diaphragmatic and skeletal muscle mitochondria of CLP-induced sepsis in $iNOS^{+/+}$ and $iNOS^{-/-}$ mice treated with aMT.

Material and methods

Chemicals

Melatonin, Nembutal, proteinase K, EGTA, HEPES, Tris-HCl, BSA, KCl, mannitol, sucrose, ammonium carbonate, potassium phosphate, succinate, rotenone, AMP, ADP, ATP, magnesium chloride, perchloric acid, trichloroacetic acid, ammonium molybdate and ferrous sulfate were purchased from Sigma-Aldrich (Madrid, Spain). Any other reagents were of highest purity available.

Animals, surgical procedures, and treatment

iNOS knockout B6.129P2-*Nos2*^{tm1Lau} mice ($iNOS^{-/-}$ derived from C57/BL/6 mice) and their respective controls C57/Bl/6 mice ($iNOS^{+/+}$) were obtained from Jackson's Laboratory through Charles River Labs (Barcelona, Spain). The animals were maintained in the University's facility in a 12 h:12 h light/dark cycle (lights on at 07 h) at 22±2 °C and on regular chow and tap water. Animals were used at 12–14 weeks of age and 25–30 g body weight. All experiments were performed according to the Spanish Government Guide and the European Community Guide for animal care. Both $iNOS^{+/+}$ and $iNOS^{-/-}$ mice were divided in the following groups: a) control; b) sham-operated; c) sepsis; d) sepsis + aMT, and e) sepsis + vehicle. The data obtained in sham-operated and vehicle-treated groups did not differ with those found in control animals. Therefore, only control, sepsis and sepsis + aMT groups are shown in the results. Sepsis was induced by CLP [44] in mice anesthetized with equithesin (1 ml/kg, i.p.). Four doses of melatonin (30 mg/kg b.w.) were injected as follows: one dose 30 min before surgery (i.p.); the second dose just after surgery (s.c.), and 4 and 8 h after surgery the other doses (s.c.). Twenty-four hours after CLP, the animals were killed and purified mitochondria from diaphragm and hind leg

skeletal muscle were immediately prepared and frozen to –80 °C until remained assays were performed.

Mitochondria isolation

Samples of diaphragm and skeletal muscle were individually processed to obtain mitochondria by differential centrifugation. All procedures were performed on cold ice. Briefly, diaphragm and skeletal muscle were excised, washed with saline, treated with proteinase K (1 mg/ml) during 30 s and 60 s, respectively, washed with buffer A (220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM HEPES and 1% BSA, pH 7.2, at 4 °C), and homogenized (1/10, w/v) in buffer A at 800 rpm with a Teflon pestle. The homogenates were centrifuged at 1,500 × *g* for 5 min at 4 °C, and the supernatants were centrifuged at 23,000 × *g* for 5 min at 4 °C. Then, the mitochondrial pellets were suspended in 1 ml buffer A and centrifuged at 10,300 × *g* for 3 min at 4 °C. The resultant pellets were used for ATP assay or for measuring ATPase activity.

ATPase activity assay

Mitochondrial ATPase activity was measured following the hydrolysis rate of ATP to ADP + Pi [13]. Briefly, mitochondria (0.25–0.35 mg/ml) were incubated in ATPase buffer (5 mM Cl_2Mg, 50 mM Tris-Cl, pH 7.5) containing 5 mM ATP during 10 min at 37 °C. The reaction was stopped after the addition of 5% trichloroacetic acid and subsequent centrifugation at 3000 × *g* for 20 min at 4 °C. The pellet was keep at –80 °C to protein determination [26]. The supernatant was diluted two times with water and ferrous sulfate-ammonium molybdate reagent was added (1.5/1 v/v) to reaction with Pi [39]. After incubation during 20 min at room temperature, the chromophore was detected at 660 nm in a microplate scanning spectrophotometer. The concentration of Pi in the samples was calculated with a standard curve [40]. ATPase-independent Pi production was also measured after incubation of the mitochondrial samples with 20 µg/ml oligomycin. ATPase activity was expressed in nmol Pi / min / mg prot.

Measurement of adenine nucleotides and ATP production

For the determination of adenine nucleotides and ATP production, diaphragm and skeletal muscle were excised, washed with saline, treated with proteinase K (1 mg/ml) during 30 s and 60 s, respectively, washed again with buffer A (220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM HEPES and 1% BSA, pH 7.2, at 4 °C), and homogenized (1/10, w/v) in the same buffer at 800 rpm at 4 °C with a Teflon pestle. The homogenates were centrifuged at 1,500 × *g* for 5 min at 4 °C, and the supernatants were centrifuged again at 23,000 × *g* for 5 min at 4 °C. Then, the mitochondrial pellets were suspended in 1 ml buffer A and centrifuged at 10,300 × *g* for 3 min at 4 °C. The resultant pellets (p1) were used for adenine nucleotides determination (see below) and for ATP production. For ATP production, p1 fractions were suspended in respiration buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-Cl, 5 mM potassium phosphate, pH 7.2, saturated with O_2, plus 5 mM succinate at 30°C) and ATP production was induced adding 125 nmol ADP. After 45 s, the samples were centrifuged at 13,000 × g for 3 min at 2 °C [28, 32] and the ATP content in the pellets (p2) and supernatants (s1) was measured. Ice-cold 0.5 M perchloric acid was rapidly added to p1, p2 and s1 fractions, mixed during 2 min in vortex and centrifuged at 25,000 × *g* for 15 min

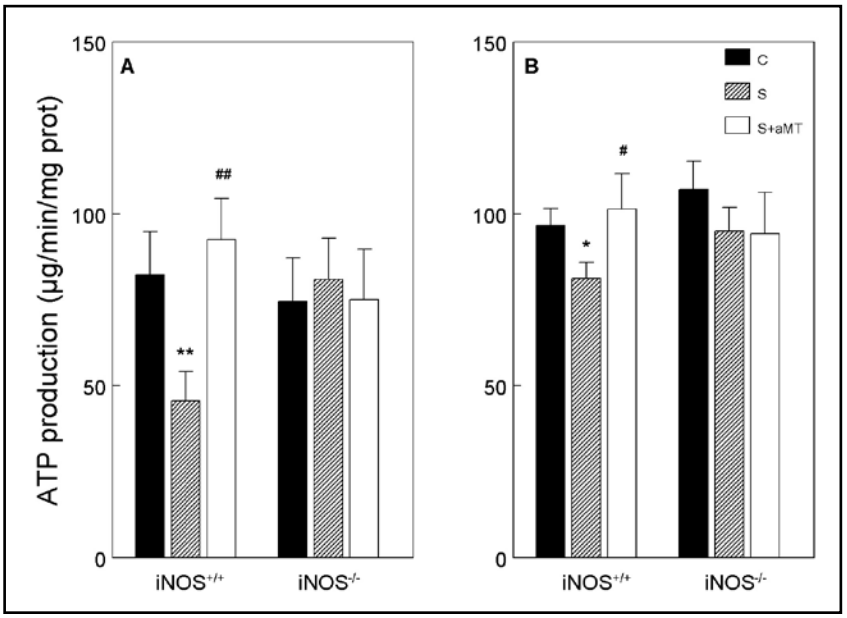

Figure 1. Changes in the mitochondrial production of ATP in hind leg skeletal muscle (A) and diaphragm (B) of $iNOS^{+/+}$ and $iNOS^{-/-}$ mice. C = control, S = sepsis and S + aMT = sepsis + melatonin. **P*<0.05 vs. control; #*P*<0.05 vs. sepsis.

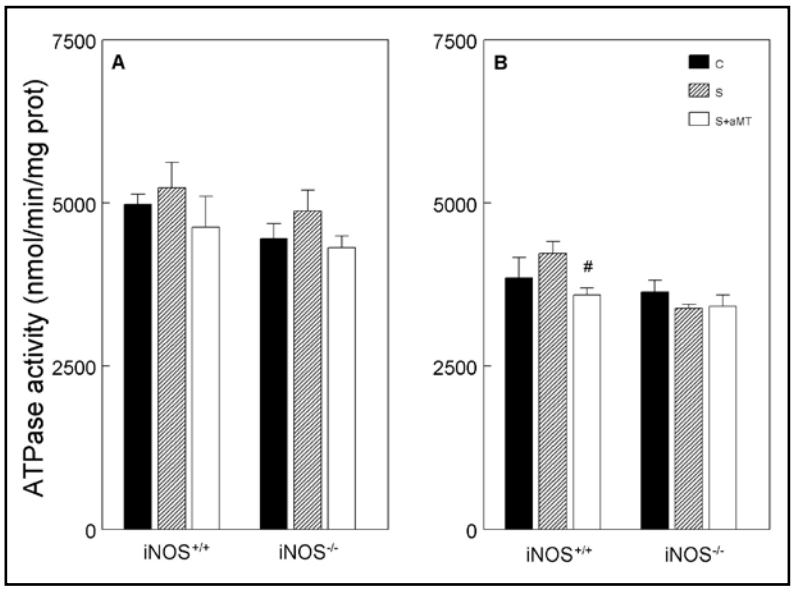

Figure 2. Changes in mitochondrial ATPase activity in hind leg skeletal muscle (A) and diaphragm (B) of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. C = control, S = sepsis and S + aMT = sepsis + melatonin.

at 2 °C to precipitate proteins. The pellets were frozen to –80 °C for protein determination [26] and the supernatants were mixed with 8 µl of 5 M potassium carbonate to neutralize the pH and centrifuged at 12,000 × *g* for 10 min at 2 °C. Adenine nucleotides were measured in the resultant supernatants by HPLC with a 4 × 250 mm ProPac PA1 column (Dionex, Barcelona, Spain) [35]. After stabilizing the column with the mobile phase, samples (20 µl) were injected onto the HPLC system. The mobile phase consisted in water (phase A) and 0.3 M ammonium carbonate pH 8.9 (phase B), and the following time schedule for the binary gradient (flow rate 1 ml/min) was used: 5 min, 50% A and 50% B; 5 min 50% to 100% B and then 100% B for 25 min; 5 min 100% to 50% B and then, another 5 min with 50% B [23]. Water was used for calibration purposes. Standard curves for AMP, ADP and ATP were constructed with 3.125 µg/ml, 6.250 µg/ml, 12.5 µg/ml and 25 µg/ml of each nucleotide. Absorbance of the samples was measured with an UV detector at 254 nm wavelength and the concentration of each nucleotide in the samples was calculated according to the peak area [35]. Adenine nucleotide levels were expressed in µg/mg prot. ATP production was expressed in µg/min/mg prot. Adenine nucleotide levels were expressed in µg/mg prot. ATP production was expressed in µg/min/mg prot. Energy charge was calculated according to the equation (ϕ) = ([ATP]+0.5[ADP])/([ATP]+[ADP]+[AMP]) [3].

Statistical analysis

Data are expressed as the mean ± S.E. of six experiments assayed in duplicate. A Student's *t*-test was used to compare the mean between groups. A *P* value of less than 0.05 was considered to be statistically significant.

Results

ATP production

Figure 1 shows the ATP production by skeletal muscle and diaphragmatic mitochondria. Sepsis reduced significantly the ATP production in skeletal muscle (Fig. 1A) and diaphragmatic (Fig. 1B) mitochondria only in iNOS$^{+/+}$ mice. Administration of aMT normalized the production of ATP by these mitochondria.

Under our conditions, less than 3% of the total nucleotides measured corresponds to AMP (data not shown), discarding extramitochondrial ATP production by adenylate kinase. Besides, our results showed that between 92% and 98% of the ATP produced was measured out of the mitochondria, suggesting an adequate ATP/ADP ratio for OXPHOS.

ATPase activity

Figure 2 shows the activity of ATPase in muscle (A) and diaphragmatic (B) mitochondria. No changes in ATPase activity were observed in any tissue during sepsis. Treatment with aMT only reduced ATPase activity in diaphragm of iNOS$^{+/+}$ mice.

Adenine nucleotide levels

Figure 3 shows the adenine nucleotide levels in skeletal muscle mitochondria. In iNOS$^{-/-}$ mice, the basal ATP/ADP ratio (Fig. 3B) and energy charge (Fig. 3D) were lower, and the basal adenine nucleotide levels (Fig. 3C) higher than in iNOS$^{+/+}$ mice. During sepsis, the ATP/ADP ratio decreased in iNOS$^{+/+}$ and increased in iNOS$^{-/-}$ mice, and these effects were counteracted by aMT administration (Fig. 3B). The energy charge increased during sepsis in iNOS$^{-/-}$ mice only, without further effect of aMT administration (Fig. 3D). ATP content (Fig. 3A) and total adenine nucleotides (Fig. 3C)

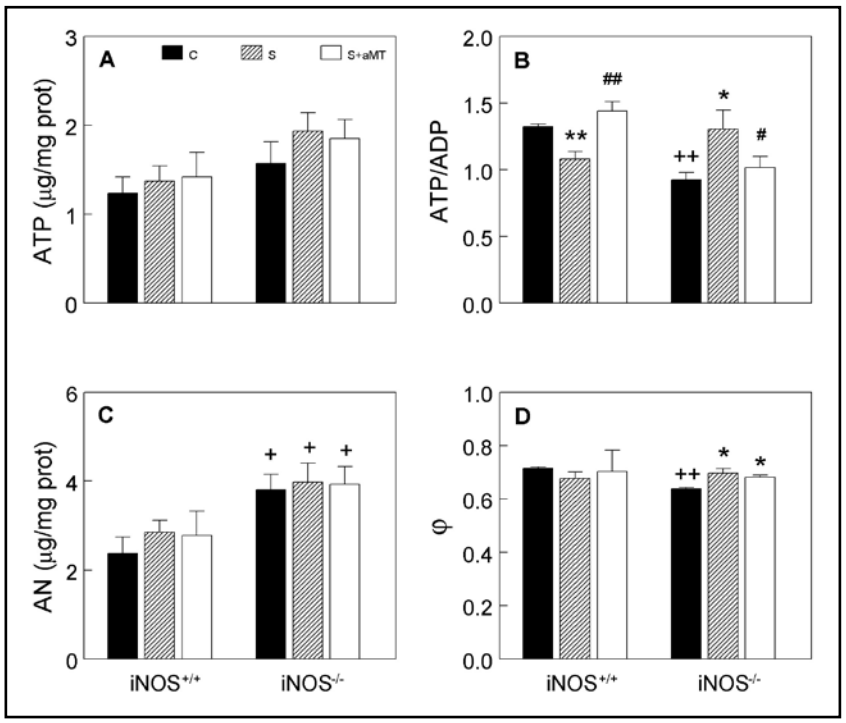

Figure 3. Changes in the mitochondrial adenine nucleotides in hind leg skeletal muscle of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice: (**A**) Changes in ATP levels; (**B**) Changes in ATP/ADP ratio; (**C**) Changes in total adenine nucleotides (AN), and (**D**) Changes in energy charge (φ), calculated as described in the Material and Methods section.
C = control, S = sepsis and S + aMT = sepsis + melatonin.
*P<0.05 and **P<0.01 vs. control; $^{\#\#}P$<0.01 vs. sepsis; ^{+}P<0.05 and ^{++}P<0.01 vs. iNOS$^{+/+}$ mice.

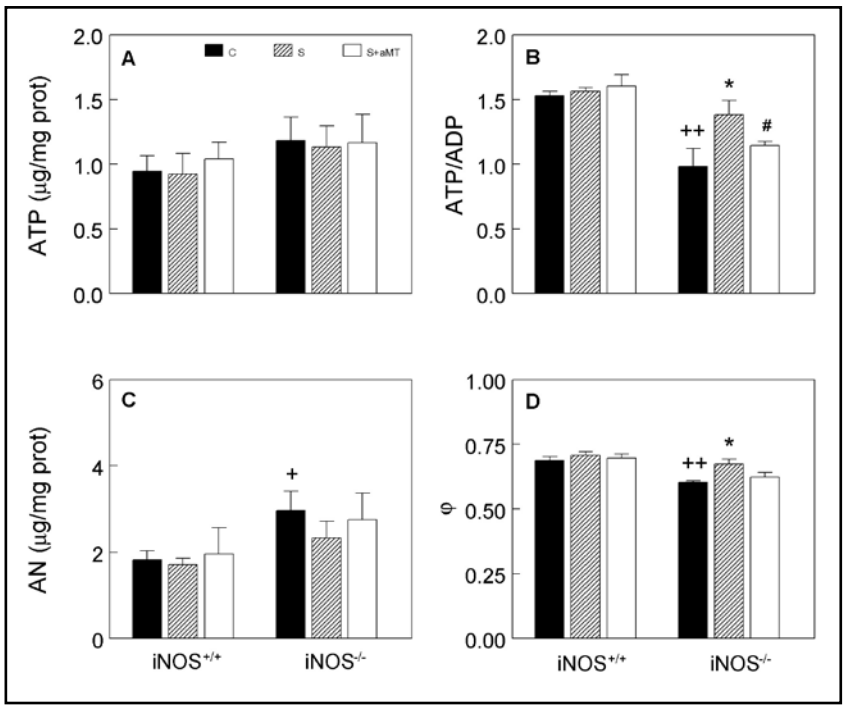

Figure 4. Changes in the mitochondrial adenine nucleotides in diaphragm of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice: (**A**) Changes in ATP levels; (**B**) Changes in ATP/ADP ratio; (**C**) Changes in total adenine nucleotides levels (AN), and (**D**) Changes in energy charge (φ), calculated as described in the Material and Methods section.
C = control, S = sepsis and S + aMT = sepsis + melatonin.
*P<0.05 and **P<0.01 vs. control; ^{+}P<0.05 and ^{++}P<0.01 vs. iNOS$^{+/+}$ mice.

did not change in any mice strain during sepsis or aMT treatment

Figure 4 shows the adenine nucleotide levels in diaphragmatic mitochondria. Similar to the data found in skeletal mitochondria, the lack of iNOS was accompanied by a reduction of basal ATP/ADP ratio (Fig. 4B) and energy charge (Fig. 4D), and an increase in basal adenine nucleotide levels (Fig. 4C) compared with $iNOS^{+/+}$ mice. During sepsis, the ATP/ADP ratio did not change in $iNOS^{+/+}$ mice, whereas it increased in $iNOS^{-/-}$ animals (Fig. 4B). aMT treatment counteracted the sepsis-induced ATP/ADP ratio in the latter. The energy charge increased during sepsis in $iNOS^{-/-}$ but not in $iNOS^{+/+}$ mice (Fig. 4D), and aMT treatment did not modified this effect. Also, mitochondrial content of ATP (Fig. 4A) and total adenine nucleotides (Fig. 4C) did not change in any mice strain during sepsis or aMT administration.

Discussion

Our results show that the ability of the ATP production by diaphragmatic and skeletal muscle mitochondria was significantly reduced during sepsis in $iNOS^{+/+}$ but not in $iNOS^{-/-}$ mice. Decreased ATP production leads to energy collapse, a finding that might be related to the diaphragmatic and skeletal muscle failure in sepsis. Our results also found that the administration of the neurohormone aMT normalized the mitochondrial production of ATP in $iNOS^{+/+}$ mice, thus providing a protective mechanism against muscle failure during sepsis.

Typically, sepsis is accompanied by an induction of iNOS, producing high amounts of NO that participate in the cell and organ failure. It was suggested that the NO produced by iNOS might be also responsible for the mitochondrial failure during endotoxemia [9]. The presence of the i-mtNOS, a NOS isoform sharing kinetic properties with iNOS, was recently confirmed in the mitochondria from several tissues including diaphragm, skeletal muscle, and liver of wild type animals [17, 18, 25]. The induction of i-mtNOS during sepsis in $iNOS^{+/+}$ mice after CLP coursed with oxidative/nitrosative stress and a reduction of the mitochondrial respiratory complexes activity [18, 25]. Because these changes did not occur in $iNOS^{-/-}$ mice, it was suggested that i-mtNOS induction, through the production of elevated amounts of NO, was the responsible for the ETC damage in sepsis [1]. Moreover, the lack of i-mtNOS expression in control and septic $iNOS^{-/-}$ mice, suggested that this isoform derives form cytosolic iNOS and it is coded by the same gene [18, 25].

Although sepsis did not modified the ATP synthase activity in any mice strain, our data showed a reduction in the mitochondrial production of ATP in $iNOS^{+/+}$ but not in $iNOS^{-/-}$ mice. The reduction in the ATP production accounted by a 44.5% and 16% in skeletal muscle and diaphragmatic mitochondria, respectively. These differences agree with the respiratory inhibition during sepsis published elsewhere [18, 25]. In fact, sepsis caused a higher reduction of the activity of the complexes I and IV in skeletal muscle. that in diaphragm. These data also correlate with the different expression of i-mtNOS during sepsis, because its activity was three times higher in skeletal muscle than in diaphragmatic mitochondria [18, 25]. Thus, CLP-induced sepsis impaired ETC but not ATP synthase, a finding also reported in the early phases of the innate inflammatory response coursing with iNOS induction [42]. Two main conclusions can be obtained from these results. Firstly, the complex V seems to be more resistant to nitrosative/oxidative stress during sepsis than the ETC complexes. This difference probably depends on the different structural composition of these enzymes, because ETC complexes but not ATP synthase contain Fe-S redox centers, that are very sensitive NO damage [34]. Secondly, in absence of ATP synthase damage, the inhibition of the respiratory chain can reduce the membrane potential and the proton-motive force, shifting the energy status [39].

It is interesting to note that $iNOS^{-/-}$ mice showed lower basal values of ATP/ADP ratio and energy charge than $iNOS^{+/+}$ animals. These findings may reflect the differences in the basal respiratory chain activity among these mice strains reported elsewhere [18, 25]. Moreover, the increase in ATP/ADP ratio and energy charge in septic $iNOS^{-/-}$ mice can reflect the slight increase in the mitochondrial respiratory complexes found in these conditions. Changes in the energy state suggest that NO produced by i-mtNOS can have additional regulatory effects on the respiratory chain than that known hitherto. Anyway, both mice strains, $iNOS^{+/+}$ and $iNOS^{-/-}$ mice, had energy charge values higher than 0.68, supporting available energy to maintain the cellular process.

Increasing evidences support that aMT reduces mitochondrial oxidative stress and increases the activity of the respiratory chain, increasing the ATP production in several models of mitochondrial dysfunction including aging and sepsis [12, 17, 18, 25, 29–31]. In our hands, aMT counteracted the sepsis-induced inhibition of the ATP production in in $iNOS^{+/+}$ mice, whereas the indoleamine had not effect on the production of ATP in $iNOS^{-/-}$ mice. Several reports supports the role of aMT to recover the ETC activity from oxidative/nitrosative damage [1, 26, 29–31]. Besides its antioxidative activity, aMT also inhibits the expression and activity of both iNOS and i-mtNOS [15, 17]. Thus, counteracting the i-mtNOS-dependent oxidative/nitrosative stress, aMT improved the ETC activity that, in absence of ATP synthase activity modification, was able to increase the ATP production [39]. The normalization of the ATP production yields energy available for muscle contraction, thus avoiding the respiratory muscle failure and reducing the mortality in sepsis [19, 21]. Moreover, the effects of aMT administration on the ATP/ADP ratio and energy charge in both tissues, further support the role of the indoleamine in mitochondrial homeostasis [1].

In conclusion, our results show that sepsis produced a decrease in the ATP production by diaphragmatic and

hind leg skeletal muscle mitochondria in iNOS$^{+/+}$ mice. These effects were not due to an impairment of the ATP synthase activity, and probably reflect the reduction of the ETC activity in these conditions. Indeed, the fact that sepsis did not affected the ATP production in iNOS$^{-/-}$ mice, supports previous data showing the relationships between endotoxemia, i-mtNOS induction and ETC failure [18, 25]. The sepsis-induced energy deficit was higher in hind leg skeletal muscle than in diaphragm, and it can be related to ventilatory muscle failure during endotoxemia [6, 14, 16, 22, 24]. Finally, administration of aMT to septic iNOS$^{+/+}$ mice restored the normal ATP production in the tissues analyzed. This action of aMT probably underlines the increase of survival in septic animals [19, 27, 43, 45] and in human neonates treated with the indolamine [21].

Acknowledgments

This study was partially supported by grants FIS01/1076, PI03/0817 and G03/137 from the Instituto de Salud Carlos III (Spain), and Consejería de Educación, Junta de Andalucía (CTS-101). LCL is a postdoctoral fellow from the Ministerio de Educación (Spain). FO is a fellow from the Instituto de Salud Carlos III (Spain).

REFERENCES

1 Acuña-Castroviejo D, Escames G, López LC, Hitos AB, León J. Melatonin and nitric oxide: two required antagonists for mitochondrial homeostasis. Endocrine 2005; **27:** 159–68.
2 Álvarez S, Bóveris A. Mitochondrial nitric oxide metabolism in rat muscle during endotoxemia. Free Radic Biol Med 2004; **37:** 1472–78.
3 Ataullakhanov FI, Vitvitsky VM. What determines the intracellular ATP concentration. Biosci Rep 2002; **22:** 501–11.
4 Boczkowski J, Lisdero C, Lanone S, Samb A, Carreras MC, Boveris A, et al. Endogenous peroxynitrite mediates mitochondrial dysfunction in rat diaphragm during endotoxemia. FASEB J 1999; **13:** 1637–46.
5 Boveris A, Costa LE, Poderoso JJ, Carreras MC, Cadenas E. Regulation of mitochondrial respiration by oxygen and NO. Ann N Y Acad Sci 2000; **899:** 121–35.
6 Brealey D, Brand M, Hargreaves I, Heales S, Land J, Smolenshi R, et al.. Association between mitochondrial dysfunction and severety and outcome of septic shock. Lancet 2002; **360:** 219–23.
7 Brealey D, Karyampudi S, Jacques TS, Novelli M, Stidwill R, Taylor V, et al. Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. Am J Physiol Regul Comp Physiol 2004; **286:** R491–R497.
8 Brown GC. Regulation of mitochondrial respiration by nitric oxide inhibition of citochrome c oxidase. Biochim Biophys Acta 2001; **1504:** 46–57.
9 Callahan LA, Supinski GS. Downregulation of diaphragm electron transport chain and glycolytic enzyme gene expression in sepsis. J Appl Physiol 2005; **99:** 1120–6.
10 Callahan LA, Supinski GS. Sepsis induces diaphragm electron transport chain dysfunction and protein depletion. Am J Respir Crit Care Med 2005; **172:** 861–68.
11 Cassina AM, Hodara R, Souza JM, Thomson L, Castro L, Ischiropoulos H, et al. Cytochrome *c* nitration by peroxynitrite. J Biol Chem 2000; **275:** 21409–15.
12 Castillo C, Salazar V, Ariznavarreta C, Vara E, Tresguerres JAF. Effect of melatonin administration on parameters related to oxidative damage in hepatocytes isolated from old wistar rats. J Pineal Res 2005; **38:** 240–46.
13 Cormier A, Morin C, Zini R, Tillement JP, Lagrue G. In vitro effects of nicotine on mitochondrial respiration and superoxide anion generation. Brain Res 2001; **900:** 72–9.
14 Crouser ED. Mitochondrial dysfunction in septic shock and multiple organ dysfunction syndrome. Mitochondrion 2004; **4:** 729–41.
15 Crespo E, Macías M, Pozo D, Escames G, Martín M, Vives F, et al. Melatonin inhibits expression of the inducible NO synthase II in liver and lung and prevents endotoxemia in lipopolysaccharide-induced multiple organ dysfunction syndrome in rats. FASEB J 1999; **13:** 1537–1546.
16 El-Dwari Q, Comtois A, Guo Y, Hussain SNA. Endotoxin-induced skeletal muscle contractile dysfunction: contribution of nitric oxide synthases. Am J Physiol 1998; **274:** C770–C779.
17 Escames G, León J, Macías M, Khaldy H, Acuña-Castroviejo D. Melatonin counteracts lipopolysaccheride-induced expression and activity of mitochondrial nitric oxide synthase in rats. FASEB J 2003; **17:** 932–934.
18 Escames G, López LC, Tapias V, Utrilla P, Reiter RJ, Hitos AB, et al. Melatonin counteracts inducible mitochondrial nitric oxide synthase-dependet mitochondrial dysfuntion in skeletal muscle of septic mice. J Pineal Res 2006; **40:** 71–78.
19 Escames G, Acuña-Castroviejo D, López LC, Tan DX, Maldonado D, Sánchez-Hidalgo M, et al. The pharmacological utility of melatonin in the treatment of septic shock: experimental and clinical evidence. J Pharm Pharmacol 2006; in press.
20 Gellerich FN, Trumbeckaite S, Opalka JR, Gellerich JF, Chen Y, Neuhof C, et al. Mitochondrial dysfunction in sepsis: evidence from bacteraemic baboons and endotoxaemic rabbits. Biosci Rep 2002; **22:** 99–113.
21 Gitto E, Karbownik M, Reiter RJ, Tan DX, Cuzzocrea S, Chiurazzi P, et al. Effects of melatonin treatment in septic newborns. Pediatr Res 2001; **50:** 756–60.
22 Hussain SNA. Respiratory muscle dysfunction in sepsis. Mol Cell Biochem 1998; **179:** 125–38.
23 Krauss GJ, Pissarek M, Blasing I. HPLC of nucleic acid components with volatile mobile phases: Part 2. Separations on polymeric supports. J. High. Resolut Chromatogr 1997; **20:** 693–96.
24 Lanone S, Mebazaa A, Heymes C, Henin D, Poderoso JJ, Panis Y, et al. Muscular contractile failure in septic patients. Role of the inducible nitric oxide synthase. Am J Respir Crit Care Med 2000; **162:** 2308–15.
25 López LC, Escames G, Tapias V, Utrilla P, León J, Acuña-Castroviejo D. Identification of an inducible nitric oxide synthase in diaphragm mitochondria from septic mice. Its relation with mitochondrial dysfunction and prevention by melatonin. Int J Biochem Cell Biol 2000; **38:** 267–78.
26 Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951; **193:** 265–75.
27 Maestroni GJM. Melatonin as a therapeutic agent in experimental endotoxic shock. J Pineal Res 1996; **20:** 284–89.
28 Manfredi G, Yang L, Gajewski CD, Mattiazzi M. Measurements of ATP in mammalian cells. Methods 2002; **26:** 317–26.
29 Martín M, Macías M, Escames G, León J, Acuña-Castroviejo D. Melatonin but not vitamins C and E maintains glutathione homeostasis in t-butyl hydroperoxide-induced mitochondrial oxidative stress. FASEB J 2000; **14:** 1677–9.
30 Martín M, Macías M, Escames G, Reiter RJ, Agapito MT, Ortiz GG, et al. Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo. J Pineal Res 2000; **28:** 242–8.

31 Martín M, Macías M, León J, Escames G, Khaldy H, Acuña-Castroviejo D. Melatonin increases the activity of the oxidative phosphorylation enzymes and the production of ATP in rat brain and liver mitochondria. Int J Biochem Cell Biol 2002; **1212:** 1–10.
32 Monteiro P, Duarte AI, Moreno A, Gonçalves LM, Providência LA. Carvedilol improves energy production during acute global myocardial ischemia. Eur J Pharmacol 2003; **482:** 245–53.
33 Nin N, Cassina A, Boggie J, Alfonso E, Botti H, Peluffo G, et al. Septic diaphragmatic dysfunction is prevented by Mn(III)porphyrin therapy and inducible nitric oxide synthase inhibition. Intensive Care Med 2004; **30:** 2171–8.
34 Pearce LL, Epperly MW, Greenberger JS, Pitt BR. Identification of respiratory complexes I and III as mitochondrial sites of damage following exposure to ionizing radiation and nitric oxide. Nitric Oxide 2001; **5:** 128–36.
35 Pissarek M, Reinhhardt R, Reichelt C, Manaenko A, Krauss GJ, Illes P. Rapid assay for one-run determination of purine and pyrimidine mucleotide contents in neocortical slices and cell cultures. Brain Res Protoc 1999; **4:** 314–21.
36 Poderoso JJ, Carreras MC, Lisdero C, Riobó N, Schöpfer F, Boveris A. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. Arch Biochem Biophys 1996; **328:** 85–92.
37 Riedemann NC, Guo RF, Ward PA. The enigma of sepsis. J Clin Invest 2003; **112:** 460–7.
38 Scheffler IE. A century of mitochondrial research: achievements and perspectives. Mitochondrion 2001; **1:** 3–31.
39 Skulachev VP. Mitochondrial physiology and pathology; concepts of programmed death of organelles, cells and organisms. Mol Aspects Med. 1999 Jun;20(3):139–84. Review.
40 Taussky HH, Shorr E. A microcolorimetric method for the determination of inorganic phosphorous. J Biol Chem 1953; **202:** 675–85.
41 Vairetti M, Ferrigno A, Bertone R, Rizzo V, Richelmi P, Berte F, et al. Exogenous melatonin enhances bile flow and ATP levels after cold storage and reperfusion in rat liver: implications for liver transplantation. J Pineal Res 2005; **38:** 223–30.
42 Vyatkina G, Vandanajay B, Gerstner A, Papaconstantinou J, Garg N. Impaired mitochondrial respiratory chain and bioenergetics during chagasic cardiomyopathy development. Biochim Biophys Acta 2004; **1689:** 162–73.
43 Wichmann MW, Haisken JM, Ayala A, Chaudry IH. Melatonin administration following hemorrhagic schock decreases mortality from subsequent septic challenge. J. Surg Res 1996; **65:** 109–14.
44 Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock-a review of laboratory models and a proposal. J Surg Res 1980; **29:** 189–201.
45 Wu CC, Chiao CW, Hsiao G, Chen A, Yen MH. Melatonin prevents endotoxic-induced circulatory failure in rats. J Pineal Res 2001; **30:** 147–56.
46 Zanella B, Giordano E, Muscari C, Zini M, Guarnieri C. Nitric oxide synthase activity in rat cardiac mitochondria. Basic Res Cardiol 2004; **99:** 159–64.