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Melatonin counteracts inducible mitochondrial nitric oxide synthase-dependent mitochondrial dysfunction in skeletal muscle of septic mice

Abstract: Mitochondrial nitric oxide synthase (mtNOS) produces nitric oxide (NO) to modulate mitochondrial respiration. Besides a constitutive mtNOS isoform it was recently suggested that mitochondria express an inducible isoform of the enzyme during sepsis. Thus, the mitochondrial respiratory inhibition and energy failure underlying skeletal muscle contractility failure observed in sepsis may reflect the high levels of NO produced by inducible mtNOS. The fact that mtNOS is induced during sepsis suggests its relation to inducible nitric oxide synthase (iNOS). Thus, we examined the changes in mtNOS activity and mitochondrial function in skeletal muscle of wild-type ($iNOS^{+/+}$) and iNOS knockout ($iNOS^{-/-}$) mice after sepsis. We also studied the effects of melatonin administration on mitochondrial damage in this experimental paradigm. After sepsis, $iNOS^{+/+}$ but no $iNOS^{-/-}$ mice showed an increase in mtNOS activity and NO production and a reduction in electron transport chain activity. These changes were accompanied by a pronounced oxidative stress reflected in changes in lipid peroxidation levels, oxidized glutathione/reduced glutathione ratio, and glutathione peroxidase and reductase activities. Melatonin treatment counteracted both the changes in mtNOS activity and rises in oxidative stress; the indole also restored mitochondrial respiratory chain in septic $iNOS^{+/+}$ mice. Mitochondria from $iNOS^{-/-}$ mice were unaffected by either sepsis or melatonin treatment. The data suggest that inducible mtNOS, which is coded by the same gene as that for iNOS, is responsible for mitochondrial dysfunction during sepsis. The results also suggest the use of melatonin for the protection against mtNOS-mediated mitochondrial failure.

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Key words: electronic transport chain, melatonin, mitochondrial nitric oxide synthase, nitric oxide, oxidative stress, sepsis, skeletal muscle

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Introduction

Sepsis is an exaggerated whole-body inflammatory response to infection that may lead to multiple organ failure. Respiratory insufficiency is considered to be the most important cause of death in patients with sepsis. Classically, respiratory deficiency was attributed to lung injury, but there is growing evidence that sepsis is associated with ventilatory pump failure [1]. The ventilatory failure depends on a reduction in contractility of the skeletal muscles involved in respiration such as the diaphragm and intercostal muscles. Both muscle types are similarly affected in sepsis and free radical species are involved in this injury [2, 3]. Specifically, the reduced muscle force in sepsis is related to the increase in the expression and activity of inducible nitric oxide synthase (iNOS) and subsequent increase of nitric oxide (NO) levels [4, 5]. The long half-life of NO, which is between 6 and 8 s, allows this gas to reach mitochondria where it induces protein nitration [6], which seems to participate in mitochondrial failure and muscle dysfunction [7, 8].

Under physiological conditions, NO regulates the activity of complex IV by competing with oxygen (O_2) for its binding site at complex IV [9]. When NO increases, it binds to the bc_1 segment of the cytochrome *c* reductase (complex III), producing similar effects to antimycin and, at higher concentrations, NO can react with ubiquinol, which leads to ubisemiquinone formation [10]. The inhibition of the electron transport chain (ETC) by NO facilitates electron leakage and the formation of the superoxide anion ($O_2^{\bullet -}$). In turn, NO reacts with $O_2^{\bullet -}$ yielding peroxynitrite ($ONOO^-$) which irreversibly inhibits the four complexes of the ETC and ATP synthase and oxidizes membrane lipids [9, 11]. Therefore, the induction of mitochondrial nitric oxide synthase (mtNOS) and the subsequent increase in intramitochondrial NO may lead to an oxidative damage of the mitochondria.

To counteract oxidative stress, mitochondria possess antioxidant mechanisms including superoxide dismutase and glutathione (GSH). High levels of melatonin were also reported in mitochondria, where they exert an efficient

antioxidant role [12–14]. Melatonin is a powerful scavenger of both reactive oxygen and nitrogen species [12, 14–16]. Melatonin also exerts an indirect antioxidant action by increasing the mitochondrial GSH pool and stimulating the activity of the enzymes for GSH synthesis [17]. The indoleamine also increases the enzymes of the GSH redox cycle, glutathione peroxidase (GPx) and reductase (GRd) [18]. Studies performed both in vitro and in vivo show that melatonin improves respiratory chain activity and increases ATP production [19–22].

The concept that mitochondrial dysfunction in sepsis depends on the cytosolic NO produced by iNOS induction should be revised [23, 24]. The characterization of a constitutive isoform of mtNOS derived from the cytosolic nNOS isoform and coded by the same gene [25] supports a role for the intramitochondrial production of NO in mitochondrial physiology. However, the existence of an inducible mtNOS isoform was recently reported [26]. The increase in the expression and activity of this inducible mtNOS may be the source of the excess of intramitochondrial NO levels noted in sepsis [26], which in turn may be responsible for mitochondrial dysfunction during inflammation.

We therefore analyzed the source and effects of the inducible mtNOS on mitochondrial function in the septic model of cecal ligation and puncture (CLP), which closely resembles human disease [27]. We studied the changes in mtNOS activity and NO production and their relation to the activities of the ETC complexes and mitochondrial oxidative stress in skeletal muscle mitochondria 24 hr after sepsis induction. The use of iNOS knockout mice allowed us to show the presence of both constitutive and inducible mtNOS isoforms, the latter being coded by the iNOS gene. We also report the involvement of induced mtNOS on skeletal muscle mitochondrial dysfunction in sepsis; furthermore, we examined the protective role of melatonin against mtNOS-induced mitochondrial failure.

Materials and methods

Chemicals

L-(2,3,4,5-^{3}H)-arginine monohydrochloride (58 Ci/mmol) was obtained from Amersham Biosciences Europe GmBH (Barcelona, Spain). Liquid scintillation cocktail (Ecolume) was purchased from ICN (Madrid, Spain). All other chemicals and reagents were purchase from Sigma-Aldrich (Madrid, Spain).

Animals, surgery, and treatment

The 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 12:12 light/dark cycle (lights on at 07:00 hr) and 22 ± 2°C, with regular chow and tap water. Animals were used at 12–14 wk 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 wild type and iNOS knockout mice were divided in the following groups comprising 48 animals per group: (a) control; (b) sham-operated; (c) sepsis; (d) sepsis + melatonin. Corresponding groups injected with vehicle were also included. 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 + melatonin groups are shown in the results. Sepsis was induced by CLP [28] in mice anesthesized 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 third and fourth doses at 4 and 8 hr after surgery (s.c.). Twenty-four hours after CLP, the animals were killed and purified mitochondria from both hind leg skeletal muscle were immediately prepared and frozen to −80°C until assays were performed.

Mitochondrial isolation and purification

Skeletal muscles were individually processed and to obtain pure mitochondria by differential centrifugation and Percoll density gradient, as previously reported [25, 29], with slight modifications [26]. All procedures were performed on ice. Briefly, skeletal muscle was excised, washed with saline, treated with proteinase K (1 mg/mL) for 60 s, washed with buffer A (250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES and 0.1% BSA, pH 7.4, at 4°C), and homogenized (1/10, w/v) in buffer A at 800 rpm with a Teflon pestle. The homogenate was centrifuged at 600 × g for 5 min at 4°C (twice), and the supernatants were mixed and centrifuged at 10,300 × g for 10 min at 4°C. Then, the mitochondrial pellets were suspended in 0.5 mL buffer A and poured into ultracentrifuge tubes containing 1.4 mL buffer B (225 mM mannitol, 1 mM EGTA, 25 mM HEPES and 0.1% BSA, pH 7.4, at 4°C) and 0.6 mL Percoll. The mixture was centrifuged at 105,000 × g for 30 min at 4°C. The fraction with a density of 1052–1075 g/mL, corresponding to a pure mitochondrial fraction was collected, washed twice with buffer A at 10,300 × g for 10 min at 4°C to remove the Percoll, and frozen to −80°C.

Assay of mtNOS activity

An aliquot of frozen mitochondria was thawed and homogenized (0.1 g/mL) in ice-cold buffer (25 mM Tris, 0.5 mM DTT, 10 μg/mL pepstatin, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM PMSF, pH 7.6) at 0–4°C. The crude homogenate was centrifuged at 2500 × g for 5 min at 4°C, and aliquots of the supernatants were either stored at −80°C for total protein determination [30] or used immediately for NOS activity determination [26]. The final incubation volume was 100 μL (2 mg/mL protein) and consisted of 10 μL sample added to prewarmed (37°C) buffer to give a final concentration of 25 mM Tris, 1 mM DTT, 30 μM H^{4}-biopterin, 10 μM FAD, 0.5 mM inosine, 0.5 mg/mL BSA, 0.1 mM $CaCl_2$, 10 μM L-arginine, and 40 nM L-^{3}arginine, pH 7.6. The reaction was initiated by the addition of 10 μL NADPH (0.75 mM final concentration) and continued for 30 min at 37°C. To determine the Ca^{+2}-independent activity of mtNOS, 10 mM EDTA was added

to the buffer before beginning of the reaction. Control incubations were performed in absence of NADPH. The reaction was stopped by adding 400 μL of cold 0.1 M HEPES containing 10 mM EGTA and 0.175 mg/mL L-citrulline, pH 5.5. The mixture was decanted onto a 2 mL column packed with Dowex −50 W ion exchanger resin (Na^+ form) and eluted with 1.2 mL of water. L-^{3}H-citrulline was quantified by liquid scintillation spectroscopy. The retention of L-^{3}H-arginine in this process was >98%. NOS activity was measured by monitoring the conversion of L-^{3}H-arginine to L-^{3}H-citrulline [31]. Enzyme activity is expressed as picomoL L-^{3}H-citrulline/min/mg protein.

Nitrite determination

Mitochondrial fractions were thawed and suspended in ice-cold distilled water and immediately sonicated to break mitochondria membranes. Aliquots of these samples were either stored at −80°C for total protein determination [30] or used to calculate nitrite levels. Nitrite levels (in nmoL nitrite/mg protein) were determined using the Griess reaction [32] which converts nitrite into a colored azo compound spectrophotometrically detected at 550 nm.

Determination of mitochondrial oxidative stress

Glutathione and GSH were measured spectrophotometrically [33]. Mitochondrial fractions were resuspended in 350 μL of 2.5% sulfosalicylic acid, sonicated and centrifuged at 1000 × g for 15 min at 4°C and the supernatants were used immediately for glutathione assay. The concentration of both GSH and GSSG is expressed in nmoL/mg protein. For GPx and GRd activities determination, mitochondrial fractions were suspended in 200 μL of phosphate buffer and sonicated. GPx activity was measured following the oxidation of NADPH for 3 min at 340 nm [34]; GRd activity was measured following NADPH oxidation for 3 min at 340 nm [34] in a UV spectrophotometer (Shimadzu Deutschland GmBH, Duisburg, Germany). The enzyme activity is expressed as nmoL/min/mg protein. For lipid peroxidation (LPO) determination, mitochondrial fractions were suspended in 20 mM Tris-HCl buffer and sonicated. Aliquots of these samples were used for malondialdehyde and 4-hydroxyalkenals determination (Bioxytech LPO-568 assay kit; OxisResearch, Portland, OR, USA) [35]. The concentration of LPO is expressed in nmoL/mg protein. Protein concentration was determined with BSA as standard [30].

Determination of respiratory complex activities

Mitochondrial pellets were thawed, suspended in 350 μL of the incubation medium corresponding to the complex to be measured and immediately sonicated to prepare submitochondrial particles. Mitochondrial protein concentration was measured using BSA as standard [30]. Complex I activity (NADH CoQ oxidoreductase, in nmoL oxidized NADH/min/mg protein) was measured in the presence of decylubiquinone and succinate as the rotenone-sensitive decrease in NADH at 340 nm [36]. The activity of complex II [succinate: 2,6-dichloroindophenol (2,6-DCIP) oxoreductase, in nmoL reduced DCIP/min/mg protein] was measured in the presence decylubiquinone plus rotenone as the antimycin A-sensitive reduction of 2,6-DCIP at 600 nm with 520 nm as reference wavelength [37]. Complex III activity (ubiquinol: cytochrome *c* oxoreductase, in nmoL reduced cytochrome *c*/min/mg protein) was measured in the presence of rotenone and decylubiquinone following the rate of reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength [37]. Complex IV activity (cytochrome *c* oxidase, in nmoL oxidized cytochrome *c*/min/mg protein) was measured as the disappearance of reduced cytochrome *c* at 550 nm [36].

Statistical analysis

All data in the figures and text are expressed as means ± S.E.M. of *n* observations, where *n* represents the number of animals studied. A two-way ANOVA followed by Dunnet's post hoc test, when appropriate, was used to compare means between groups. A *P*-value < 0.05 was considered to be statistically significant.

Results

In basal conditions, iNOS$^{+/+}$ mice constitutively express two components of mtNOS, i.e. constitutive, Ca^{2+}-dependent mtNOS activity, and inducible, Ca^{2+}-independent mtNOS activity (Fig. 1). For convenience and because the former was identified as derived from nNOS, a constitutive form of NOS, herein we use the abbreviations mtcNOS and mtiNOS for the constitutive and inducible mtNOS activities, respectively. In iNOS$^{+/+}$ mice, sepsis induced a significant increase in mtiNOS activity ($P < 0.001$), whereas mtcNOS remained unchanged. Control iNOS$^{-/-}$ mice only exhibited mtcNOS activity which was unaffected by sepsis. Melatonin administration counteracted sepsis-induced mtiNOS activity in iNOS$^{+/+}$ ($P < 0.001$), but it

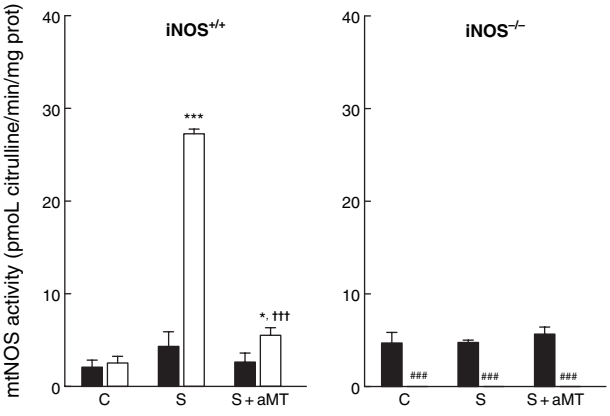

Fig. 1. Mitochondrial NOS activities measured in skeletal muscle mitochondria. Black bars represent constitutive (mtcNOS) and open bars inducible (mtiNOS) mtNOS activities. C, control, S, sepsis, S + aMT, sepsis plus melatonin. $^*P < 0.05$ and $^{***}P < 0.001$ versus control; $^{\dagger\dagger\dagger}P < 0.001$ versus sepsis, and $^{\#\#\#}P < 0.001$ versus iNOS$^{+/+}$.

Table 1. Characterization of the purity degree of skeletal muscle mitochondria

	Location	Homogenate	Mitochondria	Ratio	% Contamination
Alcaline phosphatase nmoL/min/mg protein	Plasma membrane	12.71 ± 0.26	15.15 ± 0.29	1.19	1.70
NADPH cytochrome *c* reductase nmoL/min/mg protein	Microsomes	3.28 ± 0.22	1.50 ± 0.38	0.46	0.65
Catalase μmoL/min/mg protein	Peroxisomes	0.421 ± 0.04	0.486 ± 0.09	1.73	2.00

did not affect mtcNOS activity in either mouse strain (Fig. 1).

The technique for NOS measurement specifically detects mtNOS activity [26]. In fact, addition of L-NMMA (300 μM) to the reaction mixture of mitochondrial samples from septic mice blocked the transformation of L-arginine to L-citrulline because of mtNOS inhibition (data not shown).

To guarantee the mitochondrial source of mtNOS activity, the purity of mitochondrial preparations used in our study was assessed by several procedures. Skeletal muscle mitochondria were isolated by differential centrifugation, and purified by Percoll centrifugation [26, 29]. Purified mitochondria were washed with high ionic strength solution (150 mM KCl) to yield a highly pure mitochondrial preparation without contaminating organelles and broken mitochondria, as assessed by electron microscopy (data not shown). Measurement of the activity of specific plasma membrane, microsome and peroxisome markers indicated a low percentage of mitochondrial contamination by extra-mitochondrial structures (Table 1) [25]. Finally, the presence of NOS activity and NO levels in the supernatant at the final centrifugation step yielded negative results (data not shown). The data confirmed the purity of the mitochondria used in our experiments and ensured the mitochondrial origin of the mtNOS activity measured in these samples.

Mitochondrial nitrite significantly increased ($P < 0.01$) in iNOS$^{+/+}$ mice after sepsis while melatonin treatment reduced nitrite levels to control values ($P < 0.01$) (Fig. 2). Nitrite concentration, however, remained unchanged in septic iNOS$^{-/-}$ mice ($P < 0.05$).

The LPO levels significantly increased ($P < 0.01$) in septic iNOS$^{+/+}$ mice, whereas melatonin administration counteracted this effect ($P < 0.001$) (Fig. 2). LPO levels were not modified by sepsis or melatonin administration in iNOS$^{-/-}$ mice.

Sepsis significantly ($P < 0.01$) reduced mitochondrial content of total GSH in iNOS$^{+/+}$ mice (Fig. 3), an effect mainly due to the loss of reduced GSH ($P < 0.001$). Changes in GSSG concentration in septic iNOS$^{+/+}$ mice ($P < 0.01$) caused a significant increase in the GSSG/GSH ratio ($P < 0.001$). These changes in mitochondrial GSH pool during sepsis were counteracted by melatonin administration. No changes in mitochondrial GSH were found in mice lacking the iNOS gene (Fig. 3).

The GPx activity increased in septic iNOS$^{+/+}$ mice ($P < 0.05$), and this increase was preserved after melatonin administration (Fig. 4). Septic iNOS$^{+/+}$ animals, however, showed a significant reduction in GRd activity ($P < 0.01$) while was normalized after melatonin administration ($P < 0.01$). GPx and GRd activities remained unchanged

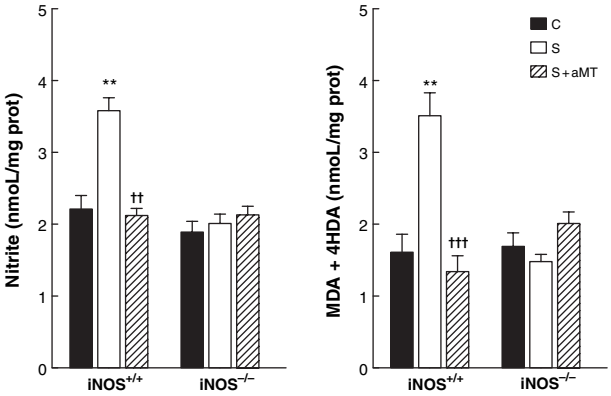

Fig. 2. Nitrite (left) and lipid peroxidation (right) concentrations measured in skeletal muscle mitochondria. C, control, S, sepsis, S + aMT, sepsis plus melatonin. $^{**}P < 0.01$ versus control; $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ versus sepsis.

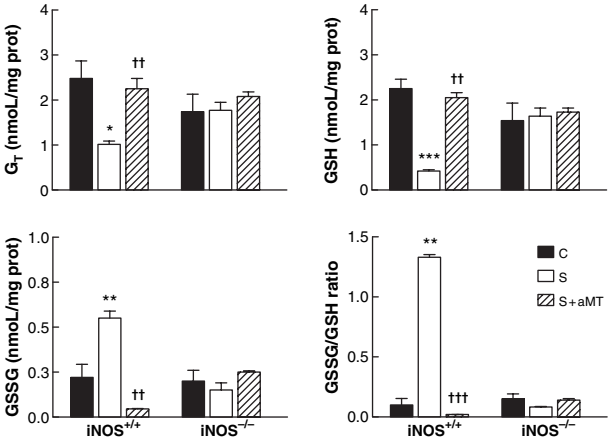

Fig. 3. Glutathione concentration and GSSG/GSH ratio measured in skeletal muscle mitochondria. C, control, S, sepsis, S + aMT, sepsis plus melatonin. G_T, total glutathione. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control; $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.005$ versus sepsis.

in septic iNOS$^{-/-}$ mice, and in this group of animals melatonin treatment was without effect on their activities (Fig. 4). Basal GPx and GRd activities were significantly lower in iNOS$^{-/-}$ mice compared with wild type strain.

Complex I, II, III and IV activities were significantly ($P < 0.001$) reduced in septic iNOS$^{+/+}$ mice by 55.8%, 40.2%, 51,8% and 40.1%, respectively (Fig. 5). Melatonin administration not only counteracted the inhibition of complex activities induced by sepsis, but increased their activities above their basal values. The activities of these

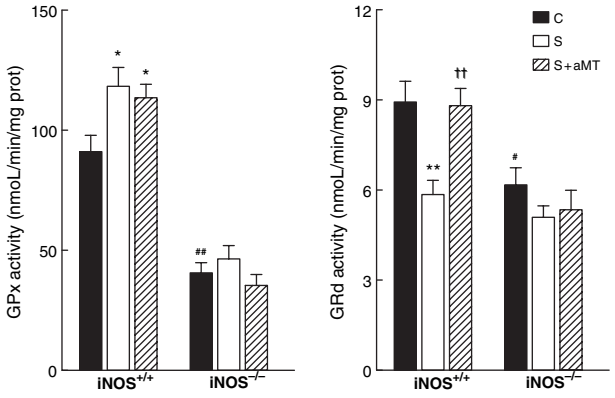

Fig. 4. Glutathione peroxidase (GPx) and reductase (GRd) activities measured in skeletal muscle mitochondria. C, control, S, sepsis, S + aMT, sepsis plus melatonin. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\dagger\dagger}P < 0.01$ versus sepsis; $^{\#\#}P < 0.01$ versus iNOS$^{+/+}$.

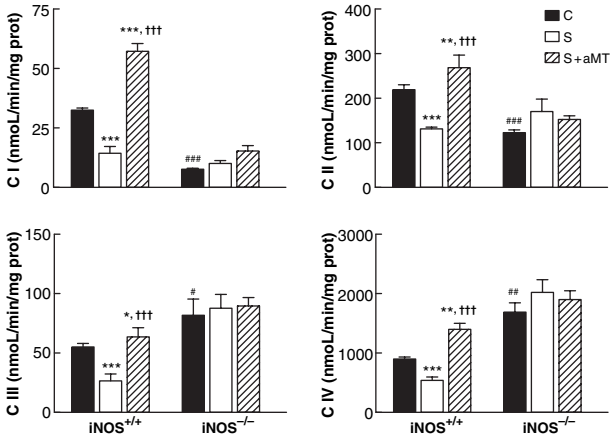

Fig. 5. Complex I, II, III and IV activities measured in skeletal muscle mitochondria. C, control, S, sepsis, S + aMT, sepsis plus melatonin. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control; $^{\dagger\dagger\dagger}P < 0.001$ versus sepsis; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus iNOS$^{+/+}$.

respiratory complexes remained unchanged in septic iNOS$^{-/-}$ mice, and they were not influenced by melatonin administration (Fig. 5). Of note, the basal activities of complex I and II were significantly lower and those of complex III and IV significantly higher in iNOS$^{-/-}$ mice than in wild type strain.

Discussion

There is evidence suggesting that mitochondrial dysfunction and subsequent bioenergetic failure are closely related to the mortality in sepsis-induced multiorgan dysfunction. In fact, an association between sepsis severity, outcome, and mitochondrial dysfunction in patients has been recently reported [7]. Among other consequences, mitochondrial dysfunction may severely negatively influence intercostal muscle contractility leading to the respiratory insufficiency in sepsis [6, 11]. In this report, we studied the behavior of skeletal muscle in a sub-chronic model of sepsis caused by CLP intervention; this model produces maximal deterioration at 24 hr and resembles closely most of the clinical features of the sepsis [27]. The results present here support the existence of two mtNOS isoforms, mtcNOS and mtiNOS in normal mitochondria for physiological regulatory purposes. From these, only the mtiNOS isoform increases in septic iNOS$^{+/+}$ mice; this isoform is not expressed in either normal and septic iNOS$^{-/-}$ mice. Thus, mtiNOS seems to be coded by the iNOS gene. Similar to mtcNOS, which derived from nNOS after a post-translational modification [25], iNOS may undergo some modification before allowing it to enter the mitochondria. Increased levels of NO produced by mtiNOS in sepsis likely are related to respiratory chain inhibition and oxidative stress, both features contributing to mitochondrial dysfunction. Our results also show that melatonin administration normalizes mtiNOS activity and reduces oxidative stress, thereby restoring respiratory chain activity after sepsis.

Although the pathophysiological pathways involved in mitochondrial damage during sepsis are uncertain, it was suggested that high amounts of NO produced by iNOS probably play a central role [6, 38]. Recent findings, however, point to an alternative explanation. The existence of a mitochondrial isoform of NOS led to the proposed that NO produced by mtNOS plays a physiological role in mitochondria [25, 26, 29, 38]. Under normal conditions, mitochondrial NO inhibits complex IV activity by a 30–40% [9]. The inhibition is reversible and competitive with O_2 and the degree of respiratory inhibition varies with the NO/O_2 ratio. It was previously reported that the enzyme responsible for NO production in mitochondria is derived from nNOSα after a specific posttranslational modification prior to its incorporation into the mitochondrion [39]. It was then proposed that mtcNOS is constitutively expressed in the mitochondria, coded by the same gene as for nNOS [39]. However, reports showing the identification of a mitochondrial NOS with antibodies against iNOS by western blot [25, 26], and its increase after sepsis [26, 40], suggested the presence of an inducible NOS isoform in the mitochondria. Thus, there is currently a controversy concerning the nature of mtNOS [23]. Here we report the existence of an mtiNOS isoform constitutively expressed in normal mitochondria and induced by sepsis in iNOS$^{+/+}$ mice. mtiNOS was also reported in lung and liver mitochondria [26], where the measurement of mtiNOS activity was accomplished by the conversion of L-arginine to L-citrulline. Liver L-citrulline is formed by pathways other than NOS such as the urea cycle, making it difficult to distinguish the source of L-citrulline. However, in muscle mitochondria, the production of L-citrulline comes exclusively from the NOS enzyme. These data, together with the absence of basal and sepsis-induced mtiNOS activity in iNOS$^{-/-}$ mice strongly support the existence of mtiNOS and its codification by the same gene as iNOS.

It was recently reported [41] that iNOS may translocate from the cytosol to mitochondria during sepsis. Our data agree with this finding, as we demonstrate that mtiNOS derives from iNOS and increases in sepsis. Our results,

however, also show that under physiological conditions mitochondria express mtcNOS and mtiNOS, although only the latter increases in sepsis. Thus, the co-expression of mtcNOS and mtiNOS in normal conditions suggests that both enzymes produce NO for regulatory purposes [24, 26]. The lack of mtiNOS in $iNOS^{-/-}$ mice could be related to the elevated basal activities of complex IV and perhaps complex III. However, the reduced basal activity of complex I and II in these mice remains unclear. The role of NO in mitochondrial biogenesis [42] may be related to the maintenance of a normal respiratory function. A positive consequence of the low complex I and II activities in $iNOS^{-/-}$ mice could be the lower oxidative stress compared with wild mice. In fact, mitochondria from control mutant mice show lower levels of GSH and lower GPx and GRd activities than do $iNOS^{+/+}$ mice. As no oxidative stress was detected after sepsis in mutant mice, the antioxidant system of these animals is sufficient to maintain the normal mitochondrial redox status.

The mtiNOS induction in sepsis is responsible for the generation of an excess of NO which affects mitochondria via several mechanisms. One is the direct inhibition of elevated NO concentrations on complex III, II and I activities in addition to complex IV inhibition [9, 10, 38]. A 20% inhibition of complex I without changes in complex IV activity was recently reported [8]. As high NO levels reversibly inhibit complex IV, it was suggested that the inhibition produced by NO in vivo may be not reproduced in vitro, because NO levels remaining in the homogenates may be too low to maintain the inhibition of complex IV [8]. Sepsis-mediated increase in protein tyrosine nitration is limited to the mitochondria, an effect previously related to iNOS [6]. We believe that in sepsis, besides reversible effects of NO, $ONOO^-$ damages respiratory complexes by irreversible nitration [6] and, thus, the in vivo modification of complex activities should be reflected in vitro. In this regard, our results show a 40–50% inhibition of all respiratory complex activities in sepsis, which was related to the NO produced in this condition, as no changes in respiratory activities were detected in iNOS deficient mice. An alternative explanation for these differences could be the different experimental models used, although in an acute model of sepsis because of administration of lipopolysaccharide, which produces a more pronounced iNOS induction, the inhibition of complex I and IV was also reported [26].

Another mechanism involved in mitochondrial dysfunction by NO is the production of free radicals. The inhibition of respiratory complexes by NO decreases membrane potential promoting the formation of $O_2^{\bullet -}$. In turn, NO reacts with $O_2^{\bullet -}$ yielding $ONOO^-$, a nitrogen-based reactant which is able to irreversibly damage the respiratory complexes [9, 10, 38]. Mitochondrial antioxidant defense includes superoxide dismutase and the glutathione redox cycling system. Whereas the former dismutates $O_2^{\bullet -}$ to hydrogen peroxide (H_2O_2), GPx transforms H_2O_2 into water. In this process, GSH is oxidized to GSSG and the enzyme GRd restores reduced GSH levels. The balance of these reactions is influenced by oxidative stress in sepsis, with a compensatory increase in GPx activity which cannot restore normal redox equilibrium because GRd activity remains inhibited possibly due to oxidative damage [18]. The increase in GSSG/GSH ratio in septic mice supports the failure of glutathione redox cycling to protect mitochondria. Also, the rate of the de novo synthesis of GSH decreases in sepsis since the total GSH content is reduced. Our data also correlate the increase in NO with that of LPO in sepsis, suggesting that NO-dependent oxidative stress in mitochondria caused ETC failure. This is supported by the observation that the markers of oxidative stress were not changed in septic $iNOS^{-/-}$ mice.

Melatonin is an indoleamine originally isolated from the pineal gland, but it is also produced by a variety of other organs and tissues [13]. Extrapineal melatonin levels are sometimes two to three orders of magnitude higher than plasma melatonin levels which reflects exclusively pineal melatonin production. Melatonin is an excellent antioxidant which scavenges both reactive oxygen and nitrogen species [13, 16], restores GSH homeostasis and mitochondrial function in organelles under oxidative stress, stimulates γ-glutamylcysteine synthase thereby increasing GSH synthesis [12, 13, 17], and increases the activity of mitochondrial complex I and IV [20, 21]. Additionally, genomic effects of melatonin include the inhibition of the expression iNOS and mtNOS [26, 43]. Considering these multiple actions it was not surprising that melatonin administration restored mitochondrial homeostasis in septic $iNOS^{+/+}$ mice. Characteristically, melatonin counteracted sepsis-induced mtiNOS activity and NO production without changes in mtcNOS. Since mtiNOS induction, but not mtcNOS, was responsible for mitochondrial damage in sepsis, the effect of melatonin is highly selective against mitochondrial impairment in sepsis. Also, melatonin counteracted mitochondrial oxidative stress and restored GRd activity and GSH pool. Reflecting these changes, LPO was reduced to below basal levels by melatonin. Additionally melatonin also counteracted the inhibition of respiratory complexes. Of note, the effect of melatonin administration yielded an activity of these complexes above their basal values, suggesting that, besides reducing oxidative stress-induced ETC damage, melatonin may play an additional role in promoting respiratory chain activities. Melatonin-mediated increase in the transcriptional activity of mtDNA may be partially responsible for the increased efficiency of ETC complexes [12].

It is interesting to note that melatonin administration seems to be effective when mitochondrial damage and oxidative stress are present. The indoleamine did not affect the mitochondria of septic $iNOS^{-/-}$ mice, which were protected against sepsis. Acting against mitochondrial dysfunction while avoiding any interference with normal mitochondrial functioning is another beneficial feature of melatonin. Thus, melatonin may be implicated in mitochondrial homeostasis [12–14] and an alteration of its circadian rhythm in critically ill patients with severe sepsis could be related to the high vulnerability of these patients to mitochondrial dysfunction [44].

In summary, we report the presence a mtiNOS isoform in mitochondria from skeletal muscle presumably coded by the iNOS gene. mtiNOS is induced during sepsis thereby increasing NO production which correlates with the rise in oxidative stress. Under these conditions, the

mitochondrial GSH pool is reduced because the reduction of its synthesis and its restoration from GSSG due to the inhibition on GRd activity. Thus, mitochondrial antioxidant protection decreases leading to the inhibition of respiratory complexes and mitochondrial dysfunction which likely causes muscle contractility failure. The absence of mitochondrial damage in mutant mice further supports a role of mtiNOS in septic dysfunction. Changes in basal activity of respiratory complexes in $iNOS^{-/-}$ mice suggest the participation of basal NO produced by mtiNOS in these phenomena, a finding that should be explored. In addition to an effect of melatonin on complex I and IV activities, melatonin also causes an improvement of the complex II and III activities, thus recovering the normal function of the respiratory chain. Because the CLP model of sepsis simulates much of the pathophysiology in human disease [27, 45, 46], and melatonin reduces mortality in septic newborns [47], the potential efficacy of melatonin could be assessed in septic adult patients.

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