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Identification of an inducible nitric oxide synthase in diaphragm mitochondria from septic mice
Its relation with mitochondrial dysfunction and prevention by melatonin

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Abstract

Sepsis provokes an induction of inducible nitric oxide synthase (iNOS) and melatonin down-regulates its expression and activity. Looking for an inducible mtNOS isoform, we induced sepsis by cecal ligation and puncture in both normal and iNOS knockout mice and studied the changes in mtNOS activity. We also studied the effects of mtNOS induction in mitochondrial function, and the role of melatonin against induced mtNOS and mitochondrial dysfunction. The activity of mtNOS and nitrite levels significantly increased after sepsis in $iNOS^{+/+}$ mice. These animals showed a significant inhibition of the respiratory chain activity and an increase in mitochondrial oxidative stress, reflected in the disulfide/glutathione ratio, glutathione redox cycling enzymes activity and lipid peroxidation levels. Interestingly, mtNOS activity remained unchanged in $iNOS^{-/-}$ septic mice, and mitochondria of these animals were unaffected by sepsis. Melatonin administration to $iNOS^{+/+}$ mice counteracted mtNOS induction and respiratory chain failure, restoring the redox status. The results support the existence of an inducible mtNOS that is likely coded by the same gene as iNOS. The results also suggest that sepsis-induced mtNOS is responsible for the increase of mitochondrial impairment due to oxidative stress in sepsis, perhaps due to the high production of $NO^{\bullet}$. Melatonin treatment prevents mitochondrial failure at the same extend as the lack of iNOS gene.

Keywords: Mitochondria; iNOS knockout mice; Respiratory chain; Oxidative stress; Melatonin; Sepsis

Abbreviations: CLP, cecal ligation and puncture; eNOS, endothelial nitric oxide synthase; ETC, electronic transport chain; GPx, glutathione peroxidase; GRd, glutathione reductase; GSH, glutathione; GSSG, disulfide glutathione; 4HDA, 4-hydroxyalkenals; iNOS, inducible nitric oxide synthase; LPO, lipid peroxidation; MDA, malondialdehyde; mtNOS, mitochondrial nitric oxide synthase; mtcNOS, mitochondrial constitutive nitric oxide synthase; mtiNOS, mitochondrial inducible nitric oxide synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species

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1. Introduction

Increasing evidence suggests that $NO^{\bullet}$ plays a central role in mitochondrial bioenergetics through the modulation of the oxygen consumption. $NO^{\bullet}$ is a reversible inhibitor of the cytochrome *c* oxidase, competing with oxygen at the same site of the enzyme (Brown, 2001). Under physiological conditions mitochondrion has a $[O_2]/[NO]$ ratio of 500/1000, which competitively inhibits cytochrome *c* oxidase by 16–26% (Boveris, Costa, Poderoso, Carreras, & Cadenas, 2000). After the first evidence supporting the production of $NO^{\bullet}$ by pure mitochondria, the existence of a mitochondrial nitric oxide synthase (mtNOS) was identified as the source of intramitochondrial $NO^{\bullet}$ (Ghafourifar & Richter, 1997). Also, the apparent absence of $NO^{\bullet}$ production in the mitochondria of mice lacking the neuronal but not the endothelial or inducible isoforms of NOS suggested that mtNOS is coded by the same gene as nNOS (Kanai et al., 2001). Soon after enzyme was identified as the nNOSα isoform through a novel alternative splicing pathway (Elfering, Sarkela, & Giulivi, 2000). Other attempts to identify mtNOS as an eNOS isoform did not report conclusive results (Brookes, 2004).

The common characteristic in these experimental studies was the use of mitochondrial preparations from tissue of normal animals. Since in these conditions iNOS is not expressed, the possibility that an iNOS isoform might be also present into the mitochondrion was not taken into account. Our laboratory reported that mitochondria contain an inducible mtNOS (mtiNOS) isoform that it is strongly expressed under stimuli such as sepsis (Escames, León, Macías, Khaldy, & Acuña-Castroviejo, 2003). Thus, mtiNOS induction in sepsis may be the source of an excess of intramitochondrial $NO^{\bullet}$ levels, that inhibits cytochrome *c* reductase and NADH CoQ oxidoreductase (Cadenas, Poderoso, Antunes, & Boveris, 2000), with the consequent increase in both reactive oxygen (ROS) and nitrogen (RNS) species and mitochondrial failure (Alvarez & Boveris, 2004; Lizasoain, Moro, Knowles, Darley-Usmar, & Moncada, 1996; Poderoso et al., 1996). The existence of an mtiNOS isoform may explain some aspects of the inflammatory process, that course with energy failure.

The impairment of the respiratory chain by high $NO^{\bullet}$ increases the production of superoxide anion ($O_2^{\bullet -}$), which reacts with $NO^{\bullet}$ yielding peroxynitrite (Poderoso et al., 1996). Peroxynitrite irreversible impairs the respiratory chain and decrease the efficiency of the oxidative phosphorylation, leading to energy depletion and cell death (Boczkowski et al., 1999; Boveris et al., 2000; Brown, 2001; Cassina et al., 2000; Poderoso et al., 1996). For counteracting free radical damage, mitochondria have the classical antioxidants superoxide dismutase and glutathione. Working together, these compounds maintain a good mitochondrial performance protecting them against attack from reactive species. Melatonin is an efficient scavenger of reactive oxygen and nitrogen species (Acuña-Castroviejo et al., 2001; León et al., 2004; Reiter et al., 2003). Melatonin increases mitochondrial glutathione (GSH) pool stimulating the activity of both γ-glutamylcysteine synthase and the enzymes of the GSH redox cycle, glutathione peroxidase (GPx) and reductase (GRd) (Martín, Macías, Escames, León, & Acuña-Castroviejo, 2000; Urata et al., 1999). After sepsis, the administration of melatonin results in a reduction of the expression and activity of cytosolic iNOS (Crespo et al., 1999). Moreover, melatonin increases the respiratory chain activity and ATP levels when mitochondria are damage by oxidative stress (Escames et al., 2003; Martín et al., 2000b, 2002).

Controversy exists among the type(s) of mtNOS in terms of their classification as constitutive or inducible (Brookes, 2004; Ghafourifar & Cadenas, 2005), and the role of the mtiNOS isoform recently reported (Escames et al., 2003) on mitochondrial function both in normal and in pathophysiological conditions is also unknown. To further clarify this situation, we examine the sepsis-induced changes in mtNOS and mitochondrial bioenergetics in diaphragm from $iNOS^{+/+}$ and $iNOS^{-/-}$ mice. The model of sepsis was chosen because it produces dramatic metabolic changes probably caused by the primary event of $NO^{\bullet}$ production due to iNOS increase. Diaphragm as source of mitochondria was used for this study since it contains abundant mitochondria and it is involved in the respiratory failure during sepsis. Since we previously found that melatonin inhibits the expression and activity of cytosolic and mitochondrial iNOS induced by sepsis, we also assessed the role of melatonin on this experimental paradigm.

2. Materials and methods

2.1. Chemicals

Melatonin, Nembutal, methanol, acetonitrile, naphtiletilen-diamine (NEDA), sulfanilamide, EGTA, EDTA, Hepes, BSA, proteinase K, Percoll, Dowex-50W resin, L-citrulline, L-arginine, 1,4-dithio-DL-threitol (DTT), leupeptin, aprotinin, pepstatin, phenylmethanesulfonyl fluoride (PMSF), (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4), FAD, inosine, NADPH, antimycin A, decylubiquinone (DB), methanesulfonic acid, rotenone, potassium cyanide (KCN), 2,6-dichloroindophenol

(DCIP), 2,4-dinitrophenol (DNP), cytochrome *c*, sodium dithionite, 5-sulfosalicylic acid, dimethyl sulfoxide (DMSO), disulfide and reduced glutathione, glutathione reductase, 5-sulfosalicylic acid, 2-vinylpiridine, cumene hydroperoxide, 5,5′-dithiolbis (2-nitrobenzoic acid) (DTNB), and mannitol were purchased from Sigma–Aldrich (Madrid, Spain); 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 reagents were of highest purity available.

2.2. Animals, surgical procedures and treatments

iNOS knockout B6.129P2-*Nos2tm1Lau* 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 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 done. 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 cecal ligation and puncture (CLP) (Wichterman, Baue, & Chaudry, 1980) 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 were immediately prepared and frozen to −80 °C until remained assays were performed.

2.3. Mitochondria purification

Diaphragms were individually processed to obtain pure mitochondria by differential centrifugation and Percoll density gradient as previously reported (Giulivi, Poderoso, & Boveris, 1998; Tatoyan & Giulivi, 1998) with slight modifications (Escames et al., 2003). All procedures were performed in cold. Briefly, diaphragmatic muscle was excised, washed with saline, treated with proteinase K (1 mg/ml) during 30 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 Stuart Scientific SS2 stirrer 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 in 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 150 mM KCl, followed by two washings with buffer A at 10 300 × *g* for 10 min at 4 °C to remove the Percoll, and frozen to −80 °C.

2.4. Assay of mitochondrial nitric oxide synthase 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 (Lowry, Rosenbrough, Farr, & Randall, 1951) or used immediately for NOS activity determination (Escames et al., 2003). The final incubation volume was 100 μl (2 mg/ml prot) 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 started by the addition of 10 μl NADPH (0.75 mM final concentration) and continued for 30 min al 37 °C. To determinate iNOS-Ca^{2+}-independent activity, 10 mM EDTA is added to the buffer before to beginning of the reaction. Control incubations were performed in absence of NADPH. The reaction was stopped 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 greater than 98%. NOS activity was mea-

sured by monitoring the conversion of L-^{3}H-arginine to L-^{3}H-citrulline (Bredt & Snyder, 1989). Enzyme activity was referred as picomol L-^{3}H-citrulline/min/mg prot.

2.5. 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 (Lowry et al., 1951) or used to calculate nitrite levels following the Griess reaction (Green, Ruiz de Luzuriaga, & Wagner, 1981) and expressed as nmol nitrite/mg prot.

2.6. Assay of respiratory enzyme activities

To prepare submitochondrial particles, mitochondrial pellets were freezing and thawed twice, sonicated, and suspended in 350 μl of the incubation medium corresponding to the complex to be measured (Martín et al., 2000). Mitochondrial protein concentration was measured by using BSA as standard (Lowry et al., 1951). Complex I activity (NADH CoQ oxidoreductase, in nmol oxidized NADH/min/mg prot) was measured in the presence of decylubiquinone and succinate as the rotenone-sensitive decrease in NADH at 340 nm (Barrientos, 2002). The activity of complex II (succinate: DCIP oxireductase, in nmol reduced DCIP/min/mg prot) 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 (Brusque et al., 2002). Complex III activity (ubiquinol: cytochrome *c* oxireductase, in nmol reduced cytochrome *c*/min/mg prot) 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 (Brusque et al., 2002). Complex IV activity (cytochrome *c* oxidase, in nmol oxidized cytochrome *c*/min/mg prot) was measured as the disappearance of reduced cytochrome *c* at 550 nm (Barrientos, 2002).

2.7. Oxidative stress

For LPO measurement, mitochondrial fractions were thawed, suspended in ice-cold 20 mM Tris–HCl buffer, pH 7.4, and sonicated to break mitochondria membranes. Aliquots of these samples were either stored at −80 °C for total protein determination (Lowry et al., 1951) or used for lipid peroxidation (LPO). For this purpose, a commercial LPO assay kit able to determine both malondialdehyde (MDA) and 4-hydroxyalkenals (4HDA) was used (Bioxytech LPO-568 assay kit, OxisResearch, Portland, OR, USA) (Esterbauer & Cheeseman, 1990). The concentration of LPO was expressed in nmol/mg prot.

Disulfide (GSSG) and glutathione (GSH) were spectrophotometrically measured (Griffith, 1985). Mitochondrial fractions were resuspended in 350 μl of 2.5% sulfosalicylic acid, sonicated and centrifuged at 1000 × *g* for 15 min at 4 °C. The pellets were used by protein determination (Lowry et al., 1951) and the supernatants were used immediately for glutathione assay. The concentration of both GSH and GSSG was expressed in nmol/mg prot.

For both glutathione peroxidase (GPx) and reductase (GRd) activities determination, mitochondrial fractions were suspended in 200 μl of buffer A (potassium-phosphate 50 mM and EDTA dipotassium 1 mM, pH 7.4) and sonicated. GPx activity was measured following the oxidation of NADPH during 3 min at 340 nm (Jaskot, Charlet, Grose, & Grady, 1983) in an UV spectrophotometer (Shimadzu Deutschland GmbH, Duisburg, Germany). GRd activity was measured following the NADPH oxidation during 3 min at 340 nm. Protein concentration was measured (Lowry et al., 1951) and the enzyme activities were expressed as nmol/min/mg prot.

2.8. Statistical analysis

Data are expressed as the mean ± S.E. of six experiments assayed in duplicate per group. 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.

3. Results

3.1. Effect of the lack of iNOS gene and/or melatonin treatment on mtNOS activity

Mitochondria from wild type mice constitutively express two components of mtNOS. These components correspond to Ca^{2+}-dependent and Ca^{2+}-independent mtNOS activities, classically referred as constitutive and inducible NOS isoforms, respectively (we propose the terms mtcNOS and mtiNOS for the constitutive and inducible forms of mtNOS, respectively). Whereas iNOS$^{+/+}$ mice show similar basal activity of the two mtNOS components (Fig. 1A), mtiNOS activity was undetectable in iNOS$^{-/-}$ mice (Fig. 1B). Twenty-four hours after sepsis induction, mtiNOS significantly increased in iNOS$^{+/+}$ animals ($P < 0.001$), whereas mtcNOS activity remained unchanged (Fig. 1A). iNOS$^{-/-}$

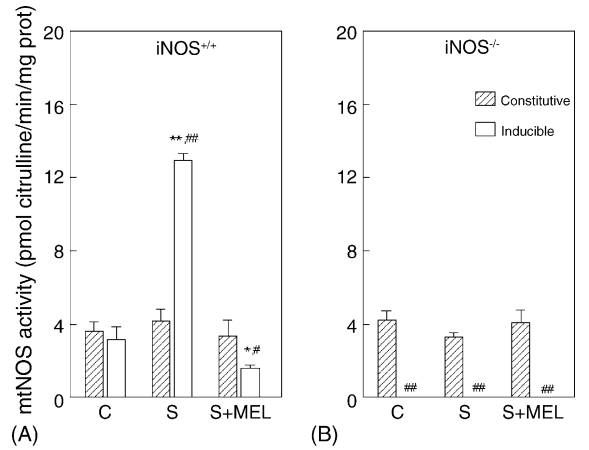

Fig. 1. Changes in the constitutive (Ca^{2+}-dependent) and inducible (Ca^{2+}-independent) components of mtNOS in mitochondria from iNOS$^{+/+}$ (A) and iNOS$^{-/-}$ (B) mice diaphragm. Sepsis was induced by CLP, mice were sacrificed 24 h postsurgery and pure mitochondria were prepared by Percoll gradient. Control, sepsis and sepsis + melatonin values are shown as bars C, S and S + MEL, respectively. Data are means ± S.E.M., $n = 8$. $^{*}P < 0.01$ and $^{**}P < 0.001$ vs. control; $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ vs. constitutive isoform.

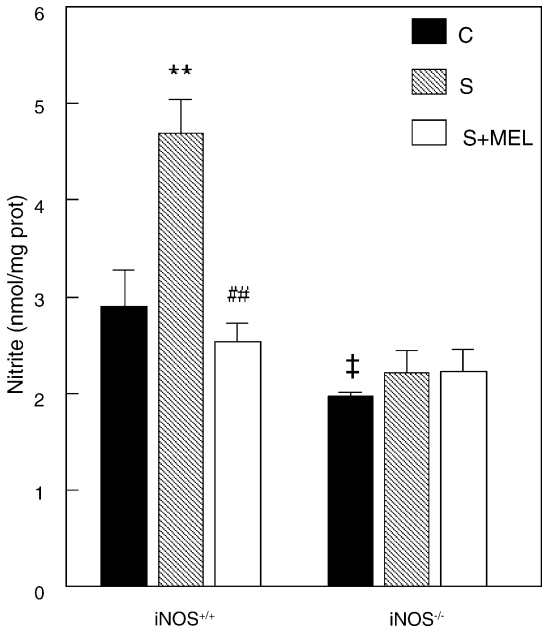

Fig. 2. Changes in the levels of nitrite in mitochondria from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Other information as in Fig. 1. $^{**}P < 0.001$ vs. control; $^{\#\#}P < 0.001$ vs. sepsis; $^{\ddagger}P < 0.01$ vs. iNOS$^{+/+}$.

mice did not show changes in mtcNOS activity in these experimental conditions (Fig. 1B).

Nitrite levels parallels the changes in mtiNOS activity, increasing significantly ($P < 0.001$) after sepsis only in iNOS$^{+/+}$ mice (Fig. 2). It is interesting to note that the basal levels of nitrite were significantly lower in iNOS$^{-/-}$ compared with iNOS$^{+/+}$ mice ($P < 0.01$). Melatonin administration (4×30 mg/kg b.w.) counteracted sepsis-induced mtiNOS activity and nitrite production in iNOS$^{+/+}$ mice, whereas no effects were found in iNOS$^{-/-}$ mice (Figs. 1 and 2).

Several procedures were used to guarantee that we are measuring NOS activity and that this activity is present into the mitochondria and it is not a cytosolic contamination. The technique for NOS measurement specifically detects mtNOS activity. Addition of L-NMMA (300 μM) to the mixture reaction of mitochondrial samples from septic mice blocked the transformation of L-arginine to L-citrulline due to mtNOS inhibition (17.04 ± 0.67 pmol citrulline/min/mg prot versus 4.82 ± 0.44 pmol citrulline/min/mg prot, CLP and CLP + L-NMMA, respectively, $P < 0.001$). The purity of the mitochondrial preparation used in the study was assessed by several procedures. Diaphragm mitochondria were isolated by differential centrifugation, and purified by Percoll centrifugation (Elfering et al., 2000; Escames et al., 2003). To remove contaminants, purified mitochondria were washed with high ionic strength solution (150 mM KCl). This protocol yields a highly pure mitochondrial fraction without contaminating organelles and broken mitochondria, as assessed by electron microscopy (data not shown). The purity of mitochondrial fraction was further assessed by two methods: (a) measuring the activity of specific markers of plasma membrane, peroxisomes and microsomes (Table 1), that suggest a low percentage of mitochondrial contamination by extramitochondrial structures (Tatoyan

Table 1
Characterization of the purity degree of diaphragm mitochondria

Marker	Location	Homogenate	Mitochondria	Ratio	% Contamination
Alkaline phosphatase	Plasma membrane	25.25 ± 0.33	29.32 ± 3.28	1.16	5.6
NADPH cytochrome *c* reductase	Microsomes	2.81 ± 0.22	0.94 ± 0.24	0.34	1.19
Catalase	Peroxisomes	1.46 ± 0.04	0.64 ± 0.06	0.43	1.78

Values are means± S.E.M.; $n = 4$. Alkaline phosphatase and NADPH cytochrome *c* reductase activities are expressed as nmol/min/mg prot; catalase activity is expressed in μmol/min/mg prot. Percentage of mitochondrial contamination was calculated as the ratio of specific activities of the enzyme in mitochondria and in the homogenate (Ratio), corrected by the protein recovered in the mitochondrial fraction (3.5%).

& Giulivi, 1998); and (b) measuring NOS activity and nitrite levels in the supernatant of the last centrifugation step, that yielded negative results (data not shown). These data confirm the purity of the mitochondria used in our experiments and ensure the mitochondrial origin of the mtNOS activities in these samples.

3.2. Effect of the lack of iNOS gene and/or melatonin treatment on mitochondrial respiratory chain activity

The activity of the respiratory chain complexes I, II, III and IV was measured. Under basal conditions, complex I and II activities were significantly lower ($P < 0.001$) in iNOS$^{-/-}$ mice than in iNOS$^{+/+}$ mice (Fig. 3A and B). In contrast, the activities of complexes III and IV were significantly higher ($P < 0.001$) in the former (Fig. 3C and D). After sepsis, the activity of the four complexes was significantly reduced in iNOS$^{+/+}$ but not in iNOS$^{-/-}$ mice (Fig. 3A–D).

Melatonin administration (4×30 mg/kg b.w.) not only counteracted ($P < 0.001$) the sepsis-induced respiratory chain inhibition but also increased it above the control levels ($P < 0.01$, Fig. 3A–D). Melatonin also increased significantly ($P < 0.01$) the activity of the complexes I and II but not III and IV in iNOS$^{-/-}$ mice (Fig. 3).

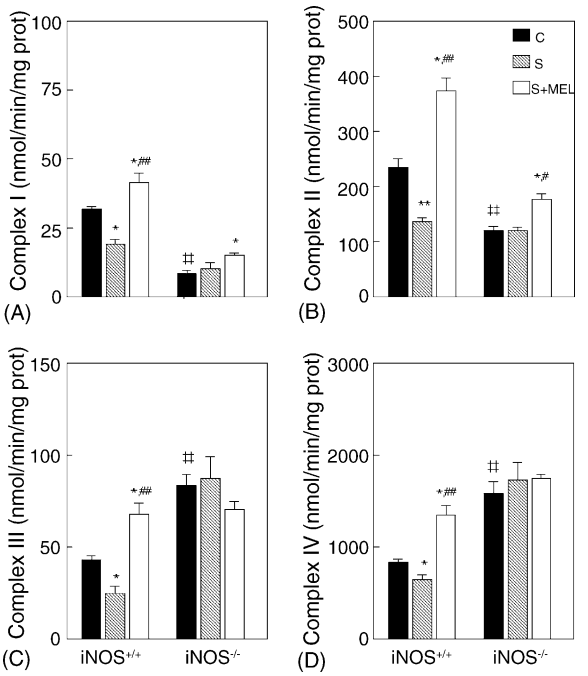

Fig. 3. Changes in the activity of the mitochondrial respiratory complex I (A), II (B), III (C) and IV (D) in diaphragm from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Other information as in Fig. 1. $^{*}P < 0.01$ and $^{**}P < 0.001$ vs. control; $^{\#\#}P < 0.001$ vs. sepsis; $^{\ddagger\ddagger}P < 0.001$ vs. iNOS$^{+/+}$.

3.3. Effect of the lack of iNOS gene and/or melatonin treatment on mitochondrial redox status

Mitochondrial lipid peroxidation (LPO) measurements confirm the existence of oxidative stress after sepsis in iNOS$^{+/+}$ mice ($P < 0.001$, Fig. 4). Again, iNOS$^{-/-}$ mice were unaffected by oxidative damage to lipids since they did not show changes in LPO levels. Melatonin counteracted the sepsis-induced LPO in iNOS$^{+/+}$ mice, and reduced the LPO levels in iNOS$^{-/-}$ mice (Fig. 4), which also showed lower basal LPO levels ($P < 0.01$) than the former (Fig. 4).

Mitochondria from both iNOS$^{+/+}$ and iNOS$^{-/-}$ mice contain comparable levels of total glutathione (Fig. 5A). However, mice lacking iNOS showed higher ($P < 0.01$) GSSG/GSH ratio than iNOS$^{+/+}$ mice (Fig. 5B), which was mainly due to increased GSSG levels in the former ($P < 0.01$, Fig. 5D). Total glutathione content in iNOS$^{+/+}$ mitochondria significantly decreases after sepsis ($P < 0.01$, Fig. 5A). In iNOS$^{+/+}$ mice, sepsis induced a strong drop in GSH levels with opposite GSSG changes ($P < 0.001$, Fig. 5C and D), leading to an increased GSSG/GSH ratio ($P < 0.001$) (Fig. 5B). Sepsis, however, did not modify glutathione levels in iNOS$^{-/-}$ mice. Melatonin administration to septic iNOS$^{+/+}$ mice prevented most of the changes in mitochondrial glu-

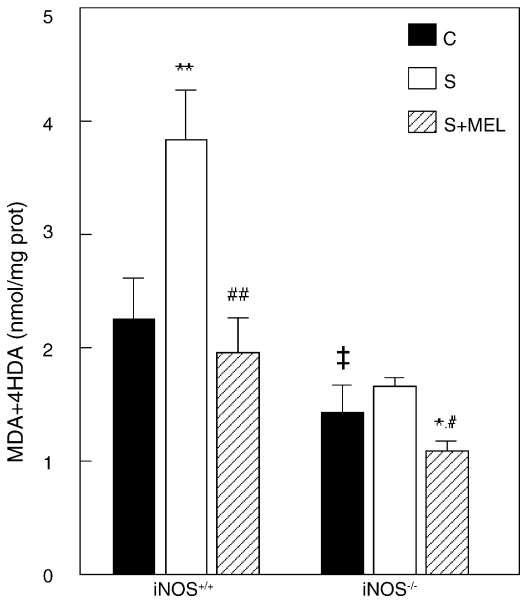

Fig. 4. Changes in the levels of LPO in mitochondria from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Other information as in Fig. 1. $^{**}P < 0.001$ vs. control; $^{\#\#}P < 0.001$ vs. sepsis.

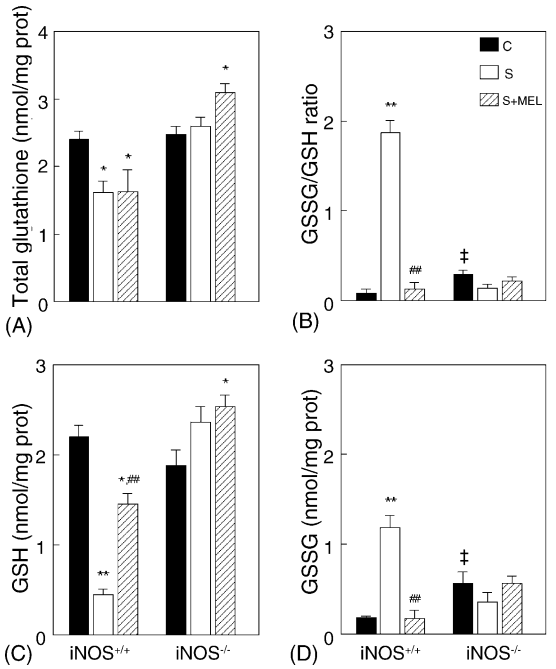

Fig. 5. Changes in total glutathione (A), GSSG/GSH ratio (B), GSH (C) and GSSG (D) levels in diaphragm mitochondria from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice after sepsis and melatonin treatment. Other information as in Fig. 1. $^{*}P < 0.01$ and $^{**}P < 0.001$ vs. control; $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ vs. sepsis; $^{\ddagger}P < 0.01$ vs. iNOS$^{+/+}$.

tathione. Although melatonin did not restore the total glutathione pool into the mitochondria, it normalized the GSSG/GSH balance impaired by sepsis ($P < 0.001$, Fig. 5B). Melatonin also increased ($P < 0.01$, Fig. 5C) the reduced glutathione content in mitochondria from iNOS$^{-/-}$ mice, leading to a total glutathione increase (Fig. 5A, $P < 0.01$).

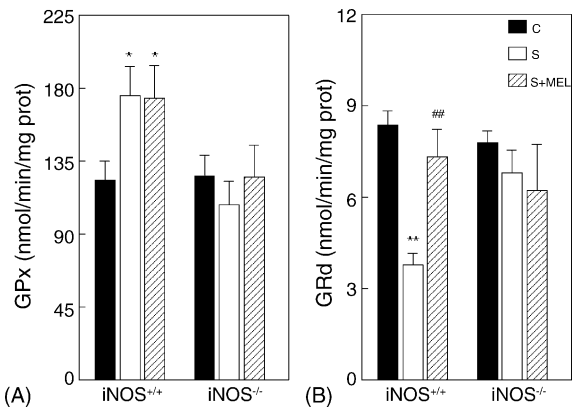

Fig. 6. Changes in the activity of GPx (A) and GRd (B) in diaphragm mitochondria from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice after sepsis and melatonin treatment. Other information as in Fig. 1. $^{*}P < 0.01$ and $^{**}P < 0.001$ vs. control; $^{\#\#}P < 0.001$ vs. sepsis.

Changes in the activity of the glutathione redox cycling enzymes, GPx and GRd, were also determined in the same experimental paradigm (Fig. 6). Basal activities of these enzymes were similar in both mice strains. In iNOS$^{+/+}$ mice sepsis significantly increased GPx activity ($P < 0.01$) and decreased that of GRd ($P < 0.001$, Fig. 6A and B). Sepsis did not affect the activity of these enzymes in iNOS$^{-/-}$. Melatonin administration counteracted the sepsis-induced GRd inhibition in iNOS$^{+/+}$ mice ($P < 0.001$), but it had not effect on iNOS$^{-/-}$ mice (Fig. 6B).

4. Discussion

The results show the existence of an mtiNOS form different from the constitutive, mtcNOS form elsewhere reported and coded by a different gene. These mtNOS isoforms differ in three main features: (a) mtcNOS corresponds to a constitutive, Ca^{2+}-dependent isoform, and mtiNOS to a inducible, Ca^{2+}-independent isoform; (b) they are coded by different genes; and (c) although both isoforms are constitutively expressed into the mitochondria, only mtiNOS isoform increases after sepsis, a known stimulus for iNOS induction. The lack of iNOS gene, that prevents the expression of cytosolic iNOS, also blunted the activity of mtiNOS in iNOS$^{-/-}$ mice, suggesting that mtiNOS derives from the same gene than iNOS. Another important conclusion from this study is the improving effect of melatonin on mitochondrial function impaired by sepsis in iNOS$^{+/+}$ mice. It is also interesting the lack of mitochondrial impairment by sepsis in iNOS$^{-/-}$ mice, suggesting that mtiNOS induction impairs mitochondria in sepsis.

Contradictory issues exist in the literature concerning the nature and functions of mtNOS (Brookes, 2004; Ghafourifar & Cadenas, 2005). Several authors have reported the presence of mtNOS in different tissues with properties of eNOS, nNOS and/or iNOS (Brookes, 2004; Elfering et al., 2000; Escames et al., 2003). Other authors, however, suggest that mtNOS does not correspond to any of these NOS isoforms (Lacza et al., 2004). Our data show that two components of mtNOS are constitutively expressed in mitochondria from iNOS$^{+/+}$ mice. These components account for the total NOS basal activity (eNOS + nNOS + traces of iNOS) in the range of 7–8 pmol/min/mg prot elsewhere described in diaphragm (Vassilakopoulos et al., 2003). Since basal iNOS activity in diaphragm was found less than 1 pmol/min/mg prot, our data suggest that most of the basal iNOS activity may be primarily localized into mitochondria as mtiNOS. The mitochondrially localized NOS activity was supported by the high purity of the

mitochondrial preparation used in our study, showing no significant extramitochondrial contamination. Another source of NOS contamination that we can discard in our study depend on the leukocyte infiltration in inflammation, that is virtually absent in the CLP model of sepsis (Lin et al., 1998). From the two mtNOS isoforms found, the Ca^{2+}-dependent mtcNOS probably corresponds to the form recently characterized in liver mitochondria as a posttranslationally modified nNOS isoform (Elfering et al., 2000; Traaseth, Elfering, Solien, Haynes, & Giulivi, 2000) and identified in rat mitochondria by western blot (Escames et al., 2003). However, because in diaphragm both constitutive nNOS and eNOS isoforms are present, it cannot be discarded the participation of eNOS in the mtcNOS in this tissue. Sepsis induced a significant increase in mtiNOS activity in $iNOS^{+/+}$ but not in $iNOS^{-/-}$ mice, suggesting that it is coded by iNOS gene. The presence of both inducible and constitutive subtypes of mtNOS in mitochondria explains its identification with antibodies against iNOS and nNOS (Elfering et al., 2000; Escames et al., 2003; Tatoyan & Giulivi, 1998).

Septic $iNOS^{+/+}$ but not $iNOS^{-/-}$ mice show a significant increase in mitochondrial nitrite levels. Under physiological conditions, $NO^{\bullet}$ regulates complex IV activity (Boveris et al., 2000; Brown, 2001; Traaseth et al., 2004). In excess, however, $NO^{\bullet}$ impairs mitochondrial function (Davies et al., 2005; Escames et al., 2003; Poderoso et al., 1996; Schapira, 1998) because it promotes the formation of $O_2^{\bullet-}$ from the respiratory chain and the subsequent production of peroxynitrite (Ghafourifar, Schenk, Klein, & Richter, 1999; Poderoso et al., 1996, 1999; Traaseth et al., 2004). Peroxynitrite irreversibly inhibit the complexes I, II and IV, and possibly III, of the electron transport chain (ETC) (Brown, 2001; Brown & Borutaite, 2004; Cadenas et al., 2000; Lizasoain et al., 1996). Although peroxynitrite may protect mitochondria because they induce Ca^{2+} release and subsequent mtNOS deactivation (Traaseth et al., 2004), this mechanism, however, only account for mtcNOS isoform that is Ca^{2+}-dependent, but not for the mtiNOS. These observations are applicable to our findings, because increased $NO^{\bullet}$ levels in septic mice were accompanied by a 30–50% reduction in the activity of the four complexes of the respiratory chain. The inhibition of the respiratory chain was associated to mtiNOS induction because mitochondrial $NO^{\bullet}$ levels and respiratory chain activity did not change in $iNOS^{-/-}$ mice after sepsis. Mice lacking iNOS gene showed control nitrite levels lower than $iNOS^{+/+}$ mice, suggesting that under physiological conditions, the intramitochondrial pool of $NO^{\bullet}$ depends on both mtNOS isoforms. In these conditions mtcNOS isoform provides almost the 68% of the mitochondrial $NO^{\bullet}$ pool, being the remained 32% from mtiNOS source. The decrease in the mitochondrial pool of $NO^{\bullet}$ in $iNOS^{-/-}$ mice, which accounts for 0.9 nmol/mg prot, was enough to increase almost two-fold the activity of the complexes III and IV in control mitochondria. Thus, a partial inhibition of complexes III and IV by the $NO^{\bullet}$ derived from the mtiNOS could be present in those studies in which the activity of mtNOS is blocked with EDTA to remove the Ca^{2+}-dependent mtcNOS. An unexpected finding was the lower activity of the complexes I and II in $iNOS^{-/-}$ mice compared with $iNOS^{+/+}$. Although we have not a rational explanation for this finding, a role for $NO^{\bullet}$ on the regulation of the activity of these complexes cannot be discarded. The control of mitochondrial functions depends on two variables, $NO^{\bullet}$ and O_2. At the steady-state levels of O_2 and $NO^{\bullet}$, the $O_2/NO^{\bullet}$ ratios of 500/1000 produce 35–15% inhibition of mitochondrial respiration, since $NO^{\bullet}$ reversibly inhibits complex IV in competition with O_2 (Boveris et al., 2000; Brown, 2001). Increasing concentrations of $NO^{\bullet}$ affects ETC by several mechanisms including: (a) impairing electron flow at the cytochrome bc_1 level and thus decreasing the activity of complex III, which may increase the production of $O_2^{\bullet-}$ (Poderoso et al., 1996); and (b) inducing reversible inhibition of complex I due to *S*-nitrosylation of thiol groups of the enzyme (Clementi, Brown, Feelisch, & Moncada, 1998). Reaction of NO with $O_2^{\bullet-}$ yields peroxynitrite, the major product of $NO^{\bullet}$ utilization into the mitochondria. Peroxynitrite also induces ubiquinol oxidation, which in turn prevents peroxynitrite mediated nitration of tyrosine residues in membrane mitochondria (Schöpfer et al., 2000). Thus, under physiological conditions, $NO^{\bullet}$-dependent nitrosylation reactions might be involved in the reversible inhibition of ETC complexes I–III, whereas complex IV is inhibited by a different pathway involving its competition with O_2. But in sepsis, when the increased levels of $NO^{\bullet}$ produce severe cytochrome oxidase inhibition, nitration reactions might be the underlying cause of inactivation of the other ETC complexes (Radi, Cassina, Hodara, Quijano, & Castro, 2002). Clearly, the inactivation of ETC complexes I–III by $NO^{\bullet}$ follows a different pathways that the fine regulation of complex IV by $NO^{\bullet}$. It should be here comment that in the presence of an excess of $NO^{\bullet}$ responsible for a severe inhibition of complex IV, the feedback self-regulatory mechanism provided by the ubiquinol/$NO^{\bullet}$ interaction appears crucial to maintain mitochondrial function (Schöpfer et al., 2000). Perhaps this mechanism sustains a minimal mitochondrial function during sepsis avoiding a dramatic mitochondrial failure. From

a physiological point of view, the increased activity of the complexes III and IV may compensate the reduction in the complex I and II activities, avoiding an excess of oxygen expend and oxidative stress.

We previously reported that melatonin inhibits both the expression and activity of iNOS and mtiNOS in liver and lungs of rats treated with lipopolysaccharide (Crespo et al., 1999; Escames et al., 2003). Our results further confirm that melatonin administration reduces CLP-induced mtiNOS activity in mice, improving the activity of the complexes I and IV (Escames et al., 2003; Martín et al., 2000b, 2002; Okatani, Wakatsuki, Reiter, & Miyahara, 2003), and show for the first time that melatonin also increases complex II and III activities reduced by sepsis. But ETC complex activities increased over the control values after melatonin administration. Although these findings may depend on the dose of indoleamine used in this study, a direct effect of melatonin on ETC complexes cannot be ruled out (Acuña-Castroviejo et al., 2003; Martín et al., 2002). Melatonin had not effect on mtcNOS and respiratory enzymes in iNOS$^{-/-}$ mice, excepting for the slight activity increase in the complexes I and II. Thus, melatonin can upregulate the mitochondrial respiratory chain when it is damaged (Escames et al., 2003; Martín et al., 2000b, 2002; Okatani et al., 2003).

The impairment of the ETC leads to electron leak and formation of more ROS including $O_2^{\bullet -}$, H_2O_2 and HO$^{\bullet}$ (Boveris, 1975; Poderoso et al., 1996) that further increase ETC damage, mitochondrial dysfunction and cell death (Brown, 2001; Escames et al., 2003; Martín et al., 2000; Piantadosi, Tatro, & Whorton, 2002). ROS induce lipoperoxidation of the mitochondrial membranes, a finding supported by the increased LPO levels in iNOS$^{+/+}$. Basal levels of LPO were lower in iNOS$^{-/-}$ than in iNOS$^{+/+}$ mice, suggesting that NO$^{\bullet}$ derived from constitutively expressed mtiNOS produces a basal free radical generation in the mitochondria. This observation agrees with the role of NO$^{\bullet}$ on the physiological production of H_2O_2 by mitochondrion for signaling purposes (Brookes, 2005; Brookes, Levonen, Shiva, Sarti, & Darley-Usmar, 2002; Poderoso et al., 1996).

It was reported an increase of mitochondrial superoxide dismutase activity in sepsis (Alvarez & Boveris, 2004), leading to an increase in H_2O_2 that should be detoxify by the glutathione system (Antunes, Han, & Cadenas, 2002). But the mitochondrial glutathione system is significantly impaired in response to sepsis. The levels of GSSG increased and those of GSH decreased in iNOS$^{+/+}$ mice under sepsis, with a decrease in the total glutathione content, which may reflect an inhibition of the GSH transport into the mitochondrion (Anderson & Sims, 2002). Changes in GSSG/GSH ratio could reflect also *S*-nitrosylation of GSH by peroxynitrite (Schrammel, Gorren, Schmidt, Pfeffer, & Mayer, 2003) and/or the reduction of peroxynitrite by the peroxynitrite reductase activity of mitochondrial glutathione peroxidase (Sies, Sharov, Klotz, & Briviba, 1997). It is unclear why control mice lacking iNOS showed higher GSSG/GSH ratio than wild type mice. An explanation may come from the biphasic effect of NO$^{\bullet}$ on mitochondrial production of oxygen radicals. In vitro, low levels of NO increase both H_2O_2 and HO$^{\bullet}$ production and high NO$^{\bullet}$ levels, that are deviate to the production of peroxynitrite, decrease their production (Poderoso et al., 1999). Thus, the GSSG/GSH ratio in iNOS$^{-/-}$ mice could reflect this situation.

Our data show an increase of GPx activity reflecting the consumption of GSH by oxidatively stressed mitochondria that cannot be recycled to GSH because the decreased activity of GRd (Anderson & Sims, 2002; Brealey et al., 2002; Martín et al., 2000). Thus, the antioxidant defense system becomes unable to protect septic mitochondria against free radical attack. Changes in redox balance were absent in mitochondria from iNOS$^{-/-}$ mice under sepsis.

Certainly, the results support the concept that increased oxidative stress and NO$^{\bullet}$ production play a central role in mitochondrial failure during sepsis (Crouser, 2004; Crouser, Julian, Blaho, & Pfeiffer, 2002). Thus, the use of antioxidants should be beneficial for counteract oxidative stress-induced mitochondrial dysfunction in sepsis. Melatonin is a well-known free radical scavenger of both ROS and RNS (Acuña-Castroviejo et al., 2001; León et al., 2004; Reiter et al., 2003). The indoleamine also maintains glutathione homeostasis in GSH depleted mitochondria (León, Acuña-Castroviejo, Escames, Tan, & Reiter, 2005; Martín et al., 2000). We now found that melatonin administration restored GSSG/GSH ratio, normalizing the GSH pool impaired during sepsis in iNOS$^{+/+}$ mice. The effect was mainly due to the increase of the GRd activity. Melatonin also restored normal membrane dynamics normalizing lipoperoxidation levels. The effects of melatonin were less pronounced in iNOS$^{-/-}$ mice. Thus, one can assume that melatonin counteracted sepsis-induced mitochondrial damage in iNOS$^{+/+}$ mice reducing the NO$^{\bullet}$-dependent free radical production through the inhibition of the iNOS/mtiNOS enzyme expression and activity (Crespo et al., 1999; Escames et al., 2003).

In conclusion, our results enhance the understanding of the mechanisms involved in mitochondrial regulation by mtNOS and how, besides ROS, NO$^{\bullet}$ contributes to the mitochondrial impairment observed in septic

mice. Mitochondria from mice diaphragm possess an mtiNOS form coded by the iNOS gene. In terms of the mitochondrial redox status, the data obtained with melatonin treatment parallel those found in iNOS$^{-/-}$ mice. Both conditions prevent mitochondrial oxidative damage under sepsis. Because melatonin reduced the expression and activity of mtiNOS induced in sepsis (Escames et al., 2003) the reduction of NO$^{\bullet}$ availability to mitochondria seems to be the primary effect of melatonin. The results of this study support the following findings reported in patients: (a) the association between NO$^{\bullet}$ overproduction, antioxidant depletion, energy failure and mitochondrial failure (Brealey et al., 2002); (b) the impaired circadian rhythm of melatonin in patients with severe sepsis (Mundigler et al., 2002); (c) the efficacy of some antioxidants to recover the levels of GSH reduced in sepsis (Ritter et al., 2004); and (d) the significant mortality reduction when septic newborns were treated with melatonin (Gitto et al., 2001). Besides counteracting mitochondrial oxidative stress (Acuña-Castroviejo et al., 2002; León et al., 2004, 2005; Martín et al., 2000, 2000b), melatonin also suppresses iNOS/mtiNOS expression and thus, the use of melatonin in combination with other antioxidants may improve the efficiency of these compounds individually used.

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