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

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ABSTRACT **We evaluated the role of melatonin in endotoxemia caused by lipopolysaccharide (LPS) in unanesthetized rats. The expression of inducible isoform of nitric oxide synthase (iNOS) and the increase in the oxidative stress seem to be responsible for the failure of lungs, liver, and kidneys in endotoxemia. Bacterial LPS (10 mg/kg b.w) was i.v. injected 6 h before rats were killed and melatonin (10–60 mg/kg b.w.) was i.p. injected before and/or after LPS. Endotoxemia was associated with a significant rise in the serum levels of aspartate and alanine aminotransferases, γ-glutamyl-transferase, alkaline phosphatase, creatinine, urea, and uric acid, and hence liver and renal dysfunction. LPS also increased serum levels of cholesterol and triglycerides and reduced glucose levels. Melatonin administration counteracted these organ and metabolic alterations at doses ranging between 20 and 60 mg/kg b.w. Melatonin significantly decreased lung lipid peroxidation and counteracted the LPS-induced NO levels in lungs and liver. Our results also show an inhibition of iNOS activity in rat lungs by melatonin in a dose-dependent manner. Expression of iNOS mRNA in lungs and liver was significantly decreased by melatonin (60 mg/kg b.w., 58–65%). We conclude that melatonin inhibits NO production mainly by inhibition of iNOS expression. The inhibition of NO levels may account for the protection of the indoleamine against LPS-induced endotoxemia in rats.—Crespo, E., Macías, M., Pozo, D., Escames, G., Martín, M., Vives, F., Guerrero, J. M., Acuña-Castroviejo, D. 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.* 13, 1537–1546 (1999)**

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MANY OF THE pathological consequences of gram-negative shock depend on the bacterial membrane component lipopolysaccharide (LPS).[2] Septic shock is characterized by hypotension, hyporeactivity to vasoconstrictor agents, inadequate tissue perfusion, vascular damage, and disseminated intravascular coagulation leading to multiple organ failure and death (1).

Nitric oxide (NO) is an important messenger regulating nervous, immune, and cardiovascular homeostasis (2). Increases in NO have been implicated in the pathogenesis of circulatory shock and inflammation (3–6). LPS-dependent induction of the inducible isoform of NO synthase (iNOS) is responsible for the overproduction of NO (3, 6, 7). NO can also react with superoxide anion ($O_2^{\cdot-}$), leading to formation the peroxynitrite anion ($ONOO^-$) (8), which oxidizes sulfhydryl groups and generates hydroxyl radical (·OH) (9). Interaction of LPS with

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[2] Abbreviations: ALP, alkaline phosphatase; aMT, melatonin (N-acetyl-5-methoxytryptamine); b.w., body weight; cNOS, constitutive nitric oxide synthase; DEPC, diethyl pyrocarbonate; dNTP, deoxyribonucleotide; DTT, DL-dithiotreitol; EGTA, ethylene glycol bis-(β-aminoethyl ether)-tetraacetic acid; eNOS, endothelial NOS; GOT, aspartate aminotransferase; GPT, alanine aminotransferase; γGT, γ-glutamyltransferase; H_2O_2, hydrogen peroxide; 4HAD, 4-hydroxyalkenals; iNOS, inducible isoform of NOS; H4-biopterin, 5,6,7,8- tetrahydro-L-biopterin dihydrochloride; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2- hydroxypropanesulfonic acid); i.p., intraperitoneal; i.v., intravenous; L-NMMA, N^G-methyl-L- arginine; LPS, lipopolysaccharide(s); MDA, malondialdehyde; NF-κB, nuclear factor kappa B; NO, nitric oxide; $O_2^{\cdot-}$ superoxide anion; ·OH, hydroxyl radical; $ONOO^-$, peroxynitrite anion; PMSF, phenylmethylsulfonylfluoride; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulfate; TLCK, Na-p-tosyl-L-lysine chloromethyl ketone; tris-HCl, tris(hydroxymethyl)aminomethane.

macrophages results in the generation of free radicals, including hydrogen peroxide (H_2O_2), $O_2^{\cdot-}$, and ·OH, leading to oxidative damage in many tissues such as lungs, liver, and kidneys (10). This progression of shock to a multiple organ dysfunction syndrome is associated with a substantial increase in mortality (11).

Nonselective inhibitors of all NOS isoforms may cause excessive vasoconstriction by endothelial NOS (eNOS) inhibition, increasing the incidence of organ ischemia, oxidative stress, and mortality (12). Thus, the beneficial effects of NOS activity inhibitors may depend on the inhibition of iNOS activity (4). However, the inhibition of NOS activity with aminoethyl-isothiourea, a relatively selective inhibitor of iNOS activity, or N^G-methyl-L- arginine (L-NMMA), a nonselective inhibitor of NOS activity, attenuates the liver dysfunction associated with LPS-induced endotoxemia in the rat without significant increase in arterial blood pressure, although eNOS activity was also inhibited (13). Thus, the production of NO and NO-dependent free radicals seems to contribute to the development of multiple organ failure in endotoxic shock.

Melatonin (N-acetyl-5-methoxytryptamine, aMT) efficiently scavenges both the hydroxyl and peroxyl radicals (14–17) acting at any subcellular component (18), thus decreasing tissue damage due to free radical generation (19, 20). The inhibition of the constitutive NOS (cNOS) activity (21–23) by aMT suggests its interaction with other NOS isoforms. In fact, both antioxidant (24, 25) and inhibitory iNOS (26) activities of aMT appear to be involved in the protective effect of the indoleamine in LPS-induced endotoxemia.

We used the present experimental paradigm, which mimics the human endotoxemia (27), to study whether aMT inhibits the expression of iNOS induced by LPS administration to rats *in vivo*. We also studied the efficacy of aMT to counteract the multiple organ failure manifestations.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (LPS from *Escherichia coli*, serotype 0127: B8), melatonin, DL-dithiotreitol (DTT), pepstatin A, phenylmethylsulfonylfluoride (PMSF), aprotinin, leupeptin, bacitracin, benzamidine, Na-p-tosyl-L-lysine chloromethyl ketone (TLCK), NADPH, 5,6,7,8- tetrahydro-L-biopterin dihydrochloride (H4-biopterin), FAD, hypoxanthine-9-β-D-ribofuranoside (inosine), bovine serum albumin, L-arginine, N-(2-hydroxyethyl)piperazine-N′-(2- hydroxypropanesulfonic acid) (HEPES), L-NMMA, ethylene glycol bis-(β-aminoethyl ether)-tetraacetic acid (EGTA), chloroform, L-citrulline, Dowex-50W (50×8–200), N-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, DTT, guanidine-thiocyanate, diethyl pyrocarbonate (DEPC), Denhardt's solution, formamide, formaldehyde, herring sperm DNA, Tween 20, sodium chloride, and sodium nitrite were purchased from Sigma Química (Spain). UV cross-linker, L-^{3}H-arginine (58 Ci/mmol), α-^{32}P-dCTP, and α-^{32}P-dATP were obtained from Amersham (Amersham, Bucks, U.K.). Tris(hydroxymethyl)-aminomethane (Tris-HCl), calcium chloride, and phosphoric acid were obtained from Merck (Spain). The Bioxytech LPO-586 kit, purchased from Cayman Chemical (Ann Arbor, Mich.), was used to measure the products of lipid peroxidation. M-MLV reverse transcriptase, *Taq* DNA polymerase, RNase inhibitor, random hexamer primers [dp$(N)_6$], dATP, dCTP, dGTP, and dTTP were obtained from Promega (Madison, Wis.). NuSieve agarose was obtained from FMC BioProducts (Rockland, Maine). Phenol/water saturated solution was obtained from Eurobio (France). The oligonucleotides and DNase FPLC pure were obtained from Pharmacia Biotech (Uppsala, Sweden). Zeta-probe nylon blotting membranes and all electrophoreses reagents were obtained from Bio-Rad (Hercules, Calif.). All other chemicals were of reagent grade.

Animals and treatments

Male Wistar rats (200–250 g) were kept in the university's animal facilities on a 12 h light/12 h dark cycle at 22°C and on regular chow and water until the day of the experiment. Three days before the experiment, the jugular vein was cannulated under intraperitoneal (i.p.) equithesin anesthesia (1 ml/kg) for the administration of LPS. Animals were divided into three experimental groups of 6–8 animals each and two control groups of 10 animals. All groups except control received an intravenous (i.v.) injection of LPS (serotype 0127:B8, 10 mg/kg body weight (b.w.) dissolved in 0.3 ml of saline) between 08:00 and 09:00 AM to minimize diurnal variations. In trial experiments, several doses of i.v. LPS were tested to find the optimal dose to induce iNOS and nitrites. A dose-dependent effect was found, but the endotoxemia caused by 30 mg/kg b.w. produced the animals death in 4–5 h. Thus, we chose the i.v. dose of 10 mg/kg b.w. as optimal to perform the experiments.

Melatonin-treated groups were i.p. injected with one to six doses of aMT (10 to 60 mg/kg body wt dissolved in 0.2 ml of 0.25% ethanol:saline). The LPS + aMT group was divided into four subgroups depending on the schedule of aMT administration: LPS + aMTb received a dose of aMT 30 min before LPS; LPS + aMTa received a dose of aMT 60 min after LPS; LPS + aMT2 received two aMT doses, one injected 30 min before LPS and the second injected 60 min after LPS; LPS + aMT6 received six injections of aMT: one injection 30 min before LPS, five injections beginning 60 min after LPS, and the last dose coinciding with 1 h before death. A saline-treated and aMT-treated groups injected with saline and aMT served as controls. There were no differences between the results obtained with six doses of saline to simulate the six doses of aMT compared with 1 dose; the data of the saline control group shown in the results correspond to the group injected with six saline injections. Similarly, six doses of aMT produced the same results as one dose, so the data of the aMT control group correspond to the group injected with six doses of aMT. Six hours after LPS injection, animals were killed by cervical dislocation and exsanguination. Lungs and livers were quickly collected, washed in cold saline, and frozen to −80°C in liquid nitrogen. Blood samples were also collected for biochemical analysis.

To assess whether the increase in organ nitrite due to LPS treatment can be blocked by NOS inhibitors, a group of six LPS-treated animals was i.v. injected with L-NMMA (3 mg/kg b.w. dissolved in 200 μl saline for each injection; flow rate of 30 μl/min through jugular vein) as follows: one injection 30 min before LPS and three injections beginning 60 min after

LPS, and the last dose 2 h before death. A saline-treated and an L-NMMA-treated group were injected with saline; L-NMMA served as controls.

Quantification of liver, kidney, and metabolic impairment

Blood samples obtained from the animals at the time of death were centrifuged (2500×*g* for 10 min) to prepare serum. All serum samples were analyzed within 24 h by the laboratory of biopathology of the Granada University's Hospital by routine methods. We assessed liver dysfunction and failure by measuring the rise in serum levels of aspartate aminotransferase (GOT, a nonspecific marker for hepatic parenchymal injury); alanine aminotransferase (GPT, a specific marker for hepatic parenchymal injury); alkaline phosphatase (ALP, a marker of the relative degree of hepatocellular damage and of obstruction, intrahepatic or extrahepatic, tends to parallel the degree of hyperbilirubinemia); total and direct bilirubin (specific markers for the development of cholestasis and of liver failure); and γ-glutamyltransferase (γGT, a marker for cholestasis). Renal dysfunction and failure were assessed by measuring the rise in serum levels of creatinine (an indicator of reduced glomerular filtration rate, hence renal failure), and urea and uric acid (an indicators of impaired excretory function of the kidney and/or increased catabolism). Metabolism impairment was assessed by measuring the changes in serum levels of cholesterol and triglycerides (as markers for lipid metabolism alteration); glucose (as marker for carbohydrate metabolism alteration); and total proteins and albumin (as markers for protein metabolism alteration).

Lipid peroxidation (LPO) assay

Soon after the animals were killed, the rats' abdomens were opened and the heart was perfused with ice-cold saline (4°C) to eliminate the excess of iron released from intracellular storage sites. Lungs and liver were removed and cooled on dry ice. Approximately 100 mg of lung and liver tissues were homogenized with a Stuart Scientific stirrer in ice-cold 20 mM Tris-HCl buffer, pH 7.4, to produce a 1/10 homogenate. The crude homogenate was centrifuged at 2500 × *g* for 30 min at 4°C. Aliquots of the supernatant were either stored at −20°C for total protein determination (28) or used to calculate LPO. Malondialdehyde (MDA) and 4-hydroxyalkenals (4HDA) concentrations provide a convenient index of lipid peroxidation (29). The Bioxytech LPO-568 kit (Cayman Chemical) was used. The kit takes advantage of a chromogenic reagent that reacts with MDA and 4HDA at 45°C, yielding a stable chromophore with maximal absorbance at the 586 nm wavelength. The light wavelength and the low temperature of incubation used for these measurements eliminate interference and undesirable artifacts.

Nitrite determination

Tissues were cooled in ice-cold distilled water and homogenized (0.1 g/ml). The crude homogenate was centrifuged at 21,000 × *g* for 20 min at 4°C, and aliquots of the supernatant were either stored at −20°C for total protein determination (28) or used to calculate nitrite levels. The amounts of nitrite in supernatants or in serum were measured following the Griess reaction (30) by incubating a 100 μl of sample with 100 μl of Griess reagent [0.1% N-(1-naphthyl)ethylenediamine dihydrochloride; 1% sulfanilamide in 5% phosphoric acid; 1:1] at room temperature for 20 min. The absorbance at 550 nm (OD 550) was measured with a Molecular Devices microplate reader. Nitrite concentration was calculated by comparison with the OD 550 of a standard solution of known sodium nitrite concentrations.

To assess whether the nitrite values measured in the tissues collected from animals were artifactually generated due to accumulated blood, previous experiments in postmortem perfused animals were undertaken. Briefly, at the end of the experiment, the rats were anesthetized with ether and a blood sample was taken from the aorta. Then the aorta was clamped, the right auricle cut, and 25 ml of cold saline was passed through the right ventricle to perfuse the organs. At the end of this procedure, lungs and livers of the animals were quickly removed, placed in cold nitrogen, and frozen to −80°C. Nitrite concentrations in perfused tissues were not significantly different from nitrite levels measured in nonperfused tissues. Thus, nitrites were routinely measured in nonperfused tissues washed with saline.

Assay of iNOS activity

Lungs were 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). Subsequent procedures were carried out at 0–4°C. The crude homogenate was centrifuged at 2500 × *g* for 5 min and aliquots of the supernatant were either stored at −20°C for total protein determination (28) or used immediately for NOS activity determination.

NOS activity was measured by monitoring the conversion of L-^{3}H- arginine to L-^{3}H-citrulline (31). The final incubation volume was 100 μl and consisted of 10 μl crude homogenate added to prewarmed (37°C) buffer to give (final concentration) 25 mM Tris, 1 mM DTT, 30 μM H4-biopterin, 10 μM FAD, 0.5 mM inosine, 0.5 mg/ml bovine serum albumin, 0.1 mM $CaCl_2$, 10 μM L-arginine, 40 nM L-^{3}H-arginine, pH 7.6. The reaction was started by the addition of 10 μl NADPH (0.75 mM final) and continued for 30 min at 37°C. Control incubations were done by NADPH omission. The reaction was stopped by adding 400 μl of cold 0.1 M HEPES, 10 mM EGTA, 0.175 mg/ml L-citrulline, pH 5.5. The reaction mixture was decanted onto a 2 ml column packed with Dowex-50W ion exchange resin (Na^+ form) and eluted with 1.2 ml of water. L-^{3}H-arginine was quantified by liquid scintillation spectroscopy. The retention of L-^{3}H-arginine in this process was greater then 98%. Specific enzymatic activity was determined by subtracting the control value, usually less than 1% of the added radioactivity. NOS activity is referred to as picomoles of L-^{3}H-citrulline produced per mg protein/min.

Analysis of iNOS protein expression

Frozen liver and lung specimens were homogenized with a Polytron homogenizer (Kinematic, Switzerland) at 4°C in HEPES 20 mM pH 7.4 containing 0.02% (W/V) bacitracin, 0.4 mM PMSF, 1 mM benzamidine, 1.5 μM pepstatin A, 0.1 mM TLCK, and 0.1 mM aprotinin. The homogenates were then centrifuged at 4000 × *g* for 10 min at 4°C and supernatants were collected to assay the protein expression. The protein content of the supernatant was determined with a protein assay kit (Bio-Rad) based on the method of Bradford (32). Samples (75 μg) were eluted from the supernatant directly into the sodium dodecyl sulfate (SDS) sample buffer for 7.5% SDS-polyacrylamide gel electrophoresis (33) and then transferred onto nitrocellulose membranes (Hoeffer, Calif.). Electroblots were blocked in 25 mM Tris-ClH buffer, pH 7.4, containing 150 mM NaCl, 0.05% (v/v) Tween 20 (TBST buffer) and 5% (w/v) dry milk. Western analysis was carried out using a 1:2,500 dilution in TBST buffer of specific polyclonal antibody against iNOS (Santa Cruz Biotechnology,

Santa Cruz, Calif.) for 2 h at room temperature. After TBST washing procedure, the blots were incubated with 1:5,000 peroxidase labeled anti-rabbit antibody (Amersham-Pharmacia Biotech, Uppsala, Sweden) in TBST for 30 min at room temperature. The immunoreaction was detected using the enhanced chemiluminescence EC system. (Amersham-Pharmacia Biotech).

Analysis of messenger RNA levels for iNOS

Total cellular RNA was purified using guanidinium thiocyanate-phenol-chloroform extraction (34). Single-stranded cDNA was synthesized from both rat liver and lung LPS-stimulated total RNA/DNase treated using the following method. Two microliters of high-quality RNA (5 μg/μl) was preincubated with 0.5 μg of $dp(N)_6$ and 26 μl Rnase-free H_2O at 85°C for 15 min, then rapidly chilled on ice. To this reaction were added 1 μl RNasin (40 U/μl), 10 μl 5× reverse transcriptase (RT) buffer, 5 μl dithiothreitol (100 mM), and 2.5 μl deoxyribonucleotide (dNTP; 10 mM each); the mixture was incubated at 39°C for 3 min. Finally, 1.5 μl Moloney murine leukemia virus reverse transcriptase (200 U/μl) was added to give a final volume of 50 μl; the reaction was incubated at 39°C for 60 min and terminated by placing it on ice after deactivation at 95°C for 5 min. Polymerase chain reaction (PCR) reactions contained 1 μM specific primers to inducible isoforms amplify a 223-bp portion of NOS (35, 36). The forward primer was TGCATGGACCAGTATAAGGCAAGC (5′ to 3′) and the reverse primer was CTTCTGGTCGATGTCATGAGCAA. For the PCR reactions (1× PCR reaction buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs), 2 μl of RT product was amplified after 'hot start' procedure, 30 PCR cycles (94°C, 1 min; 65°C, 1 min; 72°C, 1 min), followed by a final 10 min extension at 72°C (PTC-150 Minicycler (MJ Research, Watertown, Mass.). The PCR products were run on a 1.5% agarose gel, excised, purified, and random primer labeled with α-^{32}P-dCTP and α-^{32}P-dATP. RNA samples (20 μg) were electrophoresed on 1.2% agarose-formaldehyde gels, washed twice (15 min each) in DEPC-water at 65°C, transferred to zeta-probe membranes, and fixed using a UV cross-linker (750 μJ/cm^2). The blots were prehybridized (40°C, 3 h) in 5× SSPE, 0.1% SDS, 50% formamide, 2.5× Denhardt's solution, and 100 μg/ml denatured herring sperm DNA. Hybridization was performed at 42°C for 16 h with ^{32}P heat-denatured DNA probe in the prehybridization buffer, followed by washing twice (15 min each, room temperature) in 1× SSC, 0.1% SDS, and again washed twice (20 min each) to a final stringency of 0.1× SSC, 0.1% SDS at 55°C. The membranes were exposed to X-ray film with intensifying screens at −80°C and developed.

Statistical analyses

All data in the figures and text are expressed as mean ± SE of *n* observations, where *n* represents the number of animals or blood samples studied. A two-way analysis of variance, followed by a Dunnet's post hoc test when appropriate, was used to compare means between groups. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Effects of aMT on LPS-induced nitrite concentration and lipid peroxidation in liver and lungs

Baseline levels of nitrite in the lung and liver of vehicle-treated rats were 1.02 ± 0.03 and 0.68 ± 0.12 nmol/mg protein, respectively (**Fig. 1**). Similar values were found in aMT6-treated animals (1.26±0.11 and 0.90±0.01 nmol/mg protein, lung and liver, respectively). Endotoxemia was associated with a fourfold rise of nitrite levels in lungs and liver (4.18±0.54 and 2.75±0.35 nmol/mg protein, respectively, $P<0.001$). The increase in lung and liver nitrites caused by endotoxemia was significantly reduced in a dose-dependent manner in rats treated with aMT. One dose of aMT before or after LPS administration was enough to significantly reduce nitrite levels in both lungs and liver ($P<0.05$). When six doses of aMT were used, the increase in nitrite levels in both lungs and liver was abolished (1.12±0.03 and 0.92±0.06 nmol/mg protein, respectively, $P<0.001$).

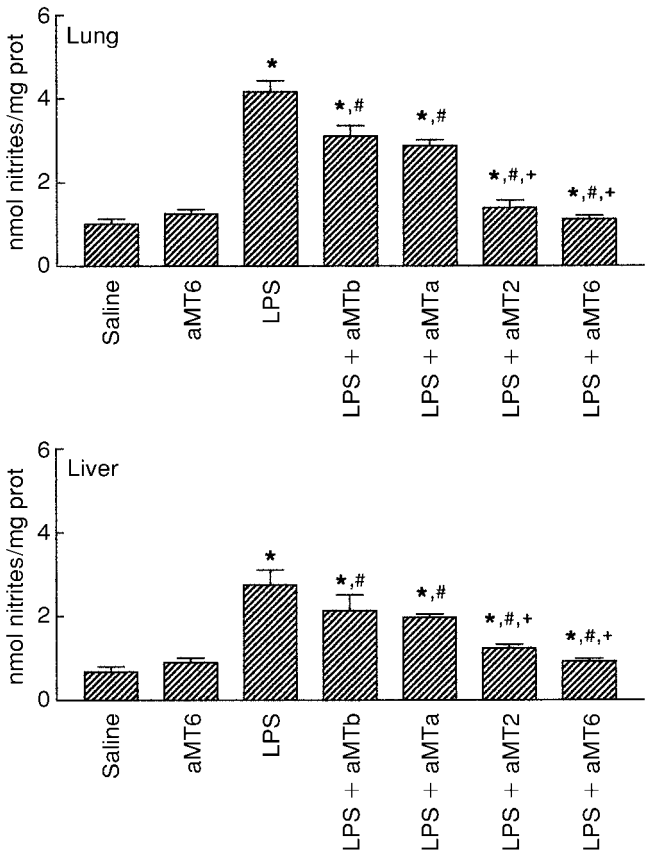

Figure 1. Dose-dependent effect of aMT on nitrite production in lung (top) and liver (bottom) of LPS-treated rats compared to saline- or aMT-injected rats. Melatonin (10 mg/kg b.w., each dose) was given in one dose 30 min before (aMTb); one dose 60 min after (aMTa); two doses, one 30 min before and another 60 min after (aMT2); or six doses, one 30 min before plus the others 60, 120, 180, 240, and 300 min after LPS (aMT6). Control rats injected with aMT alone (without LPS) were treated with one or six doses of aMT without differences in the nitrite levels; only the group treated with six doses is depicted (aMT6). Although one dose of aMT before LPS was enough to significantly inhibit nitrite levels, a most pronounced inhibition was found after two doses of aMT. Six doses of aMT reduced nitrite levels to control values. $^*P <$ 0.001 vs. control and aMT6; $^{\#}P < 0.001$ vs. LPS; $^{+}P < 0.001$ vs. aMTa, aMTb.

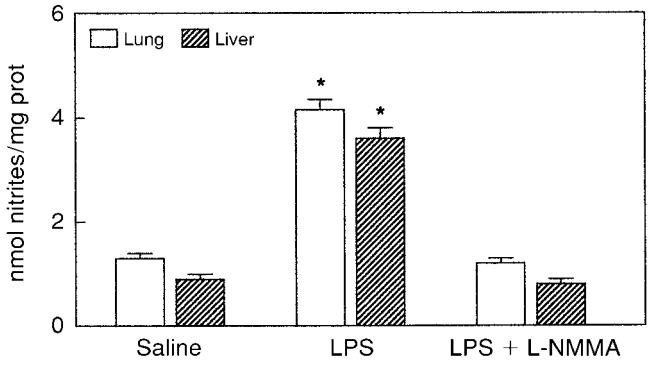

Figure 2. Effect of L-NMMA administration on nitrite production in lung and liver of LPS-treated rats compared to saline-injected animals. L-NMMA (3 mg/kg b.w. each dose, infused through jugular vein at a flow rate of 30 μl/min) was administered in one dose 30 min before LPS and three injections (beginning 60 min after LPS, the last dose coinciding 2 h before death). Four doses of L-NMMA counteracted the effect of LPS on nitrite levels. $*P < 0.001$ vs. saline- and LPS + L-NMMA-treated rats.

The LPS-induced organ nitrite depends on the iNOS activation, since administration of L-NMMA blocked the nitrite production in lung and liver due to LPS ($P<0.001$) (**Fig. 2**). At the dose used, LPS administration only induces a slight, insignificant increase in LPO in both the liver and lung. Melatonin treatment (six doses) was able to significantly decrease the lung LPO levels found in the control and LPS-treated groups ($P<0.05$), showing no effect in liver (**Fig. 3**).

Effects of aMT on the increase in LPS-induced iNOS activity in the lung

Studies of the isoform of NOS measured in our experiments show an increase in calcium-independent iNOS activity after LPS treatment, whereas no calcium-dependent cNOS was found (**Fig. 4**). A small calcium-independent iNOS activity was detectable in lung of saline-treated animals. The significant increase in LPS-induced iNOS activity (111.63±7.86 pmol citrulline/mg protein min, $P>0.001$) was significantly reduced in a dose-dependent manner by aMT treatment (**Fig. 5**). One dose of aMT 60 min after LPS injection significantly reduced the iNOS activity in lung (69.12±7.41 pmol citrulline/mg protein min, $P<0.05$). Six doses of aMT were associated with a 17-fold reduction in iNOS activity (6.62±1.54 pmol citrulline/mg protein min, $P<0.001$), although a residual activity significantly different from control iNOS activity remained.

The technique for iNOS measurement specifically detects iNOS activity. In fact, addition of L-NMMA (300 μM) to the mixture reaction of tissues from LPS-treated rats blocked the transformation of L-arginine to L-citrulline due to iNOS activity inhibition (101.23±6.98 vs. 4.26±1.23 pmol citrulline/mg protein min, LPS, and LPS + L-NMMA, respectively; $P<0.001$).

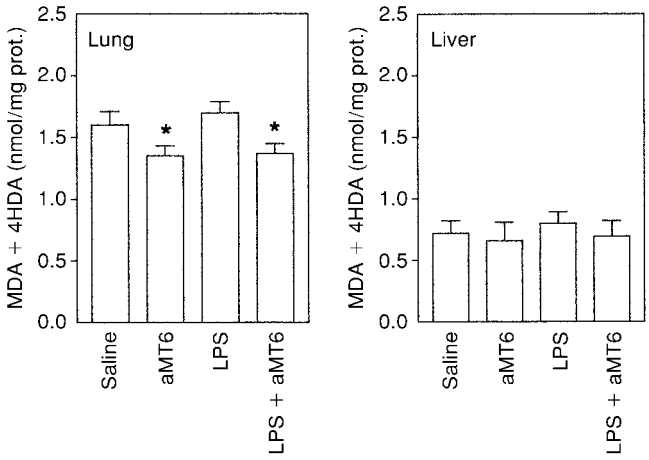

Figure 3. Effects of aMT on LPO levels in lungs (top) and liver (bottom) of LPS-treated rats. Legends are the same as in Fig. 1. Although LPS did not change LPO levels *per se*, six doses of melatonin were able to significantly decrease LPO levels in lungs, but not in liver.

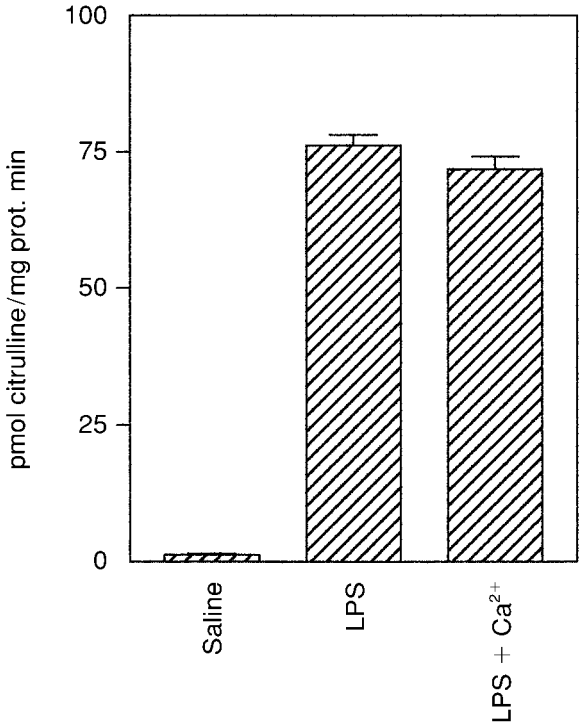

Figure 4. LPS effect on NOS activity in rat lungs. The presence of calcium did not change the activity of NOS found, suggesting that most of the activity corresponded to iNOS.

Effects of aMT on the LPS-induced expression of iNOS mRNA in lung and liver

To gain insight into the molecular mechanisms involved in the inhibitory effect of aMT on iNOS

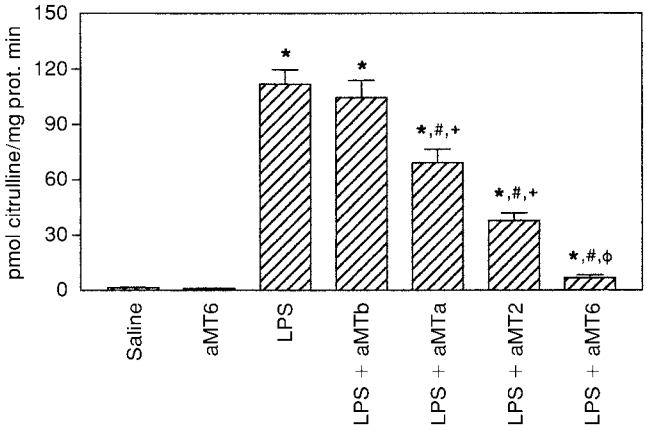

Figure 5. Dose-dependent effect of aMT on iNOS activity in lungs of LPS-treated rats compared to saline- or aMT-injected rats. Melatonin was given following the schedule of Fig. 1. Control rats injected with aMT alone (without LPS) were treated with one or six doses of aMT without differences in the iNOS activity; therefore, only the group treated with six doses is depicted in the figure (aMT6). One dose of aMT after LPS was enough to significantly inhibit iNOS activity. The most pronounced inhibition was found after six doses of aMT, which restored iNOS activity to near control values. $^{*}P < 0.001$ vs. control and aMT6; $^{\#}P < 0.001$ vs. LPS; $^{+}P < 0.001$ vs. aMTb, aMT2, and aMT6; $^{\Phi}P < 0.001$ vs. aMT aMTa, aMTb, and aMT2.

activity, we performed Northern blot analysis to analyze whether aMT affects the steady-state levels of iNOS mRNA. No detectable levels of iNOS mRNA were noted from unstimulated LPS or melatonin alone (data not shown) by RT-PCR. Blots were probed with a radiolabeled iNOS cDNA generated by RT-PCR (see Materials and Methods). Northern blot showed a single band in RNA isolated from lungs and liver treated with LPS. An inhibitory effect of six doses of aMT could be seen after just 6 h of treatment (65.2% and 58.2% in lung and liver, respectively) (**Fig. 6**), indicating transcriptional or posttranscriptional mechanisms of modulation by aMT.

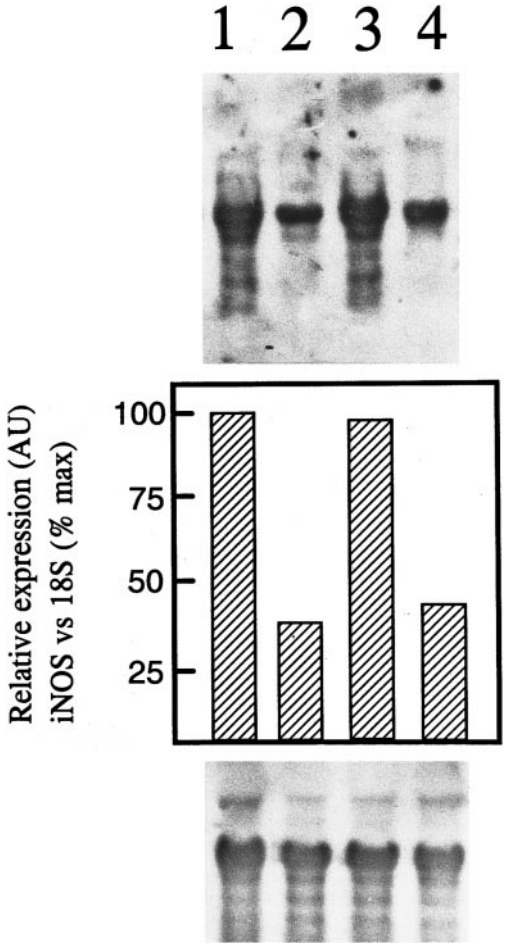

Figure 6. *In vivo* inhibitory effects of aMT on iNOS mRNA steady-state levels in both lung and liver. LPS-treated rats were i.p. injected with six doses of aMT (lane 2, lung; lane 4, liver) or vehicle (lane 1, lung; lane 3, liver). The top panel shows Northern blot analysis of total RNA (20 μg/lane) probed with a radiolabeled rat iNOS cDNA. Expression of rat 18S rRNA in the same membrane is shown at bottom; densitometric analysis of the iNOS Northern blot corrected for 18S rRNA is depicted in the middle panel. Representative data from one of two independent blots are shown.

Effects of aMT on the LPS-induced iNOS protein expression in lung and liver

Western blot studies confirmed the presence of iNOS protein and agreed with measurements of nitric oxide. Immunoblot using an anti-iNOS antibody revealed a single component corresponding to the 130 kDa monomer of iNOS (**Fig. 7**). The results show that the increase of iNOS activity was accompanied with a significant increase of iNOS protein expression in rat lung and liver of LPS-treated animals, with a marked reduction after aMT administration.

Effect of aMT on LPS-induced liver dysfunction

As expected from the LPS dose used, levels of GOT ($P<0.005$), GPT ($P<0.05$), γGT ($P<0.005$), ALP ($P<0.005$), and total ($P<0.05$) and direct ($P<0.005$) bilirubin increase with LPS treatment. **Table 1** shows that a unique dose of aMT before or after LPS was enough to significantly decrease the serum levels of GOT and GPT, respectively ($P<0.05$). Normal serum values of these hepatic enzymes were reached with two aMT doses ($P<0.005$). γGT and ALP were more resistant to aMT treatment, and two doses of the indoleamine were necessary to significantly counteract the effect of LPS ($P<0.005$). Regarding total and direct bilirubin one dose of aMT after or before, respectively, counteracts the LPS effect ($P<0.05$). In all cases except γGT, the rise in serum levels of the hepatic markers caused by LPS was abolished by treatment with two doses of aMT. Additional aMT doses do not further decrease the levels of these enzymes.

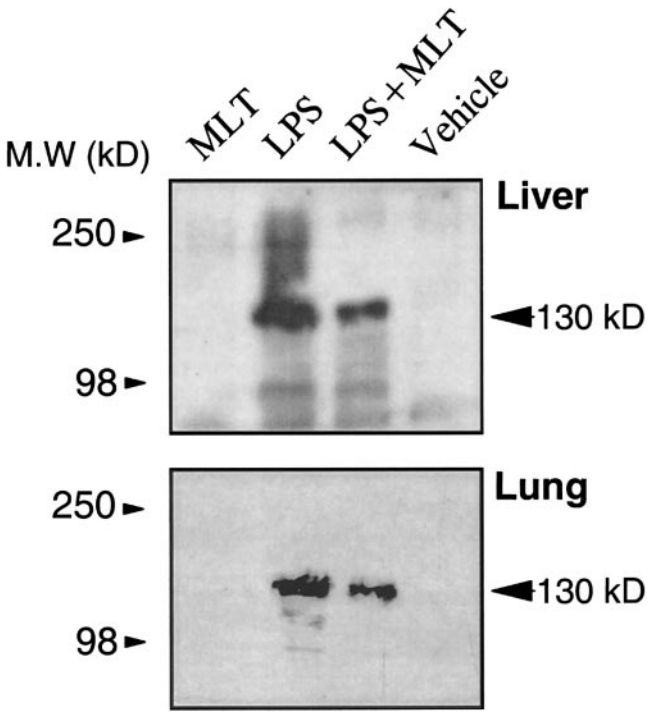

Figure 7. Effect of melatonin on iNOS protein expression in the rat liver (upper gel) and lung (bottom gel) treated with LPS. Lane 1: melatonin; lane 2: LPS; lane 3: LPS + melatonin; lane 4: vehicle.

Effect of aMT on LPS-induced renal dysfunction

The dose of LPS used here also impaired renal function. **Table 2** shows the significant increase in serum levels of creatinine ($P<0.05$), urea ($P<0.005$), and uric acid ($P<0.05$) after i.v. injection of 10 mg/kg body wt of LPS. Although two doses of aMT significantly counteract the effect of LPS on urea levels ($P<0.005$), six doses of the indoleamine were necessary to normalize serum levels of both creatinine and urea. One dose of aMT before LPS was enough to counteract LPS-dependent increases of uric acid levels ($P<0.05$), the effect being more valuable with two doses of aMT.

Effect of melatonin on LPS-induced metabolism impairment

Because of the organ dysfunction caused by endotoxemia, an alteration in metabolic routes is expected. **Table 3** shows the changes in some parameters measured as index of lipid (cholesterol and triglycerides), carbohydrate (glucose), and protein (total protein and albumin) metabolism. Uric acid, which may be used as an index of nucleic acid metabolism, was included with markers of renal dysfunction. Cholesterol ($P<0.05$) and triglycerides ($P<0.005$) significantly increase after LPS treatment. One dose of aMT significantly normalizes cholesterol levels ($P<0.05$), whereas two doses of the indoleamine were necessary to normalize triglyceride values. LPS also induced changes in carbohydrate metabolism, decreasing the glucose levels ($P<0.005$). In this case, six doses of aMT were able to abolish the LPS effect ($P<0.05$). Regarding protein metabolism, no significant changes were found in total protein after LPS treatment. Only a slight decrease in albumin was found after LPS, an effect counteracted by six doses of aMT ($P<0.05$).

DISCUSSION

This study clearly demonstrates for the first time that aMT is able to inhibit the expression of the iNOS mRNA levels induced by LPS in lungs and liver of rats *in vivo* in a dose-related manner. As expected, we also observed a dose-dependent inhibition of iNOS activity and nitrite production by aMT in the same experimental paradigm. These data further support and extend to *in vivo* conditions the recently reported inhibition of the LPS-induced iNOS expression in murine macrophages in culture by aMT (37).

Gram-negative bacterial endotoxin (LPS) induces a variety of biological responses and diseases. It is believed that at least two mechanisms are involved in these responses: the production of anaphylatoxin and the release of biochemical mediators such as

TABLE 1. *Effects of melatonin on LPS-induced increases in the serum activities of aspartate aminotransferase (GOT), alanine aminotransferase (CGT), γ-glutamyl-transferase (γGT), alkaline phosphatase (ALP), and both total (Tbil) and direct (Dbil) bilirubin*[a]

Group	*n*	GOT, iu l^{-1}	GPT, iu l^{-1}	γGT, iu l^{-1}	ALP, iu l^{-1}	Tbil, mg/dl	Dbil, mg/dl
Saline	10	329.5 ± 14.0	75.7 ± 2.9	0.7 ± 0.3	411.8 ± 67.2	0.1 ± 0.02	0.02 ± 0.01
aMT6	6	315.3 ± 61.7	69.3 ± 11.5	0.8 ± 0.1	594.8 ± 53.7	0.08 ± 0.002	0.02 ± 0.005
LPS	5	840.0 ± 179.0**	190.2 ± 48.2*	14.2 ± 2.9**	1665.2 ± 333.3**	0.21 ± 0.06*	0.14 ± 0.05**
LPS + aMTb	6	446.0 ± 88.0†	182.5 ± 48.8	14.8 ± 6.0	1788.8 ± 589.6	0.16 ± 0.01	0.05 ± 0.008†
LPS + aMTa	6	495.1 ± 85.7†	104.1 ± 23.4†	11.5 ± 1.4	1240.5 ± 236.5	0.12 ± 0.02†	0.05 ± 0.02†
LPs + aMT2	6	310.0 ± 28.7††	52.5 ± 4.3††	3.1 ± 0.1††	561.8 ± 7.4††	0.11 ± 0.002†	0.05 ± 0.003†
LPS + aMT6	6	241.6 ± 9.3††	59.3 ± 2.9††	5.0 ± 0.8††	645.0 ± 106.6††	0.12 ± 0.006†	0.038 ± 0.003†

[a] Data are mean ± SE, *n* = number of observations. Values from LPS-treated animals that are statistically different from those from controls and aMT6-treated ones are shown: * ($P<0.05$) and ** ($P<0.005$). Values from LPS-treated rats injected with different doses of aMT that are statistically different from LPS-treated ones are shown: † ($P<0.05$) and †† ($P<0.005$).

TABLE 2. *Effects of melatonin on LPS-induced increases in the serum leves of creatinine, urea, and uric acid*[a]

Group	*n*	Creatinine, mg/dl	Urea, mg/dl	Uric acid, mg/dl
Saline	10	0.42 ± 0.04	31.1 ± 5.3	0.77 ± 0.14
aMT6	5	0.43 ± 0.04	25.1 ± 4.1	0.70 ± 0.09
LPS	6	0.68 ± 0.1*	91.6 ± 8.1**	1.46 ± 0.38*
LPS + aMTb	6	0.45 ± 0.02	67.3 ± 13.6	0.81 ± 0.17†
LPS + aMTa	6	0.45 ± 0.02	81.0 ± 6.7	0.93 ± 0.19
LPs + aMT2	6	0.46 ± 0.02	57.0 ± 3.8††	0.78 ± 0.15†
LPS + aMT6	6	0.40 ± 0.03†	41.6 ± 4.6††	0.75 ± 0.02†

[a] Data are mean ± SE, *n* = number of observations. Values from LPS-treated animals that are statistically different from those from controls and aMT6-treated ones are shown: * ($P<0.05$) and ** ($P<0.01$). Values from LPS-treated rats injected with different doses of aMT that are statistically different from LPS-treated ones are shown: † ($P<0.05$) and †† ($P<0.01$).

histamine, serotonin, kinins, platelet-activating factors by the reticuloendothelial system, and cytokines such as tumor necrosis factor-α, interleukin 1β, and interferon-γ (1, 26, 38). In addition, LPS directly inhibits both glucose (39) and lipid metabolism (40) and causes hepatotoxicity, renal failure, and lipid peroxidation via the induction of free radicals (10, 24, 25, 41, 42). Our data agree with these organ failure and metabolic alterations caused by LPS. Our results also demonstrated that aMT administration counteracted the LPS-induced multiple organ dysfunction syndrome in rats, protecting the animals against endotoxemia and death.

Is the inhibitory effect of aMT on the expression of iNOS mRNA levels the same mechanism by which the indoleamine counteracted multiple organ dysfunction caused by LPS? Previous reports showed that aMT inhibits the activity of cNOS (21–23). It was suggested that with nonselective inhibitors of all isoforms of NOS such as L-NMMA, the concomitant inhibition of eNOS activity causes excessive vasoconstriction and thus increases the incidence of organ ischemia and mortality (43). Consequently, the beneficial effects of NOS inhibitors may be due to inhibition of iNOS activity, whereas the adverse effects reported may be due to the inhibition of eNOS and/or neuronal NOS activities (4, 44). However, in a recent report, the use of a iNOS-selective inhibitor (aminoethyl-isothiourea) and a nonselective NOS inhibitor (L-NMMA) yielded similar beneficial results in LPS-induced endotoxemia in rats (13), without adverse effects. In our experiments, we cannot know the degree (if any) of inhibition of constitutive eNOS isoform by aMT. Nevertheless, our results demonstrate that melatonin not only prevents LPS toxicity in terms of iNOS activity and iNOS mRNA levels, but also promotes recovery from LPS effects in rats.

Expression of iNOS activity impairs hepatocyte cell function, leading to a reduction in protein, prostaglandins, and interleukin 6 synthesis (45, 46), glycogen and lipid metabolism alterations (39, 40), and inhibition of mitochondrial respiratory chain complex I and II (47). These metabolic alterations may contribute to the injury caused by NO after the induction of iNOS. NO may combine with $O_2^{\cdot-}$ to form the more potent, reactive oxygen metabolite peroxynitrite, which decomposes to form the ·OH. Both peroxynitrites and ·OH are responsible for cellular lipid peroxidation, protein oxidation, and mitochondrial impairment function. Melatonin is a very efficient scavenger of peroxynitrites (48) and hydroxyl radicals (15–17, 49), counteracting lipid peroxidation produced by toxins, including LPS (24, 25). Although at the dose used here LPS produced a significant increase in lipid peroxidation in lungs,

TABLE 3. *Effects of melatonin on LPS-induced changes in the serum levels of cholesterol, triglycerides, glucose, total protein, and albumin*[a]

Group	*n*	Cholesterol, mg/dl	Triglycerides, mg/dl	Glucose, mg/dl	Total protein, g/dl	Albumin, g/dl
Saline	10	62.1 ± 1.6	142.4 ± 19.9	138.0 ± 5.4	5.8 ± 0.1	3.3 ± 0.1
aMT6	6	57.6 ± 3.8	113.5 ± 17.2	142.3 ± 7.1	5.9 ± 0.2	3.6 ± 0.09
LPS	5	75.8 ± 10.5*	211.4 ± 35.6**	100.2 ± 12.0**	5.6 ± 0.2	3.1 ± 0.2*
LPS + aMTb	6	64.3 ± 1.4	183.3 ± 33.4	73.1 ± 19.7	5.6 ± 0.1	3.06 ± 0.08
LPS + aMTa	6	58.1 ± 1.4†	192.8 ± 19.8	77.3 ± 10.9	5.7 ± 0.06	2.9 ± 0.07
LPs + aMT2	6	59.1 ± 0.8†	111.3 ± 8.8††	102.3 ± 3.8	5.5 ± 0.06	2.8 ± 0.05
LPS + aMT6	6	57.3 ± 1.8†	114.5 ± 11.1††	125.0 ± 2.5†	5.6 ± 0.08	3.2 ± 0.07†

[a] Data are mean ± SE, *n* = number of observations. Values from LPS-treated animals that are statistically different from those from controls and aMT6-treated ones are shown: * ($P<0.05$) and ** ($P<0.005$). Values from LPS-treated rats injected with different doses of aMT that are statistically different from LPS-treated ones are shown: † ($P<0.05$) and †† ($P<0.005$).

aMT significantly decreased it, suggesting that some of the beneficial effects of melatonin depend on its antioxidant properties. aMT significantly increased the activity of the mitochondrial respiratory chain complex I and IV in liver of normal rats (50).

Thus, the ability of aMT to inhibit the expression of iNOS mRNA levels, and thus iNOS activity and NO production, may be the main effect of the indoleamine to protect rats against endotoxemia. However, both the antioxidant and stimulatory mitochondrial respiratory chain roles of aMT seems to participate in the recovery of multiple organ failure in LPS-treated animals. How aMT inhibits the expression of iNOS mRNA? Melatonin exhibits remarkable functional versatility. Besides the antioxidant, nonmediated receptor effects of the indoleamine, some it effects are related to the cell nucleus. A nuclear receptor for melatonin has been characterized, and it was shown that nuclear binding sites may be a family of orphan receptors (51, 52). Several genomic actions of melatonin have been reported (53, 54). Thus, melatonin may inhibit iNOS mRNA expression acting at nuclear level. Our results show that the changes in iNOS mRNA steady-state levels measured by Northern blot were lower than enzymatic activity, suggesting a regulatory role of aMT in the transcriptional rate. However, from our results, a role of aMT in the stability and/or translation efficiency cannot be discarded. A recent study showed that aMT inhibits NO production mainly by inhibiting the expression of iNOS (37). The authors show that aMT inhibits iNOS promoter activation and that the inhibition of iNOS expression was associated with inhibition of activation of the transcription factor nuclear factor kappa B (NF-κB) (37). NF-κB has been found to be regulated by the intracellular redox state (55). Critical steps in the signal transduction cascade are sensitive to oxidants and antioxidants (56), and the endogenous glutathione system may therefore be considered to effectively regulate redox-sensitive gene expression. Since both glutathione and glutathione peroxidase are influenced by aMT (53, 57), the changes in cellular redox status by aMT may underlie the inhibition of iNOS mRNA expression due to the pineal hormone. The possibility that aMT acts as a regulator of redox-sensitive gene expression opens new perspectives in the study of its mechanism of action.

A last consideration is the dose of aMT used to counteract LPS damage in our study. Endogenous aMT levels reach 1 nM during nocturnal peak, and this concentration is enough to reduce cNOS activity in a 20% (21–23). In rats, a dose of 200 μg/kg of aMT may be regarded as within the physiological range (58). Thus, the doses of aMT used here (from 10 to 60 mg/kg) will increase plasma melatonin levels up to 50–300 nM. These values are significantly lower than those reported elsewhere (1 μM–1 mM) showing aMT inhibition of iNOS expression in murine macrophages in culture (35). These results suggest that aMT is more efficient in counteracting LPS-induced iNOS expression in *in vivo* than in *in vitro* conditions. Melatonin plasma half-life is approximately 40 min, and this value is significantly reduced in the presence of free radicals that quickly oxidize the indoleamine. Because including high doses of aMT seems to lack negative side effects (59), the results of this study may support the clinical use of melatonin in endotoxemia. FJ

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REFERENCES

1. Barron, R. L. (1993) Pathophysiology of septic shock and implications for therapy. *Clin. Pharm.* **12,** 829–845
2. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109–142
3. Moncada, S., and Higgs, A. (1993) The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329,** 2002–2012
4. Thiemermann, C. (1994) The role of the L-arginine: nitric oxide pathway in circulatory shock. *Adv. Pharmacol.* **28,** 45–79
5. Nathan, C., and Xie, Q. W. (1994) Nitric oxide synthases: roles, trolls and controls. *Cell* **78,** 915–918
6. Szabó, C. (1995) Alterations in nitric oxide production in various forms of circulatory shock. *New Horiz.* **3,** 2–32
7. Iuvone, T., D'Acquisto, F., Carnuccio, R., and Di Rosa, M. (1997) Nitric oxide inhibits LPS-induced tumor necrosis factor synthesis in vitro and in vivo. *Life Sci.* **59,** PL207–PL211
8. Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature (London)* **364,** 626–632
9. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87,** 1620–1624
10. Sugino, K., Dohi, K., Yamada, K., and Kawasaki, T. (1989) Changes in the levels of endogenous antioxidants in the liver of mice with experimental endotoxemia and the protective effects of the antioxidants. *Surgery* **105,** 200–206
11. Deitsch, E. A. (1992) Multiple organ failure: pathophysiology and potential future therapy. *Ann. Surg.* **216,** 117–134
12. Wright, C. E., Rees, D. D., and Moncada, S. (1992) Protective and pathological roles of nitric oxide in endotoxic shock. *Cardiovasc. Res.* **26,** 48–57
13. Thiemermann, C., Ruetten, H., Wu, C. C., and Vane, J. R. (1995) The multiple organ dysfunction syndrome caused by endotoxin in the rat: attenuation of liver dysfunction by inhibitors of nitric oxide synthase. *Br. J. Pharmacol.* **116,** 2845–2851
14. Pieri, C., Marra, M., Moroni, F., Recchioni, R., and Marcheselli, F. (1994) Melatonin: a peroxyl radical scavenger more effective than vitamin E. *Life Sci.* **55**, 271–276
15. Reiter, R. J., Melchiorri, D., Sewerynek, E., Poeggeler, B., Barlow-Walden, L., Chuang, G. G., Ortiz, G., and Acuña-Castroviejo, D. (1995) A review of the evidence supporting melatonin as antioxidant. *J. Pineal Res.* **18,** 1-l 1
16. Acuña-Castroviejo, D., Escames, G., Macías, M., Muñóz Hoyos, A., Molina Carballo, A., Arauzo, M., Montes, R., and Vives, F. (1995) Cell protective role of melatonin in the brain. *J. Pineal Res.* **19,** 57–63

17. Reiter, R. J., Guerrero, J. M., Escames, G., Pappolla, M. A., and Acuña-Castroviejo, D. (1997) Prophylactic actions of melatonin in oxidative neurotoxicity. *Ann. N.Y. Acad. Sci.* **825,** 70–78
18. Menendez-Pelaez, A., and Reiter, R. J. (1993) Distribution of melatonin in mammalian tissues: The relative importance of cytosolic versus nuclear localization. *J. Pineal Res.* **15,** 9–165
19. Melchiorri, D., Reiter, R. J., Attia, A., Hara, M., Burgos, A., and Nístico, G. (1995) Potent protective effect of melatonin on in vivo paraquat-induced oxidative damage in rats. *Life Sci.* **56,** 83–89
20. Vijayalaxmi, Reiter, R. J., and Meltz, M. L. (1995) Melatonin protects human blood lymphocytes from radiation-induced chromosome damage. *Mutat. Res.* **346,** 23–3 l
21. Bettahi, I., Pozo, D., Osuna, C., Reiter, R. J., Acuña-Castroviejo, D., and Guerrero, J. M. (1996) Melatonin reduces nitric oxide synthase activity in rat hypothalamus. *J. Pineal Res.* **20,** 205–210
22. Pozo, D., Reiter, R. J., Calvo, J. R., and Guerrero, J. M. (1997) Inhibition of cerebellar nitric oxide synthase and cyclic GMP production by melatonin via complex formation with calmodulin. *J. Cell. Biochem.* **65,** 430–442
23. León, J., Vives, F., Crespo, E., Camacho, E., Espinosa, A., Gallo, M. A., Escames, G., and Acuña-Castroviejo, D. (1998) Modification of nitric oxide synthase activity and neuronal response in rat striatum by melatonin and kynurenine derivatives. *J. Neuroendocrinol.* **10,** 297–302
24. Sewerynek, E., Melchiorri, D., Chen, L., and Reiter, R. J. (1995) Melatonin reduces both basal and bacterial lipid peroxidation in vitro. *Free Rad. Biol. Med.* **19,** 903–909
25. Sewerynek, E., Melchiorri, D., Reiter, R. J., Ortiz, G., and Lewinski, A. (1995) Lipopolysaccharide-induced hepatotoxicity is inhibited by the antioxidant melatonin. *Eur. J. Pharmacol.* **293,** 327–334
26. Maestroni, G. J. M. (1996) Melatonin as a therapeutic agent in experimental endotoxic shock. *J. Pineal Res.* **20,** 84–89.
27. Danner, R. L., Elin, R. J., Hossein, J. M., Wesley, R. A., Reilly, J. M., and Parillo, J. E. (1991) Endotoxemia in human septic shock. *Chest* **99,** 169
28. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275
29. Esterbauer, H., and Cheeseman, K. H. (1990) Determination of aldehydic lipid peroxidation products: maionaldehyde and 4-hydroxynonenal. *Methods Enzymol.* **186,** 407–42l
30. Green, L. C., Ruiz de Luzuriaga, K., and Wagner, D. A. (198l) Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. USA* **78,** 7764–7768
31. Bredt, D. S., and Snyder, S. H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA* **86,** 9030–9033
32. Bradford, M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–252
33. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature (London)* **263,** 789–798
34. Chomczynski, P., and Sacchi, N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159
35. Nunokawa, Y., Ishida, N., and Tanaka, S. (1993) Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **191,** 89–94
36. Mohaupt, M. G., Elzie, J. L., Ahn, K. Y., Clapp, W. L., Wilcox, C. S., and Kone, B. C. (1994) Differential expression and induction of mRNAs encoding two inducible nitric oxide synthases in rat kidney. *Kidney Int.* **46,** 653–655
37. Gilad, E., Wong, H. R., Zingarelli, B., Virag, L., O'Connor, M., Salzman, A. L., and Csabó, C. (1998) Melatonin inhibits expression of the inducible isoform of nitric oxide synthase in murine macrophages: role of inhibition of NF 54 6B activation. *FASEB J.* **12,** 685–693
38. Tracey, K. J. (1992) The acute and chronic pathophysiologic effects of TNF: mediation of septic shock and wasting (cachexia). In *Tumor Necrosis Factors: The Molecules and their Emerging Role in Medicine* (Beutler, B., ed) pp 255–273, Raven, New York
39. McCallum, R. E., and Berry, L. J. (1973) Effects of endotoxin on gluconeogenesis, glycogen synthesis and liver glycogen synthase in mice. *Infect. Immunol.* **7,** 642–654.
40. Sakaguchi, O., and Sakaguchi, S. (1979) Alterations of lipid metabolism in mice injected with endotoxin. *Microbiol. Immunol.* **23,** 71–85
41. Sakaguchi, S., Kanda, N., Hsu, C. C., and Sakaguchi, 0. (1981) Lipid peroxide formation and membrane damage in endotoxin poisoned mice. *Microbiol. Immunol.* **25,** 229–244
42. Parks, D. A., Bulkey, G. B., and Granger, D. N. (1983) Role of oxygen free radicals in shock, ischemia, and organ preservation. *Surgery* **94,** 428–432
43. Harbrecht, R. G., Stadler, J., Demetris, A. J., Simmons, R. L., and Billiar, T. R. (1994) Nitric oxide and prostaglandins interact to prevent hepatic damage during murine endotoxemia. *Am. J. Physiol.* **266,** G1004–G1010
44. Tracey, W. R., Tse, J., and Carter, G. (1995) Lipopolysaccharide-induced changes in plasma nitrite and nitrate concentrations in rats and mice: pharmacological evaluation of nitric oxide synthase inhibitors. *J. Pharmacol. Exp. Ther.* **272,** 1011–1015
45. Billiard, T. R., Curran, R. D., Stuehr, D. J., West, M. A., Bentz, B. G., and Simmons, R. L. (1989) An L-arginine-dependent mechanism mediated Kupffer cell inhibition of hepatocyte protein synthesis in vitro. *J. Exp. Med.* **169,** 1467–1472
46. Stadler, J., Harbrecht, B. G., Di Silvo, M., Curran, R. D., Jordan, M. L., Simons, R. L., and Billiar, T. R. (1993) Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin 6 by rat Kupffer cells. *J. Leukoc. Biol.* **53,** 165–172
47. Morris, S. M. Jr., and Billiard, T. R. (1993) New insights into the regulation of inducible nitric oxide synthesis. *Am. J. Physiol.* **266,** E829–E839
48. Gilad, E., Cuzzocrea, S., Zingarelli, B., Salzman, A. L., and Csabó, C. (1997) Melatonin is a scavenger of peroxynitrite. *Life Sci.* **60,** 169–174
49. Reiter, R. J. (1995) Oxidative processes and antioxidative defense mechanisms in the aging brain. *FASEB J.* **9,** 526–533
50. Martín, M., Crespo E., Macías M., Aguilera P., Arauzo M., León, J., Escames G., and Acuña-Castroviejo, D. (1997) Melatonin counteracts the ruthenium red cytotoxicity. *J. Physiol. Biochem.* **53,** 179
51. Acuña-Castroviejo, D., Reiter, R. J., Menendez-Pelaez, A., Pablos, M. I., and Burgos, A. (1994) Characterization of high-affinity melatonin binding sites in purified cell nuclei of rat liver. *J. Pineal Res.* **16,** 100–112
52. Becker-Andre, M., Wiesenberg, I., Scaeren-Wiemer, N., Andre, E., Missbach, M., Saurat, J. H., and Carlberg, C. (1994) Pineal hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. *J. Biol. Chem.* **269,** 28531–28534
53. Steinhilber, D., Brungs, M., Werz, O., Wiesenberg, I., Danielsson, C., Kalen, J. P., Nayeri, S., Schader, M., and Carlberg, C. (1995) The nuclear receptor for melatonin repress 5-lipoxygenase gene expression in human B lymphocytes. *J. Biol. Chem.* **270,** 7037–7040
54. Antolin, I., Rodriguez, C., Sainz, R. M., Mayo, J. C., Uria, H., Kotler, M. N., Rodriguez-Colunga, M. J., Tolivia, D., and Menendez-Pelaez, A. (1996) Neurohormone melatonin prevents cell damage: Effect on gene expression for antioxidant enzymes. *FASEB J.* **10,** 882–890
55. Sen, C. K., and Packer, L. (1996) Antioxidant and redox regulation of gene transcription. *FASEB J.* **10,** 709–720
56. Vesley, D. L. (1997) Signal transduction: activation of the guanylate cyclase-cyclic guanosine-3′-5′ monophosphate system by hormones and free radicals. *Am. J. Physiol.* **314,** 311–323
57. Pablos, M. I., Agapito, M. T., Gutierrez, R., Recio, J. M., Reiter, R. J., Barlow-Walden, L., Acuña-Castroviejo, D., and Menendez-Pelaez, A. (1995) Melatonin stimulates the activity of the detoxifying enzyme glutathione peroxidase in several tissues of chicks. *J. Pineal Res.* **19,** 111–115
58. Lesions in suprachiasmatic nuclei simulate effects of pinealectomy on prolactin release in ovariectomized, and sulpiride-treated female rats. *Experientia* **45,** 739–742
59. Molina-Carballo, A., Muñoz-Hoyos, A., Reiter, R. J., Sanchez-Forte, M., Moreno-Madrid, F., Rufo-Campos, M., Molina-Font, J. A., and Acuña-Castroviejo, D. (1997) Utility of high doses of melatonin as adjunctive anticonvulsant therapy in a child with severe myoclonic epilepsy: two years' experience. *J. Pineal Res.* **23,** 97–105

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