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Effect of Methylguanidine in a Model of Septic Shock Induced by LPS

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Septic shock, a severe form of sepsis, is characterized by cardiovascular collapse following microbial invasion of the body. The progressive hypotension, hyporeactivity to vasopressor agents and vascular leak leads to circulatory failure with multiple organ dysfunction and death. Many inflammatory mediators (e.g. TNF-α, IL-1 and IL-6) are involved in the pathogenesis of shock and, among them, nitric oxide (NO). The overproduction of NO during septic shock has been demonstrated to contribute to circulatory failure, myocardial dysfunction, organ injury and multiple organ failure. We have previously demonstrated with *in vitro* and *in vivo* studies that methylguanidine (MG), a guanidine compound deriving from protein catabolism, significantly inhibits iNOS activity, TNF-α release and carrageenan-induced acute inflammation in rats.

The aim of the present study was to evaluate the possible anti-inflammatory activity of MG in a model of septic shock induced by lipopolysaccharide (LPS) in mice. MG was administered intraperitoneally (i.p.) at the dose of 30 mg/kg 1 h before and at 1 and 6 h after LPS-induced shock. LPS injection (10 mg/kg in 0.9% NaCl; 0.1 ml/mouse; i.p.) in mouse developed a shock syndrome with enhanced NO release and liver, kidney and pancreatic damage 18 h later. NO_x levels, evaluated as nitrite/nitrate serum levels, was significantly reduced in MG-treated rats (78.6%, $p < 0.0001$; $n = 10$). Immunohistochemistry revealed, in the lung tissue of LPS-treated group, a positive staining for nitrotyrosine and poly(adenosine diphosphate [ADP] ribose) synthase, both of which were reduced in MG-treated mice. Furthermore, enzymatic evaluation revealed a significant reduction in liver, renal and pancreatic tissue damage and MG treatment also improved significantly the survival rate. This study provides evidence that MG attenuates the degree of inflammation and tissue damage associated with endotoxic shock in mice. The mechanisms of the anti-inflammatory effect of MG is, at least in part, dependent on the inhibition of NO formation.

Keywords: Methylguanidine; LPS-induced septic shock; Reactive oxygen species; Organ damage

INTRODUCTION

Sepsis is a heterogeneous class of syndromes caused by a systemic inflammatory response to infection. The prevalence of this pathology is continuously increasing due to the extended longevity of patients with chronic illnesses, the increased occurrence of immunosuppression, and the more frequent use of invasive procedures.[1] Septic shock, a severe form of sepsis, continues to be the major cause of morbidity and mortality in the United States.[1] Sepsis results from the generalized activation of inflammatory cascades following invasion of the blood stream by bacteria, viruses or parasites, with the systemic release of various toxic products.[2] These products include bacterial cell-wall components, such as endotoxin, as lipopolysaccharide (LPS) membrane component of gram-negative bacteria, lipoteichoic acid forms gram-positive organisms and various endotoxins.[3,4] Sepsis induced by gram-negative is accompanied by

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systemic shock and the initial symptoms of sepsis encompass those usually associated with acute inflammation, including fever or hypothermia, tachypnea, tachycardia, high white blood cell counts, pulmonary edema and, finally multiple organ dysfunction syndrome (MODS) and death.[5] Many of the pathological consequences of gram-negative shock are attributable to the bacterial membrane component, LPS, which induces experimental endotoxemia and has become a valuable experimental model for septicemia and has been studied extensively in laboratory animals. Most effects of LPS act via endogenous mediators, mainly produced by mononuclear phagocytes.[6] Among these endogenous mediators several cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1 and IL-6 have been implicated in the pathogenesis of shock.

Recent studies reported that an overproduction of nitric oxide (NO) may be a common mechanism by which microbial products and several cytokines bring about their deleterious actions on the cardiovascular system, such as hypotension, vascular hyporesponsiveness and death, suggesting that excessive NO production plays an important role in septic shock.[7–9] LPS-dependent induction of the inducible isoform of NO synthase (iNOS) is responsible for the overproduction of NO in circulatory shock.[10] This isoform can be expressed in many cell types, including macrophages, neutrophils, endothelial cells and vascular smooth muscle cells, subsequently to the induction by many inflammatory agents (e.g. LPS, interferon-γ, TNF-α, etc.). Recently, it has been suggested that some of the cytotoxic effects of NO are tightly related to the production of peroxynitrite anion ($ONOO^-$), a high-energy oxidant derived by the rapid reaction of NO with superoxide anion ($O_2^{\cdot-}$).[11–13] It is assumed that LPS may activate macrophages and cause free radicals generation with macrophages to cause the generation of free radicals, including hydrogen peroxide (H_2O_2), $O_2^{\cdot-}$ and hydroxyl radical ($^{\cdot}OH$) leading to oxidative damage in many tissues.[14] This progression of circulatory failure to a MODS is associated with a substantial increase in mortality.[15] Previous studies have demonstrated that inhibition of iNOS activity attenuates liver, renal and pancreatic dysfunctions associated with LPS-induced endotoxemia in the rat.[16] Thus, NO overproduction and NO-dependent free radicals seems to contribute to the development of MODS in endotoxic shock.

A new class of iNOS inhibitors has been developed around the guanidine group of the amino acid L-arginine such as guanidine, aminoguanidine, mercaptoethylguanidine and methylguanidine (MG) which is known to be a product of protein catabolism.[17–19] We have previously demonstrated that MG attenuates TNF-α release in a model of LPS-induced shock[20] and carrageenan-induced acute inflammation in rats.[21]

In the present study, we have investigated the effects of MG on endotoxic shock induced by LPS. In particular, we have investigated the effect of MG on (1) NO release, (2) nitration of tyrosine residues (an indicator of the formation of peroxynitrite by immunohistochemistry), (3) poly(adenosine diphosphate [ADP] ribose) synthase (PARS) activation, (4) kidney, liver and pancreatic organ damage, (5) mortality rate and (6) lung damage (histology).

MATERIALS AND METHODS

Animals

Male CD mice (25–35 g) were purchased from Harlan Nossan, Italy. The mice were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations (D.M. 116192) on protection of animals used for experimental and other scientific purpose, as well as with the EEC regulations (O.J. of E.C. L 358/1 18/12/1986).

Experimental Groups

The mice were randomly allocated into the following groups.

(i) *LPS + vehicle group*: Mice were subjected to LPS-induced shock and received the vehicle for MG (saline solution i.p. 1 h before and 3 and 6 h after LPS; $n = 10$).
(ii) *MG group*: Mice were treated as the LPS + vehicle group but MG (30 mg/kg i.p.) was administered 1 h before and 3 and 6 h after LPS injection; $n = 10$).
(iii) *Sham + saline group*: Sham-operated group in which identical procedures to the LPS + vehicle group was performed, except that saline was administered instead of LPS.
(iv) *Sham + MG group*: Identical to Sham + saline group except for the administration of MG (30 mg/kg i.p.) 1 h before and 3 and 6 h after saline injection; $n = 10$).

After 18 h from LPS injection mice were sacrificed and histological and biochemical parameters were evaluated.

Materials

Unless stated otherwise, all reagents and compounds used were obtained from Sigma Chemical Company (Sigma, Milan, Italy).

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Quantification of Organ Function and Injury

Eighteen hours after LPS or saline injection, blood samples were collected from all animals ($n = 10$ for each group). The blood sample was centrifuged (1610*g* for 3 min at room temperature) to separate plasma. All plasma samples were analyzed within 24 h by a veterinary clinical laboratory using standard laboratory techniques. The following marker enzymes were measured in the plasma as biochemical indicators of multiple organ injury/dysfunction: (1) liver injury was assessed by measuring the rise in plasma levels and AST; (2) renal dysfunction was assessed by measuring the rises in plasma levels of creatinine and bond urea nitrogen (BUN) (an indicator of reduced glomerular filtration rate, and hence, renal failure); (3) serum levels of lipase and amylase were determined as an indicator of pancreatic injury.

Light Microscopy

Lung samples were taken 18 h after LPS injection. The tissue slices were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Sections (thickness 7 μm) were deparaffinized with xylene, stained with hematoxylin/eosin and observed in Dialux 22 Leitz microscope.

Immunohistochemical Localization of Nitrotyrosine and PARS

Tyrosine nitration and PARS activation were detected as previously described[22] in lung sections using immunohistochemistry. At 18 h after LPS or saline injection, tissues were fixed in 10% (w/v) PBS-buffered formalin and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with 1:1000 dilution of primary anti-nitrotyrosine antibody (DBA), anti-poly(ADP-ribose) (PAR) antibody (DBA), or with or with control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated specific secondary anti-IgG and avidin–biotin peroxidase complex (DBA). In order to confirm that the immunoreaction for the nitrotyrosine was specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for PARS, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections indicating that the immunoreaction was positive in all the experiments carried out.

Cell Lines and Cell Culture

The murine macrophage cell line, J774.A1, was obtained from American Tissue Culture Collection (ATCC). J774.A1 cells were maintained in DMEM supplemented with $NaHCO_3$ (42 mM), penicillin (100 units/ml), streptomycin (100 u/ml), glutamine (2 mM) and fetal calf serum (10%) at 37°C in a 95% air and 5% CO_2 atmosphere. To induce iNOS, fresh culture medium containing LPS (6×10^3 u/ml) was added. NO, evaluated as nitrite (NO_2^-), accumulation in the cell culture medium and Western blot analysis for iNOS expression on cell lysates were performed 24 h after LPS stimulation while nuclear extracts for nuclear factor-κB (NF-κB) activity were obtained 90 min after LPS induction. MG (0.1, 1 and 10 mM) was added 30 min before LPS.

MTT Assay for Cell Viability

To exclude a possible interference of MG on cell viability the MTT assay was performed. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT to formazan and cell viability was assessed according to the method of Mosmann.[23]

J774.A1 (3.5×10^4) macrophages were plated in 96-well plates and allowed to adhere at 37°C in a 95% air and 5% CO_2 atmosphere, for 2 h. Thereafter, the medium was replaced with fresh medium or medium containing increasing concentrations of MG (0.1, 1 and 10 mM). Cells were then incubated for a further 72 h and thereafter 25 μl of (MTT, 5 mg/ml) was added for 3 h. Following this time, cells were lysed and formazan crystals solubilized with 100 μl of a solution containing 50% (v/v) *N*,*N*-dimethylformamide, 20% (w/v) SDS with an adjusted pH of 4.5.[24] The optical density (OD_{620}) of each well was measured with a microplate spectrophotometer (Multiskan EX, Dasit, UK). The viability of J774.A1 cells in response to treatment

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with MG was calculated as

$$\%\ \text{dead cells} = 100 - \left(\frac{\text{OD treated}}{\text{OD control}}\right) \times 100.$$

Analysis of NO_2^- Production

Cells (1.5×10^5 cells/P30 dishes) were activated with LPS (6×10^3 u/ml) alone or in combination with MG (0.1, 1 and 10 mM). NO release was determined 24 h after LPS-activation measuring nitrite levels in the culture medium by Griess reagent.[25]

Western Blot Analysis for INOS Expression

J774.A1 cells (1.5×10^5 cells/P30 dishes) were pre-incubated with MG (0.1, 1 and 10 mM) for 30 min and then incubated with either DMEM alone or DMEM containing LPS (6×10^3 u/ml). After 24 h of incubation, cells were scraped off, washed with ice-cold PBS and centrifuged at 5000*g* for 10 min at 4°C. The cell pellet was lysed in a buffer containing 20 mM of Tris–HCl (pH 7.5), 1 mM of sodium orthovanadate, 1 mM of phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, 10 mM of NaF, 150 mM of NaCl, 10 mg/ml of trypsin inhibitor and 1% of Tween-20. Protein concentration was estimated by the Bio-Rad protein assay using BSA as standard. Equal amounts of protein (70 μg) were dissolved in Laemmli's sample buffer, boiled and run on a SDS-PAGE minigel (8% polyacrylamide) and then transferred for 40 min at 5 mA cm^2 into 0.45 μm hybond polyvinylidene difluoride membrane. Membranes were blocked for 40 min in PBS and 5% (w/v) non-fat milk and subsequently probed overnight at 4°C with mouse monoclonal anti-iNOS (1:10,000) antibody (in PBS, 5%, w/v non-fat milk and 0.1% Tween-20). Blots were then incubated with horseradish peroxidase conjugated goat anti-mouse IgG (1:5000) for 1 h at room temperature. Immunoreactive bands were visualized using ECL detection system according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of iNOS on X Omat films were quantified by scanning densitometry (Imaging Densitometer GS-700, Bio-Rad, USA).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

J774.A1 cells (1.0×10^7 cells/P60 dishes) were pre-incubated with MG (0.1, 1 and 10 mM) for 18 h or 30 min and then incubated with either DMEM alone or DMEM containing LPS (6×10^3 u/ml) for 90 min. Nuclear extracts were prepared[26] by cell pellet homogenization in 2 vol of 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride and 10% glycerol. Nuclei were centrifuged at 1000*g* for 5 min, washed and resuspended in 2 vol of the solution specified above. KCl (3 M) was added to reach a concentration of 0.39 M KCl. Nuclei were extracted at 4°C for 1 h and centrifuged at 10,000*g* for 30 min. The supernatants were clarified by centrifugation and stored at −80°C. Protein concentration, of the supernatant containing nuclear extracts, was determined using Bio-Rad protein assay. The double-stranded oligonucleotides containing the NF-κB recognition sequence (5′-AGTTGAGGG-GACTTTCC-CAGGC-3′) were end-labeled with [γ-^{32}P] ATP. Nuclear extracts containing 5 μg of proteins were incubated at room temperature for 20 min with radiolabeled oligonucleotides (2.0×10^{-4}–5.0×10^{-4} cpm/μg) in the presence of 1 μg of poly (dI-dC) in 20 μl of a buffer containing 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 5% glycerol. Nuclear protein–oligonucleotide complexes were resolved by electrophoresis on a 6% polyacrylamide gel and run in a 0.25× Tris–borate/EDTA buffer at 200 V for 3 h. The gel was dried and exposed to X-ray films at −80°C. Subsequently, the relative bands were quantified by densitometric scanning with a GS 700 Imaging Densitometer (Bio-Rad).

Data Analysis

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) of *n* observations. For the *in vivo* studies, *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analyzed by one-way ANOVA followed by a Bonferroni post-test for multiple comparisons. A *p*-value of less than 0.05 was considered significant. Statistical analysis for survival data was calculated by Fisher's exact probability test. For such analyses, $p < 0.05$ was considered significant. The Mann–Whitney test was used to examine differences between the body weight and organ weights of control and experimental groups. When this test was used, $p < 0.05$ was considered significant.

RESULTS

Effect of MG on NO Release in LPS-treated Mice

Endotoxemia was associated with a significant rise in serum levels of NO_x (Fig. 1). The administration of MG (30 mg/kg, i.p.) significantly attenuated serum

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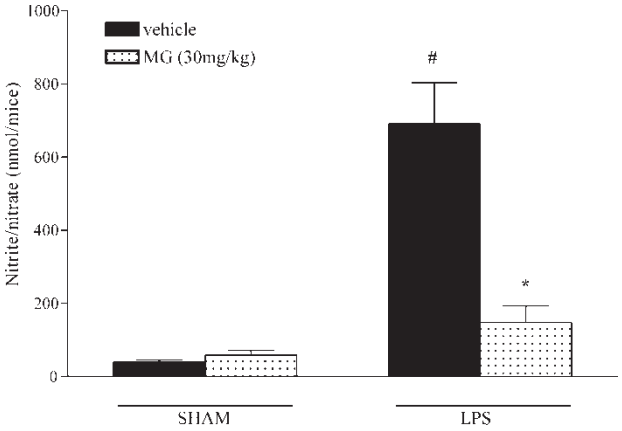

FIGURE 1 Effect of MG on NO_x serum levels at 18 h after LPS administration. NO_x levels in LPS treated mice were significantly increased vs. vehicle group. MG (30 mg/kg i.p.) significantly reduced the LPS-induced elevation of NO_x levels. Data are means of mean ± SEM from $n = 10$ mice for each group. *$p < 0.001$ vs. LPS group, #$p < 0.001$ vs. vehicle group.

levels of NO_x. The administration of MG without subsequent injection of LPS had no effects on serum NO_x levels (Fig. 1).

Effect of Nitrotyrosine Formation and PARS Activation

At 18 h following i.p. administration of LPS, lung sections were also analyzed for the evidence of nitrotyrosine formation. Immunohistochemical analysis, using a specific anti-nitrotyrosine antibody, revealed a positive staining in lung from LPS-treated mice (Fig. 2A). A marked reduction in nitrotyrosine staining was found in the lung of the LPS-treated mice that had been treated with MG (Fig. 2B). Immunohistochemical analysis of lung sections obtained from mice treated with LPS also revealed a positive staining for PAR (Fig. 3A) indicating PARS activation. In contrast, no positive staining for PAR was found in the lung of LPS-treated mice, which had been treated with MG (Fig. 3B). There was no staining for either nitrotyrosine or PAR in lung obtained from sham/vehicle mice (Figs. 2C and 3C).

Organ Failure Caused by LPS is Reduced by MG Treatment

Effects on the Renal Dysfunction

No significant alterations in the plasma levels of creatinine and BUN were observed in the sham/vehicle and sham/MG groups (Fig. 4A,B). When compared with sham treated mice (sham/vehicle and sham/MG), LPS administration in mice resulted in significant rises in plasma levels of creatinine and BUN, demonstrating the development of renal dysfunction. MG treatment significantly reduced the renal dysfunction caused by LPS-induced shock.

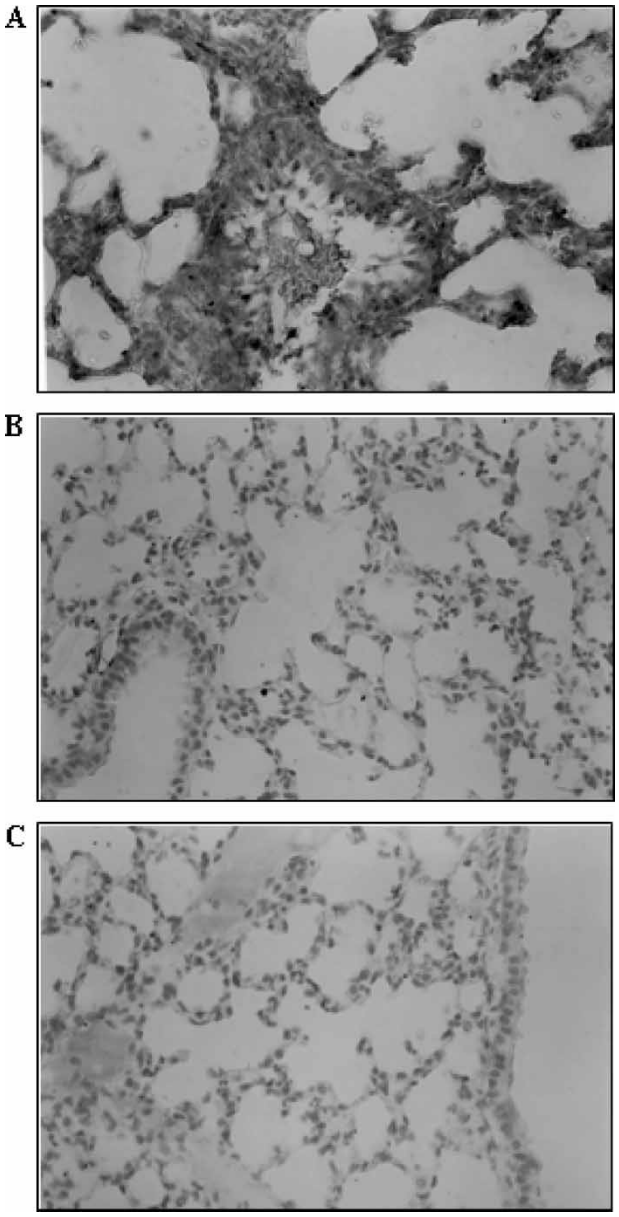

FIGURE 2 Effect of MG on nitrotyrosine formation. Eighteen hours after LPS challenge, positive staining for nitrotyrosine was observed in the lungs of LPS-treated mice (A). A marked reduction in the immunostaining for nitrotyrosine was observed in the lungs of mice treated with MG (30 mg/kg i.p.) (B). No staining was observed for nitrotyrosine in lung from vehicle group (C). Original magnification: 150×. This figure is representative of at least 3 experiments performed on different experimental days.

The detected amount of creatinine and BUN in the MG group was in fact undistinguishable from the control group (Fig. 4A,B).

Effects on the Liver Injury

No significant alteration in the plasma levels of AST was observed in the sham/vehicle and sham/MG groups. When compared with sham treated mice, LPS administration in rats resulted in significant rises in the plasma levels of AST demonstrating the development of hepatocellular injury (Fig. 5).

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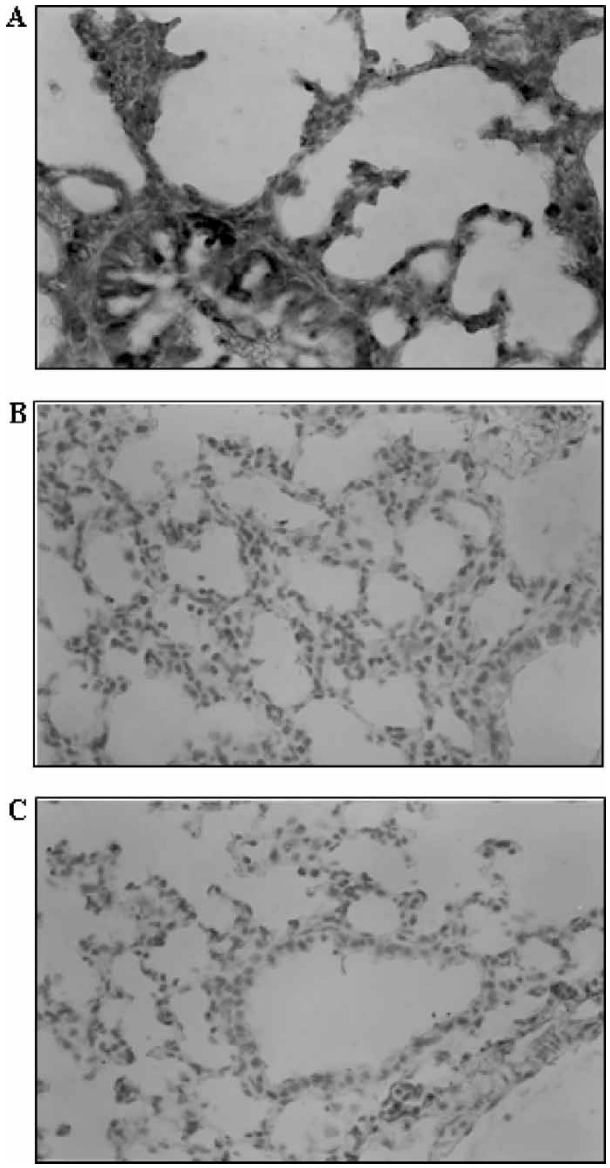

FIGURE 3 Effect of MG on PARS activation. Eighteen hours after LPS challenge, positive staining for PARS was observed was observed in the lungs of LPS-treated mice (A). A marked reduction in the immunostaining for PARS was observed in the lungs of MG (30 mg/kg i.p.)-treated mice (B). No staining was observed for PARS in lung from vehicle group (C). Original magnification: 150×. This figure is representative of at least 3 experiments performed on different experimental days.

MG treatment attenuated the liver injury caused by LPS-induced shock (Fig. 5).

Effects on Pancreatic Injury

No significant alterations in the plasma levels of lipase and amylase were observed in the sham/vehicle and sham/MG groups (Fig. 6A,B). When compared with sham-treated mice, LPS administration in mice resulted in significant rises in the plasma levels of lipase and amylase, demonstrating the development of pancreatic injury (Fig. 6A,B). MG treatment abolished the abolished the pancreatic injury caused LPS (Fig. 6A,B).

Histological Evaluation of Lung Injury

At 18 h after LPS administration, the tissue injury in lung was evaluated by histology. At histological examination, the lung (see representative sections at Fig. 7) revealed pathological changes. The examination of the lung biopsies revealed extravasation of red cells and neutrophils and macrophage accumulation (Fig. 7A). MG treatment resulted in a significant reduction of pulmonary injury (Fig. 7B). No histological alteration was observed in sham/vehicle rats (Fig. 7C).

Effect of MG on Mortality Rate

LPS administration caused a severe illness in mice characterized by a systemic toxicity. At 18 h from LPS injection, 40% of LPS-treated mice were dead. MG treatment (30 mg/kg, i.p.), significantly reduced mortality rate (11%) caused by LPS-induced shock. MG alone did not cause significant changes mortality rate of sham treated mice (data not shown).

In Vitro Studies

MG-induced Inhibition of NO Production in LPS-stimulated J774.A1

LPS increased NO production in the medium of J774.A1 macrophages. In unstimulated cells, the concentration of NO_2^- was 0.05 ± 0.03, whereas in LPS-stimulated cells it was significantly increased ($16.64 \pm 0.58\,\mu M$ NO_2^-). As shown in Fig. 8, MG addition to the culture medium 30 min before LPS reduced in a concentration-dependent manner NO production.

Effect of MG on iNOS

Since elevated production of NO in LPS-activated macrophages require induction of iNOS enzymes, we evaluated MG effect on their protein expression. Lysates prepared from the same cells used to obtain data showed in Fig. 1 were subjected to SDS-page and analyzed for iNOS expression by Western blot analysis.

In lysates from LPS-stimulated cells, the iNOS antibodies were recognized as a 130 kDa (iNOS) protein band, respectively, whereas no bands were evident in lysates from unstimulated cells (Fig. 9B).

iNOS protein levels, determined by scanning densitometry, were significantly reduced in a concentration-dependent manner in lysates obtained from J774.A1 cells pre-incubated with MG 30 min before LPS (Fig. 9B). A representative blot is shown in Fig. 9A.

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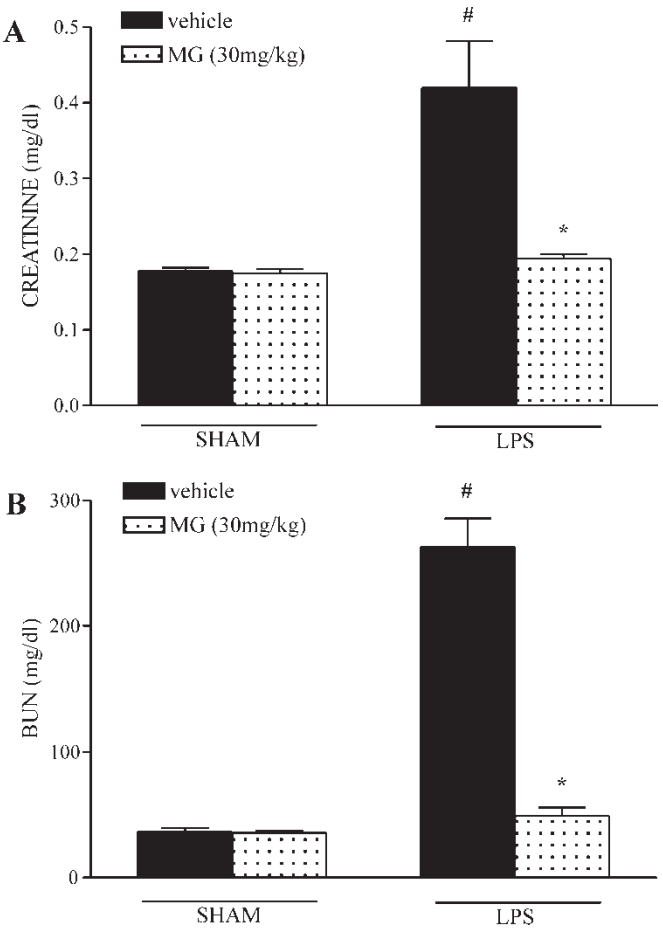

FIGURE 4 Effect of MG on renal injury. Creatinine (A) levels and BUN (B) resulted significantly increased in LPS treated mice. MG (30 mg/kg i.p.) treatment significantly decrease all these parameters in plasma treated mice. *$p < 0.01$ vs. LPS group, #$p < 0.01$ vs. vehicle group.

Effect of MG on NF-κB Activity

To examine whether the effect of MG inhibition on iNOS protein expression could be due to a modification of the activity of NF-κB, EMSA were conducted. In LPS-treated cells, a time dependent increase in NF-κB activity was evident and reached a peak between 1 and 2 h approaching baseline values after 24 h (data not shown). Addition of MG (0.1, 1 and 10 mM) to cells 30 min before LPS resulted in a significant decrease in NF-κB activity as measured at 90 min (Fig. 10B). A representative EMSA is shown in Fig. 10A.

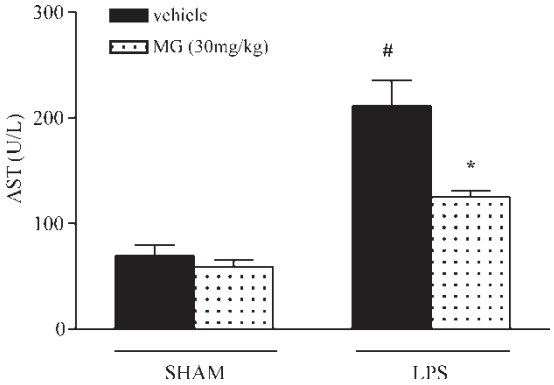

FIGURE 5 Effect of MG on liver injury. AST plasma levels resulted significantly increased in LPS treated mice. MG (30 mg/kg i.p.) treatment significantly decrease this parameter in plasma treated mice. *$p < 0.05$ vs. LPS group, #$p < 0.05$ vs. vehicle group.

Effect of MG on Cell Viability

To exclude a possible interference of MG on cell viability, we examined the effect of increasing concentrations of MG (0.1, 1 and 10 mM) on J774.A1 cell viability. MG treatment did not affect significantly mitochondrial reduction of MTT to formazan indicating that the observed effects on iNOS expression and protein metabolites NO could not be due to any significant inhibitory effect on cell viability (data not shown).

DISCUSSION

Gram-negative septicemia is often accompanied by systemic shock and it is a serious and often fatal medical condition. The initial symptoms of sepsis

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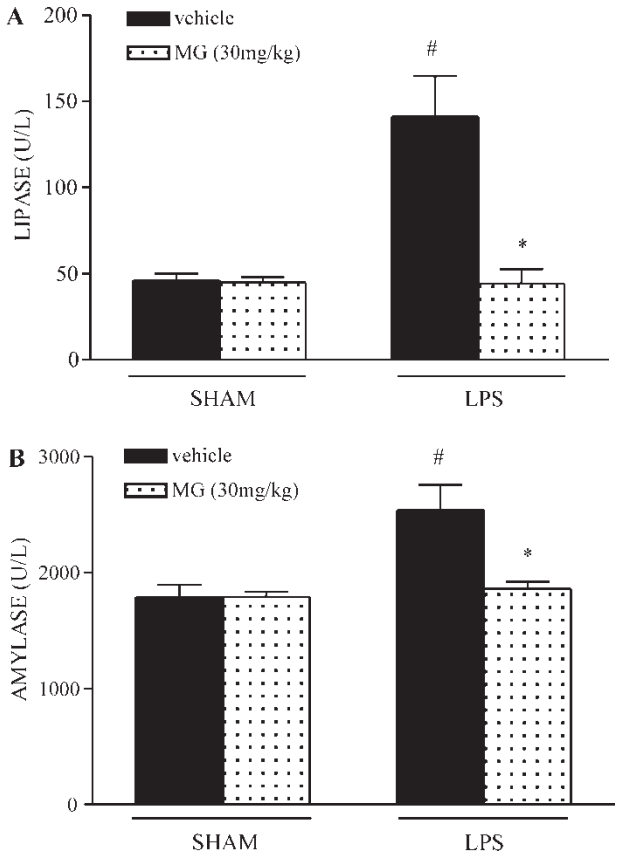

FIGURE 6 Effect of MG on pancreatic injury. Lipase (A) and amylase (B) plasma levels resulted significantly increased in LPS treated mice. MG (30 mg/kg i.p.) treatment significantly decrease all these parameters in plasma treated mice. *$p < 0.05$ vs. LPS group, #$p < 0.05$ vs. vehicle group.

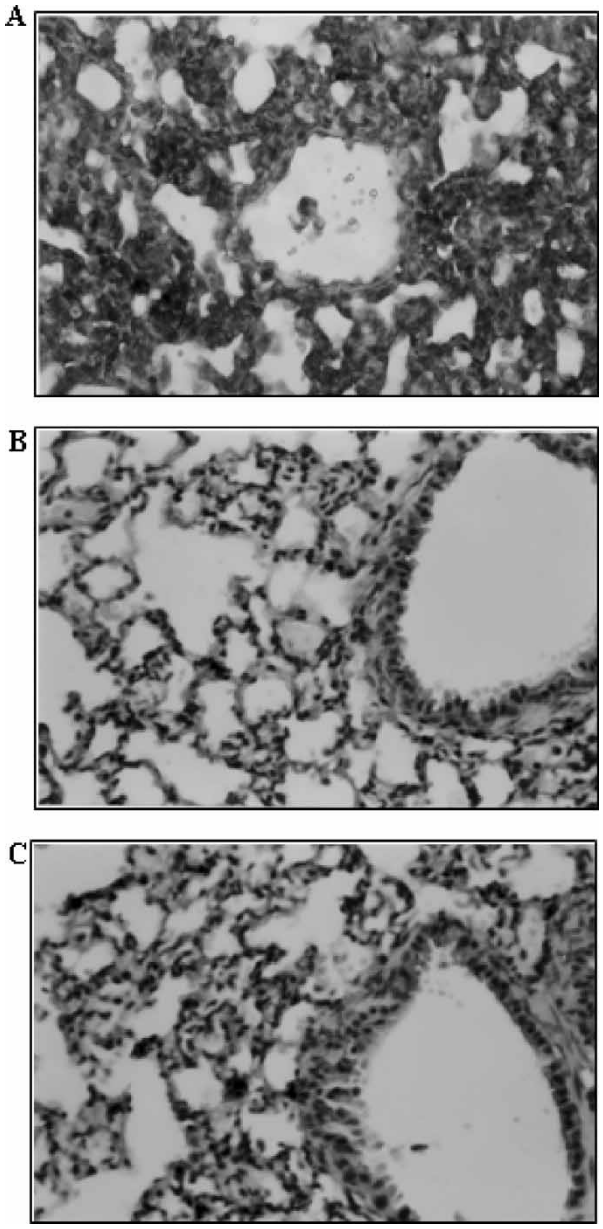

FIGURE 7 Effect of MG on lung injury. Lung section from an LPS-treated rats (A) demonstrating interstitial hemorrhage and polymorphonuclear neutrophil accumulation. Lung section from a LPS-treated mice after administration of MG (30 mg/kg) (B) demonstrating reduced interstitial hemorrhage and cellular infiltration. A representative lung section from a vehicle treated mouse is shown in (C). Original magnification: 125 × . This figure is representative of at least 3 experiments performed on different experimental days.

encompass those usually associated with acute inflammation including, finally, MODS. In some occasions, endotoxins are considered to be responsible for these manifestations[27] due, primarily, to activation of phagocytic cells.[28,29] Endotoxin induces a variety of biological responses, including hypertension, vascular hyporeactivity to vasoconstrictor agents and septic shock. This is caused by the release of biochemical mediators, such as histamine, kinins, platelet-activating factor (PAF) by the reticuloendothelial system (e.g. liver), and cytokines, such as TNF-α, interleukins and interferon-γ, since in the presence of inhibitors of these mediators, the deleterious hemodynamic change induced by endotoxin was ameliorated.[30] In recent years, NO has been implicated in the significant hypotension and vascular hyporesponsiveness often associated with sepsis and/or endotoxemia.[8–10] Evidence supporting this hypothesis comes from reports indicating that mediators (e.g. TNF-α and interleukins) produced by endotoxin challenge can induce iNOS expression and produce large amounts of NO.[31,32]

There is good evidence that septic or endotoxic shock is also associated with the generation of reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide and peroxynitrite.[33] Furthermore, it has been demonstrated that the production of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage.[34] Our results demonstrated that, in an experimental model of septic shock induced by LPS, MG (1)

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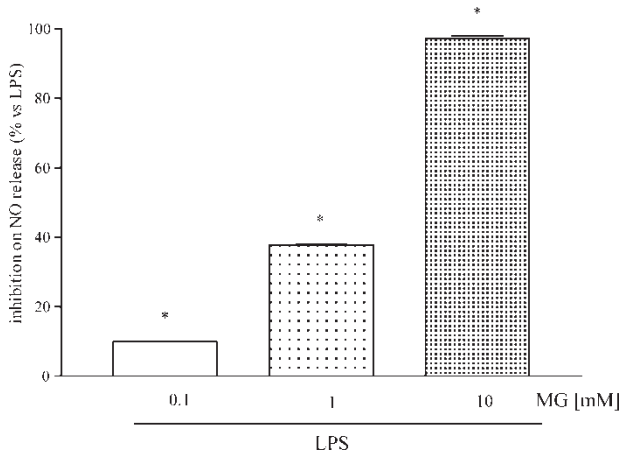

FIGURE 8 Effect of MG on LPS-induced NO_2^- production by J774.A1 macrophages. Cells were incubated with LPS (6×10^3 u/ml) alone (control) or in combination with MG (0.01–10 mM). MG was added 30 min before LPS challenge. Accumulated NO_2^- in the culture medium was determined 24 h after LPS addition by the Griess reaction. Results are expressed as % inhibition vs. LPS response (16.6 ± 0.6 μM NO_2^-/24 h) and values are means ± SEM of at least 3–6 independent experiments with 5 replicates in each. *$p < 0.001$ vs. LPS.

reduces the formation of NO_x, (2) attenuates MODS caused by bacterial endotoxin, (3) reduces morphological lung injury, (4) nitrotyrosine (an indicator of nitrosative stress in inflammation) and PAR immunostaining in endotoxin shock as well as (5) mortality rate. In order to evaluate the possible mechanism of action of MG here we also reported that MG treatment (1) attenuates in a murine macrophage cell line NO_x levels and iNOS expression and (2) reduces, in the same experimental condition, NF-κB activity.

MG is a guanidine compound that has been found in meat extracts, muscle autolyzates and in various tissue and biological fluids.[35] This compound is a product of protein catabolism[36] synthesized from creatinine by active oxygen generated not only by chemical reagents but also by isolated rat hepatocytes and accumulates in chronic renal failure.[37] Previous studies reported that MG attenuates NO production by both constitutive and inducible iNOS[38] and we showed that MG, both *in vitro* and *in vivo*, significantly inhibited LPS-induced TNF-α production.[20]

There is a large amount of evidence that the production of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage.[33,34] In LPS-induced shock, in addition to NO, peroxynitrite was also generated and sulfhydryl groups that were oxidized generated hydroxyl radicals ($^{\cdot}OH$).[13] It is assumed that LPS may act with macrophages to cause the generation of free radicals hydrogen peroxide (H_2O_2), O_2^- and $^{\cdot}OH$, leading to oxidative damage in many tissues such as liver.[14] This progression of circulatory failure to a MODS is associated with a substantial increase in mortality.[15]

Therefore, in this study we clearly demonstrate that MG treatment prevented the production of

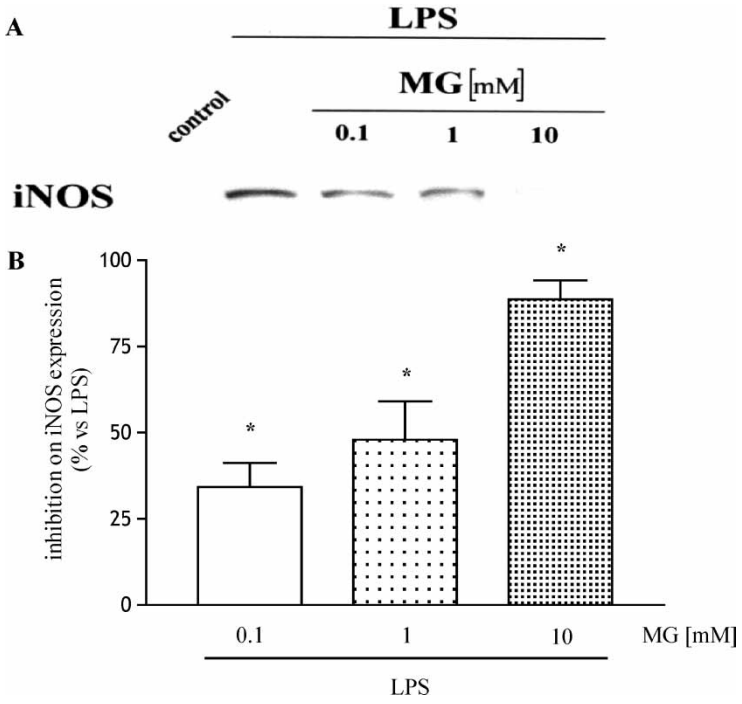

FIGURE 9 Effect of MG on iNOS protein expression in J774.A1 macrophages activated with LPS (6×10^3 u/ml) under control conditions or in the presence of MG (0.1–10 mM). Protein expression was analyzed by Western blot (A), and bands were quantified by densitometry (B). Data are represented as means ± SEM density, expressed as a percentage of inhibition vs. LPS response. *$p < 0.001$ vs. LPS.

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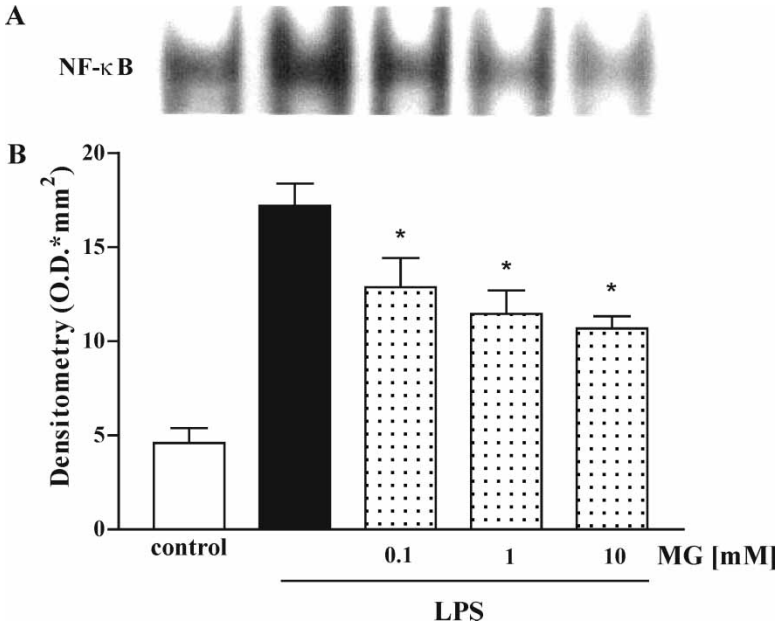

FIGURE 10 Effect of MG on NF-kB activity in J774.A1 macrophages activated with LPS (6×10^3 u/ml) in the absence and in presence of increasing concentrations of MG (0.1–10 mM) added 30 min before LPS. NF-κB activity was measured by EMSA 90 min after activation with LPS (A) and bands were analyzed by densitometry (B). Data are represented as the mean ± SEM density, expressed as a percentage of inhibition on NF-κB activity with LPS alone. *$p < 0.05$ vs. LPS.

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NO and the formation of peroxynitrite. ROS produce strand breaks in DNA which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARS resulting in the depletion of its substrate NAD^+ *in vitro* and a reduction in the rate of glycolysis. In glycolysis and in the tri-carboxylic acid cycle, NAD^+ acts as a cofactor and its depletion leads to a rapid fall in intracellular ATP. This process has been termed "the PARS suicide hypothesis".[39] There are evidences that the activation of PARS may also play an important role in inflammation[39–41] and we demonstrate here that MG treatment reduced the activation of PARS during LPS-induced septic shock in the lung. Thus, we propose that the anti-inflammatory effects of MG may be, at least in part, due to the prevention of the activation of PARS. All these beneficial effects of MG resulted in a significant reduction in lung tissue damage, as demonstrated by histological examination, in a substantial reduction in MOD and finally, in a significant reduction in mortality rate.

In order to investigate the possible mechanism of action of MG effect, we tested MG *in vitro*. Our results demonstrated that MG was able, also *in vitro*, to inhibit both iNOS activity and expression. Moreover, in this study we report that MG pre-incubation was also able to reduce NF-κB activity. The mechanism by which wall fragments of gram-negative or -positive bacteria induce NOS involves, after TNF-α, IL-1 and IFNγ release, NF-κB activation which leads to the binding of iNOS gene promoter region and then iNOS transcription. Translation of iNOS mRNA and the subsequent assembly of iNOS protein is associated with an enhanced formation of NO, which in turn may contribute either to host defense or to the pathophysiology of septic shock.

In conclusion, this study provides the first evidence that MG causes a substantial reduction of LPS-induced shock in mice. Thus, we demonstrate here that the mechanisms underlying the protective effects of MG are dependent by a reduction of (i) the expression of iNOS and the nitration of proteins by peroxynitrite, (ii) the formation of the pro-inflammatory cytokines and (iii) the NF-κB reduced activity.

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