

EXPERIMENTAL / LABORATORY STUDIES

**Reversal of Endotoxin-Induced Hypotension by Inhibition of Inducible Nitric Oxide Synthase Activity is Associated with Improved Oxidative Status in Rat Heart, Aorta and Mesenteric Artery**

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**Abstract:** Overproduction of reactive oxygen and nitrogen species leads to oxidative stress and decreased total antioxidant capacity, which is responsible for high mortality from several diseases such as endotoxic shock. Nitric oxide (NO) produced by inducible NO synthase (iNOS) during endotoxemia is the major cause of vascular hyporeactivity, hypotension and multiple organ failure. We investigated whether NO-mediated oxidative stress in rat heart, aorta and mesenteric artery is involved in the attenuation of endotoxin-induced hypotension by inhibition of iNOS. In conscious male Wistar rats, injection of endotoxin (10 mg/kg, i.p.) caused a gradual fall in mean arterial pressure (MAP) for 4 hours and increased serum and tissue nitrite levels. These effects of endotoxin were prevented by selective inhibition of iNOS with phenylene-1,3-bis[ethane-2-isothiourea] dihydrobromide (1,3-PBIT) (10 mg/kg, i.p.; 1 hour after endotoxin). Myeloperoxidase (MPO) activity was increased in the heart and aorta and decreased in the mesenteric artery by endotoxin which was reversed by 1,3-PBIT. Endotoxin caused a decrease in products of lipid peroxidation in the tissues, which is prevented by 1,3-PBIT. These data suggest that NO-mediated decrease in MAP during endotoxemia is associated with decreased oxidative stress in the heart, aorta and mesenteric artery and that the beneficial effects of iNOS inhibitors on the endotoxin-induced hypotension may be due to restoration of total antioxidant capacity.

**Key Words:** Rat, endotoxin, hypotension, inducible nitric oxide synthase, lipid peroxidation

## Introduction

Oxidative stress results from an oxidant/antioxidant imbalance, an excess of oxidants and/or a depletion of antioxidants. The excessive production of reactive oxygen and nitrogen species (ROS and RNS, respectively) associated with inflammation leads to oxidative stress, which is involved in the high mortality from several diseases such as endotoxic shock (1-4). There is a considerable body of evidence for redox imbalance and oxidative stress in endotoxic shock. It has been reported that endotoxemia increases markers of oxidative stress

and tissue injury in different animal models and in humans. This effect of endotoxin has been found to be correlated with decreased total antioxidant potential and blood levels of several antioxidants such as  $\alpha$ -tocopherol, retinol, vitamin E, vitamin C and  $\beta$ -carotene in humans with sepsis and septic shock (1-4). It has also been reported that although total antioxidant capacity was decreased in patients with sepsis, it was increased in patients with septic shock (5). Increased xanthine oxidase, superoxide dismutase and glutathione peroxidase activity has also been reported in patients with sepsis,

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suggesting increased production of ROS (6). However, malondialdehyde (MDA) (an index for lipid peroxidation) levels were also increased, suggesting that the elevations of these antioxidant enzymes were not effective enough to prevent cellular damage.

Among RNS, nitric oxide (NO) derived from inducible NO synthase (iNOS) is considered to be the major cause of systemic hypotension, vascular hyporeactivity, multiple organ failure and high mortality rate associated with septic shock (4,7). Although the role of NO is controversial in tissue injury with oxidative DNA damage in endotoxemia, most of the clinical trials demonstrate that iNOS inhibitors may serve as a potentially effective pharmacological agents in alleviating endotoxin-induced decrease in antioxidant capacity and tissue injuries (1, 4, 7).

The purpose of this study was to determine whether NO-mediated oxidative stress in the heart, aorta and mesenteric artery from endotoxemic rats is involved in the attenuation of systemic hypotension by selective inhibition of iNOS.

## Materials and Methods

### Chemicals

Endotoxin (lipopolysaccharide, *Escherichia coli* O111:B4), phenylene-1,3-bis[ethane-2-isothioureia] dihydrobromide (1,3-PBIT), sodium nitrite, bovine serum albumin (BSA) and Bradford reagent were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Other chemicals were obtained from Merck (Darmstadt, Germany). All drugs were prepared daily in distilled water or saline.

### Animals and endotoxic shock model

Male Wistar rats weighing 250 to 350 g were fed standard chow. They were synchronised by maintenance of controlled environmental conditions throughout the duration of the experiments. The circadian rhythmicity of the animals were entrained by a standardised 12 h light and 12 h dark period. All animal experiments were carried out according to the proposal of the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the ethics committee of Mersin University School of Medicine. Conscious rats received either endotoxin (10 mg/kg, i.p., sublethal dose) or saline (4 ml/kg, i.p.) at time 0 and

mean arterial blood pressure (MAP) and heart rate were measured using the tail-cuff method at 0, 1, 2, 3 and 4 hours. Separate groups of rats were treated with endotoxin alone or in combination with the highly selective iNOS inhibitor 1,3-PBIT (10 mg/kg, i.p.) at 1 hour after injection of saline or endotoxin. Rats were sacrificed 4 hours after the endotoxin challenge, and blood from the heart, aorta and mesenteric artery were collected. Sera were obtained from blood samples by centrifugation at 18.000 rpm for 15 minutes at 4 °C and stored at -20 °C until analyzed for the measurement of nitrite levels. The tissues were homogenized in ice-cold buffer (1 ml) (mM: HEPES 20 [pH 7.5],  $\beta$ -glycerophosphate 20, sodium pyrophosphate 20; sodium orthovanadate 0.2, EDTA 2, sodium fluoride 20, benzamidine 10, dithiothreitol 1, leupeptin 20 and aprotinin 10) (8). Cell debris was removed by centrifugation at 18.000 rpm for 15 minutes at 4 °C followed by sonication for 15 seconds on ice with 50  $\mu$ L ice-cold Tris (50 mM, pH 8.0) and KCl (0.5 M). The samples were centrifuged at 18.000 rpm for 15 minutes at 4 °C and then supernatants were removed and stored at -20 °C until analyzed for the measurement of myeloperoxidase (MPO) activity and protein, nitrite and malondialdehyde (MDA) levels.

### Measurement of tissue protein content

The protein content in the tissue homogenates was determined according to Coomassie blue method using BSA for standard (9). Briefly, Bradford reagent (200  $\mu$ l) was added to the mixture of tissue homogenate (5  $\mu$ l) and distilled water (795  $\mu$ l). Samples (100  $\mu$ l) were then pipetted into 96 well microtiter plates and absorbance was measured at 620 nm with a microplate reader (Organo Teknika Microwell System, Holland). Linear regression analysis was used to calculate the protein amount in the tissue homogenates from the standard calibration curves of BSA.

### Measurement of serum and tissue nitrite levels

In biological systems conversion of NO in aqueous solution to nitrite and nitrate is thought to favour nitrite production (10). It has been reported that nitrite is the only stable end-product of the autooxidation of NO in aqueous solution (11) and measurement of nitrite concentrations in the serum and tissue homogenates is widely accepted as an index of NOS activity (7, 12-14).

Therefore, concentrations of nitrite in serum and tissue homogenates were measured by using the diazotization method based on the Griess reaction, which is an indirect assay for NO production (12). Briefly, samples (50  $\mu$ l) were pipetted into 96 well microtiter plates and an equal volume of Griess reagent (1% sulphonylamide (25  $\mu$ l) and 0.1% N-1-naphtylethylenediamine dihydrochloride (25  $\mu$ l) in 2.5% orthophosphoric acid) was added to each well. After incubation for 10 minutes at room temperature, absorbance was measured at 540 nm with a microplate reader (Organo Teknika Microwell System, Holland). Linear regression analysis was used to calculate the nitrite concentrations in the sera and the tissue homogenates from the standard calibration curves of sodium nitrite. Serum and tissue nitrite levels were expressed as  $\mu$ M or mmol/mg protein, respectively.

#### Measurement of tissue MPO activity

MPO is a haem-containing enzyme within the azurophil granules of neutrophils and MPO activity was measured as a simple quantitative method of detecting leukosequestration. The determination of MPO activity as an index of neutrophil infiltration in tissue homogenates depends on the fact that oxidized hydrogen peroxide reduces o-dianisidine (15). Reduced o-dianisidine was measured at 410 nm by spectrophotometer. One unit of MPO activity was defined as that degrading 1  $\mu$ mol of hydrogen peroxide to water per minute at 25 °C. Tissue MPO activity was expressed as U/mg protein.

#### Measurement of tissue MDA levels

As an index of lipid peroxidation, the levels of MDA in tissue homogenates were determined by thiobarbituric acid reaction according to Yagi (16). The method depends on the measurement of the pink color produced by interaction of the barbituric acid with MDA caused by lipid peroxidation. Linear regression analysis was used to calculate the MDA levels in the tissue homogenates from the standard calibration curves of 1,1,3,3-tetraethoxypropane. Tissue MDA levels were expressed as mmol/mg protein.

#### Statistical analysis

All data were expressed as means  $\pm$  SEM. Data were analyzed by one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons and

unpaired Student's t or Mann-Whitney U tests when necessary. P value of  $< 0.05$  was considered to be statistically significant.

## Results

### Endotoxin-induced fall in MAP is mediated by NO produced by iNOS

Injection of endotoxin caused a gradual fall in MAP reaching from  $134 \pm 4$  mmHg (n = 8) at 0 time to  $109 \pm 5$  mmHg (n = 6) at 4 hours (P < 0.05) (Table 1). Endotoxin also caused a gradual increase in heart rate reaching from  $339 \pm 11$  beats/min (n = 12) at 0 time to  $422 \pm 14$  beats/min (n = 7) at 4 hours (P < 0.05) (Table 1). We chose this time point in all our experiments so that we could measure changes in endotoxin-induced decrease in MAP and increase in heart rate caused by various interventions.

In order to evaluate the contribution of iNOS-derived NO to the endotoxin-induced hypotension, the selective inhibitor of iNOS, 1,3-PBIT, was injected to the animals alone or in combination with endotoxin. Endotoxin-induced decrease in MAP (Figure 1A) and increase in heart rate (Figure 1B) were blunted by the administration of 1,3-PBIT. While 1,3-PBIT alone had no effect on MAP (Figure 1A), heart rate was decreased by 1,3-PBIT (Figure 1B).

### Endotoxin-induced rise in systemic and tissue nitrite levels is decreased by iNOS inhibitor, 1,3-PBIT

In order to evaluate the contribution of iNOS-derived NO to the endotoxin-induced decrease in MAP and increase in heart rate, nitrite levels were measured in serum and tissues from 1,3-PBIT-injected animals. The endotoxin-induced decrease in MAP and increase in heart rate were associated with an increase in the nitrite levels in serum (Figure 2A), heart (Figure 2B), aorta (Figure 2C) and mesenteric artery (Figure 2D). Endotoxin-induced increase in nitrite production in serum and the tissues was blunted by the administration of 1,3-PBIT (Figure 2). 1,3-PBIT had no effect on basal serum and tissue nitrite levels (Figure 2).

### Endotoxin produces different effects on tissue MPO activity

To investigate the effect of endotoxin on neutrophil infiltration as an index for the development of

Table 1. Effect of 1,3-PBIT on mean arterial pressure (MAP) and heart rate in endotoxemic rats.

	Time after endotoxin injection (hours)				
	0	1	2	3	4
Control					
MAP (mmHg)	128 ± 8 (n = 6)	149 ± 13 (n = 5)	136 ± 4 (n = 6)	136 ± 6 (n = 5)	138 ± 5 (n = 5)
Heart rate (beats/min)	343 ± 8 (n = 6)	336 ± 10 (n = 6)	319 ± 4 (n = 6)	336 ± 10 (n = 6)	344 ± 10 (n = 6)
ET					
MAP (mmHg)	134 ± 4 (n = 8)	107 ± 8*■ (n = 8)	106 ± 6*■ (n = 8)	111 ± 8*■ (n = 5)	109 ± 5*■ (n = 6)
Heart rate (beats/min)	339 ± 11 (n = 12)	397 ± 17*■ (n = 10)	420 ± 9*■ (n = 7)	419 ± 13*■ (n = 5)	422 ± 14*■ (n = 7)
1,3-PBIT					
MAP (mmHg)	139 ± 3 (n = 6)	142 ± 1 (n = 6)	140 ± 3 (n = 6)	140 ± 2 (n = 6)	146 ± 3 (n = 6)
Heart rate (beats/min)	319 ± 8 (n = 6)	318 ± 5 (n = 6)	307 ± 2* (n = 6)	300 ± 5* (n = 6)	301 ± 9* (n = 6)
ET+1,3-PBIT					
MAP (mmHg)	140 ± 4 (n = 6)	121 ± 4*▲■ (n = 6)	124 ± 1**▲■ (n = 6)	129 ± 2*▲● (n = 6)	129 ± 7*▲ (n = 5)
Heart rate (beats/min)	318 ± 1 (n = 6)	384 ± 3*▲■ (n = 6)	383 ± 15*▲■ (n = 5)	366 ± 8*▲■ (n = 6)	380 ± 10*▲■ (n = 6)

MAP and heart rate measurements were performed in rats treated with saline (control) or endotoxin (ET; 10 mg/kg, i.p.) or in endotoxemic rats treated with 1,3-PBIT (10 mg/kg, i.p.). The inhibitor, alone or in combination with ET, were injected at 1 hour after endotoxin. Data are expressed as mean ± SEM (n = 5-12). \* P < 0.05 vs. saline-treated group (vehicle) determined by using unpaired Student's t or Mann-Whitney U tests. + P < 0.05 vs. ET-treated group determined by using unpaired Student's t or Mann-Whitney U tests. ▲ P < 0.05 vs. 1,3-PBIT-treated group determined by using unpaired Student's t or Mann-Whitney U tests. ■ P < 0.05 vs. time 0 value determined by using one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons test. ● P < 0.05 vs. time 1 value determined by using one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons test.

inflammation, MPO activity was measured in the tissues from endotoxemic animals. Endotoxemia increased MPO activity in heart (Figure 3A) and aorta (Figure 3B), which is prevented by 1,3-PBIT. On the other hand, endotoxin caused a decreased MPO activity in mesenteric artery which is also blunted with 1,3-PBIT (Fig 3C). 1,3-PBIT had no effect on tissue MPO activity (Figure 3).

### Endotoxin-induced increase in systemic and tissue nitrite levels are associated with decreased oxidative stress

To investigate the effect of endotoxin on oxidative stress, one of the products of lipid peroxidation, MDA, was measured in the tissues from endotoxemic animals. Endotoxin decreased MDA levels in the heart (Figure 4A),

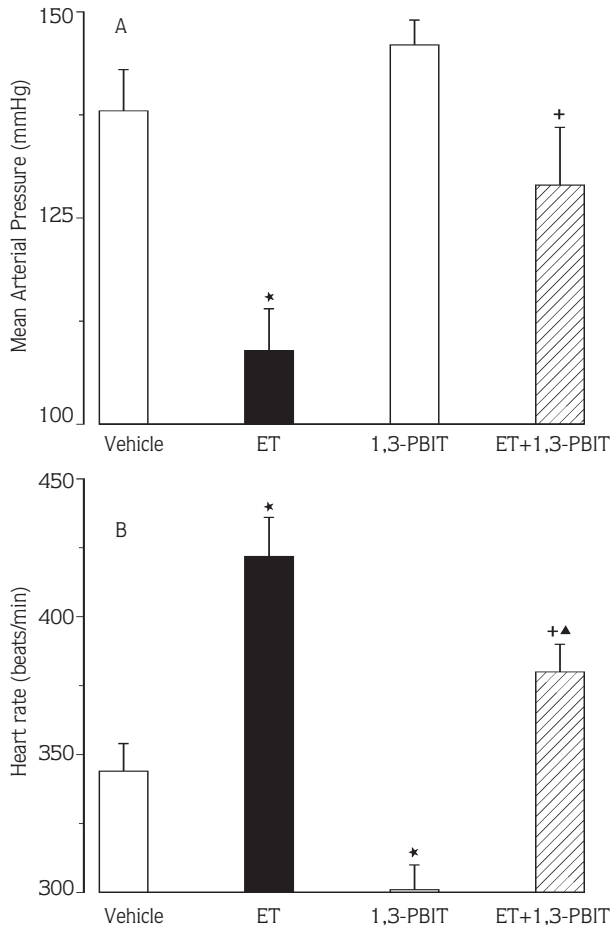


Figure 1. Effect of iNOS inhibition by 1,3-PBIT on mean arterial pressure (MAP) (A) and heart rate (B) 4 h after saline (vehicle) or endotoxin (ET) injection in conscious rats. 1,3-PBIT was given at 1 h after ET injection. Values are expressed as means  $\pm$  SEM from 5-7 rats per treatment group. \*  $P < 0.05$  vs. saline-treated group (vehicle) determined by using unpaired Student's t test. +  $P < 0.05$  vs. ET-treated group determined by using unpaired Student's t test. ▲  $P < 0.05$  vs. 1,3-PBIT-treated group determined by using unpaired Student's t test.

aorta (Figure 4B) and mesenteric artery (Figure 4C) which is prevented by 1,3-PBIT. 1,3-PBIT had no effect on basal MDA levels in the tissues (Figure 4).

## Discussion

This study demonstrates that increased NO production by iNOS 4 hour after endotoxin injection causes hypotension associated with cardiac and aortic

inflammation in conscious rats, and NO-derived from the heart, aorta and mesenteric artery contributes to these effects of endotoxin. More importantly, we showed that endotoxemia-induced increase in NO production suppresses lipid peroxidation in cardiac and vascular tissue and selective inhibition of iNOS with 1,3-PBIT restores tissue antioxidant capacity and consequently MAP, presumably due to decreased levels of antioxidant molecules or activities of antioxidant enzyme systems.

Expression of iNOS protein and mRNA expression approximately 3 hours after systemic endotoxin administration to animals has been reported in several tissues, including several blood vessels, heart, kidney and lung associated with significant increase in serum/plasma and tissue nitrite levels and decrease in MAP (7,17-19). In the present study, administration of endotoxin to rats decreased MAP at 4 hour. This was associated with an increase in the serum nitrite levels that was prevented after selective inhibition of iNOS with 1,3-PBIT. Endotoxin-induced increase in heart, aorta and mesenteric artery nitrite production was also blunted by 1,3-PBIT. These observations suggest that NO produced mainly by activation of iNOS contributes to the endotoxin-induced hypotension in conscious rats.

There are contradictory reports in the literature concerning the role of NO as an antiinflammatory or proinflammatory agent (7,17,18). In the present study, we found that endotoxin caused an increased level of nitrite in serum, heart and aorta as well as increased MPO activity prevented by the iNOS inhibitor, 1,3-PBIT. However, increased levels of nitrite in the mesenteric artery was associated with decreased MPO activity, also reversed by 1,3-PBIT. These results suggest that NO produced by iNOS, expressed in, at least the heart and aorta, acts as a proinflammatory mediator in this endotoxemia model in rat. On the other hand, endotoxin-induced decrease in MPO activity in the mesenteric artery suggests that iNOS-derived NO acts as an anti-inflammatory mediator to protect small resistance arteries which are responsible for maintaining blood pressure under pathological conditions from detrimental effects of endotoxin.

It is well known that oxidative stress results from an oxidant/antioxidant imbalance, an excess of oxidants and/or a depletion of antioxidants (4). Although increased activity of antioxidant enzymes such as xanthine oxidase, superoxide dismutase and glutathione peroxidase has

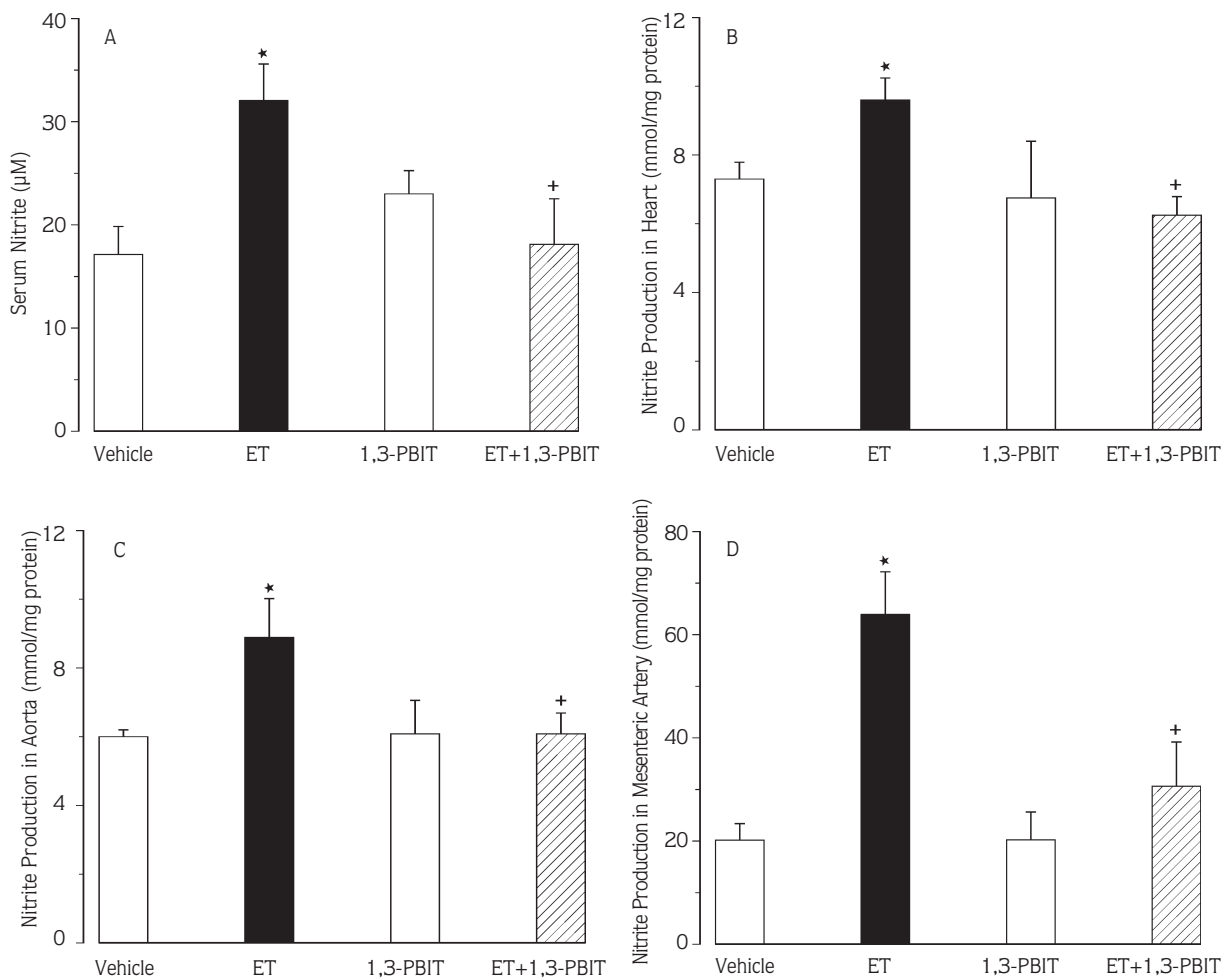


Figure 2. Effect of iNOS inhibition by 1,3-PBIT on changes in serum (A), heart (B), aorta (C) and mesenteric artery (D) nitrite levels 4 h after saline or endotoxin (ET) injection in conscious rats. Values are expressed as means  $\pm$  SEM from 3-10 rats per treatment group. \*  $P < 0.05$  vs. saline-treated group (vehicle) determined by using unpaired Student's t or Mann-Whitney U tests. +  $P < 0.05$  vs. ET-treated group determined by using unpaired Student's t test.

been reported in patients with sepsis, MDA levels have also been shown to be increased, suggesting that the elevations of these antioxidant enzymes were not sufficiently effective to prevent cellular damage (5). Moreover, Mirochnitchenko et al. (20) have reported that transgenic mice overexpressing 2 major forms of human glutathione peroxidases (GPs), intra- and extracellular GP, are able to modulate host response during endotoxemic conditions. They also showed that these animals had decreased hypotension and increased survival rate after administration of a high dosage of endotoxin. In the present study, endotoxin-induced

increase in the nitrite levels in serum, heart, aorta and mesenteric artery was associated with decreased MDA levels in these tissues and prevented by 1,3-PBIT. Although there are conflicting reports on the effect of NO on lipid peroxidation and oxidative stress (21-24), our results are also consistent with the observations that NO behaves as a potent antioxidant. Indeed, it has been demonstrated that addition of NO as a saturated aqueous solution to human leukemia cells (25) and rat brain homogenates under aerobic conditions (26) decreases lipid peroxidation. Levels of MDA have also been shown to be decreased during NO inhalation in rats (27). NO

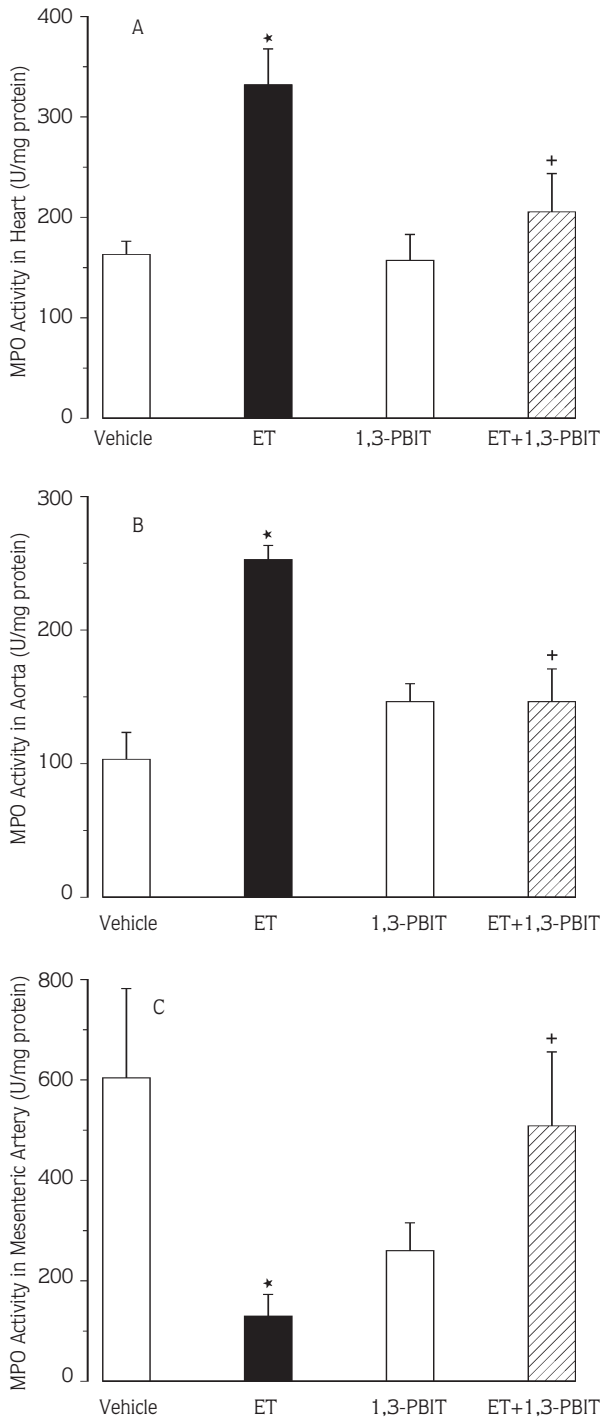


Figure 3. Effect of iNOS inhibition by 1,3-PBIT on changes in heart (A), aorta (B) and mesenteric artery (C) myeloperoxidase (MPO) activity 4 h after saline or endotoxin (ET) injection in conscious rats. Values are expressed as means  $\pm$  SEM in 3-9 rats per treatment group. \*  $P < 0.05$  vs. saline-treated group (vehicle) determined by using unpaired Student's t test. +  $P < 0.05$  vs. ET-treated group determined by using unpaired Student's t test.

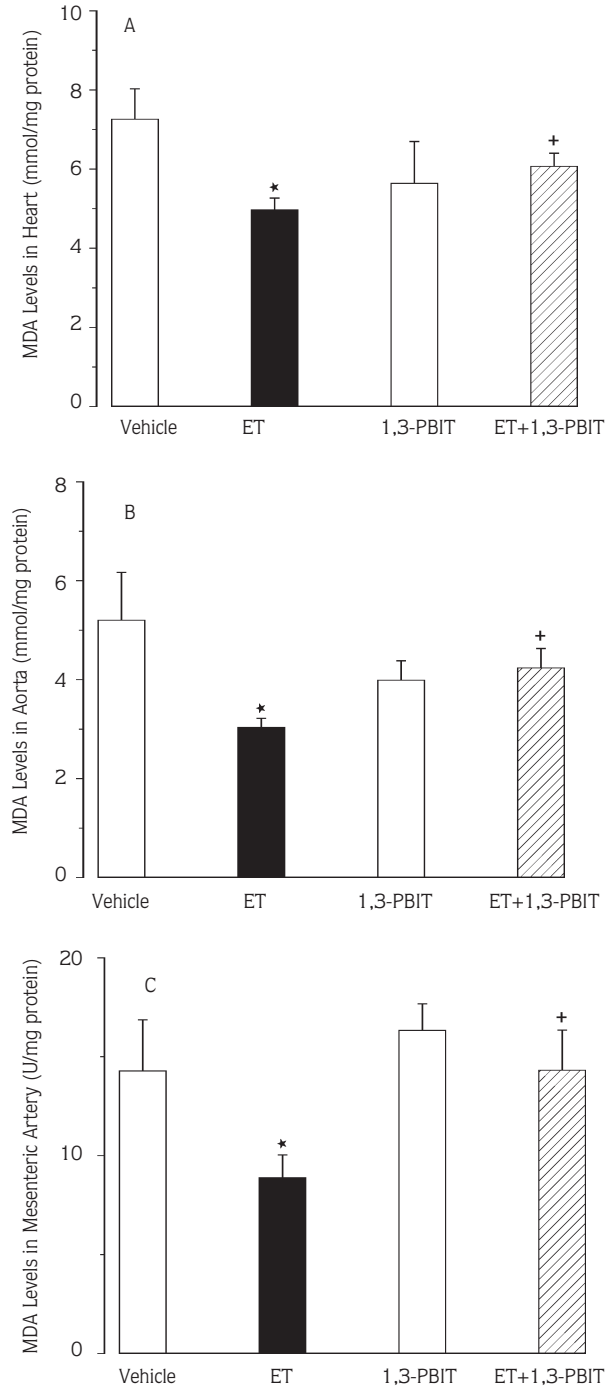


Figure 4. Effect of iNOS inhibition by 1,3-PBIT on changes in heart (A), aorta (B) and mesenteric artery (C) malondialdehyde (MDA) levels 4 h after saline or endotoxin (ET) injection in conscious rats. Values are expressed as means  $\pm$  SEM in 4-8 rats per treatment group. \*  $P < 0.05$  vs. saline-treated group (vehicle) determined by using unpaired Student's t or Mann-Whitney U tests. +  $P < 0.05$  vs. ET-treated group determined by using unpaired Student's t test.

donors, such as nitroglycerine in coronary artery bypass surgery (28), stress-induced gastric mucosal damage (29) and physical training (30), glyceryltrinitrate in ferric nitrilotriacetate-induced oxidative stress (31) stress-induced gastric mucosal damage (32), sodium nitroprusside in carbon tetrachloride-induced liver injury (33), 3-morpholinopyrrolidine in stress-induced gastric mucosal damage (29), S-nitroso-N-acetylpenicillamine in stress-induced gastric mucosal damage (29), hypoxia/regeneration-induced damage of brain-blood barrier cells (34) iron-catalyzed brain lipid peroxidation (35), diethylamine NONOate in rat brain homogenates under aerobic conditions (26), molsidomine in intestinal ischemia-reperfusion injury (36), O(2)-vinyl 1-(pyrrolidin-1-yl) diazen-1-ium-1,2-diolate in acetaminophen nephrotoxicity (37) or nitric oxide-releasing aspirin in stress-induced gastric mucosal damage (29,32,38) have also been reported to cause an attenuation of lipid peroxidation as documented by a decrease of tissue MDA levels in rats and mice as well as in humans. Moreover the NO precursor, L-arginine has been shown to decrease blood and/or tissue MDA levels in several disease models in animals with hypercholesterolemia and atherosclerosis (39), hypoxia/reoxygenation-induced intestinal injury (40), burn injury (41), hypoxia/reoxygenation-induced necrotizing enterocolitis, warm ischemia-reperfusion injury (42), hypoxia-induced tissue damage (43) and hypoxemic/reoxygenation injury (44) as well as in humans during coronary artery bypass surgery (45). On the other hand, tissue and/or plasma MDA levels have been reported to increase after in vitro incubation of tissues or systemic administration of NO donors, such as cyanamide (29), SNP (46-48), isosorbide dinitrate (49) or SNAP (50) in rats. Tissue MDA levels have also been shown to be increased after L-arginine treatment in the renal ischemia-reperfusion model in rat (51,52), 2,4,6-trinitrobenzenesulfonic acid induced colitis in rat (53) and myocardial stunning in dogs (54). There are several reports indicating that systemic administration of inhibitors of lipid peroxidation, anthocyanins (55), potent antioxidants such as ascorbic acid (56) and melatonin (57), a dual vitamin E-like antioxidant and inhibitor of nuclear factor- $\kappa$ B, IRFI 042 (58), ethyl pyruvate (59), U-

83836E (60), an alpha/beta-adrenoceptor and serotonergic receptor blocker, eugenosedin-A (56) and the tyrosine kinase inhibitor genistein (61), result in improved hemodynamic status and reversal of endotoxin shock. The protective effects of these agents have been attributed to their antioxidant properties and also inhibition of cytokine and/or NO overproduction. Based on our findings and previous studies, therefore, it can be concluded that NO produced by iNOS mediates the endotoxin-induced increased total antioxidant capacity and consequently decreases lipid peroxidation.

In summary, overproduction of NO during endotoxemia leads to cardiac and aortic inflammation and suppression of lipid peroxidation in cardiac and vascular tissue. Selective inhibition of iNOS with 1,3-PBIT restores tissue inflammation and antioxidant capacity and consequently MAP, presumably due to decreased levels of antioxidant molecules or activities of antioxidant enzyme systems. Impairment of cardiovascular function is critically involved in the pathophysiological sequelae in septic shock finally resulting in multiorgan failure and death; restoration of these impaired functions should improve therapeutic benefit. Our results suggest that treatment with the selective iNOS could improve the cardiovascular function associated with inflammation and increased antioxidant capacity in patients during septic shock.

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