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# Amelioration by mercaptoethylguanidine of the vascular and energetic failure in haemorrhagic shock in the anesthetised rat

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**Abstract**

The effects of mercaptoethylguanidine, a dual inhibitor of the inducible nitric oxide (NO) synthase and cyclooxygenase with scavenging effect on peroxynitrite, was studied on the delayed vascular decompensation and cellular energetic failure in a rat model of haemorrhagic shock. Shock was induced by bleeding of the animals to a mean arterial blood pressure of 50 mmHg. At 3 h, animals were resuscitated with Ringers-lactate and monitored for a subsequent 3 h period. In the treated group mercaptoethylguanidine (10 mg/kg/i.v. bolus, followed by 10 mg/kg/i.v. infusion) was administered from the beginning of the resuscitation. Haemorrhagic shock resulted in the upregulation of both the constitutive and the inducible NO synthase, as measured in the lung. In shocked rats mercaptoethylguanidine prevented the increase in plasma nitrite/nitrate and 6-keto-prostaglandin $F_{1\alpha}$ levels, ameliorated the decrease in mean arterial blood pressure, and inhibited the development of vascular hyporeactivity of the thoracic aorta ex vivo. A significant nitrotyrosine staining, an indicator of peroxynitrite formation, was found in thoracic aortic rings from shocked animals, which was prevented by mercaptoethylguanidine treatment. In ex vivo experiments in peritoneal macrophages obtained from shocked rats, treatment with mercaptoethylguanidine prevented the reduction in the intracellular $NAD^+$ content, ameliorated the suppression of mitochondrial respiration and reduced the development of DNA single strand breaks. Our data suggest that mercaptoethylguanidine may be an useful tool for the experimental therapy of haemorrhagic shock. © 1997 Elsevier Science B.V.

*Keywords:* Mercaptoalkylguanidine; Haemorrhagic shock; Nitric oxide (NO); 6-Keto-prostaglandin-1$\alpha$; Peroxynitrite

## 1. Introduction

Complex interactions of various endogenous mediators are known to contribute to the pathophysiology of haemorrhagic shock. Recently enhanced formation of nitric oxide (NO), a potent vasodilator with cytotoxic effects produced by the inducible NO synthase, has been demonstrated after haemorrhage as shown by measurement of increased NO production (Naziri et al., 1995) and increased enzyme activity for the inducible NO synthase ex vivo (Thiemermann et al., 1993; Kelly et al., 1997). In the delayed phase of shock (the period of vascular decompensation), induction of the inducible NO synthase in various tissues and in the vasculature has been suggested to contribute to the progressive vasodilatation seen after haemorrhage (Thiemermann et al., 1993). In this regard, numerous studies have demonstrated beneficial effects of non-isoform selective NO synthase inhibitors in haemorrhagic shock (Szabó and Thiemermann, 1994). These effects include an increase in blood pressure (Zingarelli et al., 1992; Thiemermann et al., 1993; Vromen et al., 1996; Yao et al., 1996), cardiac output (Klabunde et al., 1993), an improvement of renal blood flow and glomerular filtration rate (Lieberthal et al., 1991) and protection against the development of pulmonary injury, gastric lesions and death (Zingarelli et al., 1992; Yao et al., 1996). Several experimental studies demonstrated detrimental effects with non-isoform selective NO synthase inhibitors in haemorrhagic shock (Brown et al., 1995; Harbrecht et al., 1995), while selective inhibition of the inducible isoform of NO synthase provided benefit in the same experimental models (Menezes et al., 1997). We have recently proposed that some of the effects of non-isoform-selective inhibition of NO synthase (both beneficial and detrimental) may be related to inhibition of the constitutive isoform of NO

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synthase (Vromen et al., 1996). It is, in fact, well known that NO derived from this latter isoform, serves physiological purpose in the maintenance of the tissue blood flow since it inhibits platelet aggregation, neutrophil activation and adhesion and maintains the vessels in a physiological vasodilator tone (Szabó and Thiemermann, 1994).

The restoration of oxygen supply by transfusion or by improvement of the cardiovascular performance, while providing blood redistribution to the organs, may amplify the tissue injury by the production of oxygen free radicals (Hamano et al., 1993; Tan et al., 1993; Kapoor and Prasad, 1994). Simultaneous generation of both NO and superoxide anion radicals favors the development of a toxic reaction product, the oxidant peroxynitrite (Beckman et al., 1990; Crow and Beckman, 1995). Both NO and peroxynitrite have been shown to exert cytotoxic effects by direct and indirect inhibition of energy-generating intracellular processes (Radi et al., 1994; Szabó et al., 1996a,b). The evidence that peroxynitrite is produced and plays an important role in cardiovascular shock is supported by recent studies on endotoxic shock (Wizemann et al., 1994; Szabó et al., 1996b; Zingarelli et al., 1996). Our group has recently demonstrated that prevention of the formation of peroxynitrite by a superoxide dismutase mimetic and peroxynitrite scavenger compound improves vascular contractility and cellular energetic status in endotoxic shock (Zingarelli et al., 1997a).

Because of the complexity of the pathophysiology of cardiovascular shock, major efforts have recently been focusing on identifying anti-inflammatory drugs which can prevent proinflammatory processes without interfering with the physiological homeostasis of the organism. Recently, certain isothiourea compounds, such as *S*-methyl-isothiourea and aminoethyl-isothiourea have been identified as potent inhibitors of NO synthase, with selectivity towards the inducible isoform in rodent models and with protective effects in endotoxic shock (Garvey et al., 1994; Southan et al., 1995). The isoform-selective principle of aminoethyl-isothiourea is mercaptoethylguanidine, the rearrangement product of aminoethyl-isothiourea in aqueous solutions (Southan et al., 1996). In vitro studies have demonstrated that mercaptoethylguanidine also possesses unique additional properties of direct inhibition of cyclooxygenase activity and peroxynitrite scavenging (Szabó et al., 1997; Zingarelli et al., 1997b).

Because of the multiple anti-inflammatory modes of mercaptoethylguanidine, in the present study we have investigated the potential protective effect of this agent in haemorrhagic shock. In specific, we studied the effect of this inhibitor on the vascular contractile and cellular energetic failure in a rat model of delayed haemorrhagic shock. In addition, by performing nitrotyrosine immunohistochemistry (a marker of peroxynitrite) in blood vessels ex vivo, we have investigated the production of peroxynitrite in haemorrhagic shock, and the effect of mercaptoethylguanidine on this process.

## 2. Materials and methods

### 2.1. Haemorrhagic shock model

Male Wistar rats (Charles River Laboratories, Wilmington, MA, USA) were anaesthetized with sodium pentathol (120 mg/kg) intraperitoneally (i.p.). The trachea was cannulated to facilitate respiration and temperature was maintained at 37°C using a homeothermic blanket. The right carotid artery was cannulated and connected to a pressure transducer for the measurement of phasic and mean arterial blood pressure and heart rate which were digitized using a Maclab A/D converter (AD Instruments, Milford, MA, USA) and stored and displayed on a Macintosh personal computer. The left femoral vein was cannulated for the administration of drugs and the right femoral artery was cannulated for withdrawal of blood. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 10 min. After recording baseline haemodynamic parameters, haemorrhagic shock was induced by withdrawing blood from the femoral artery into a reservoir until mean arterial blood pressure stabilized at 50 mmHg (Thiemermann et al., 1993). In this model, the initial rate of blood withdrawal was approximately 0.3 ml/min and the initial volume of blood withdrawn was not different between individual groups. Thereafter, additional blood was withdrawn as necessary to maintain mean arterial blood pressure at 50 mmHg. At the end of the 3 h, the total volume withdrawn was $9.2 \pm 0.7$ ml and was not significantly different between groups.

At 3 h, the animals subjected to bleeding ($n = 11$) were randomly divided into two groups. In the first group ($n = 5$), treatment with vehicle (saline) started, whereas in the second group ($n = 6$), treatment with mercaptoethylguanidine (10 mg/kg intravenous bolus and 10 mg/kg per h infusion) started. In both groups, resuscitation was performed with Ringers-lactate (volume of the shed blood), which was injected at 3 h over a 5 min period. After resuscitation, blood pressure was monitored for an additional 3 h. Vascular decompensation was evidenced as a gradual fall in blood pressure after resuscitation. At 3 h after resuscitation, animals were killed and blood was taken for the measurement of plasma nitrite/nitrate and 6-keto-prostaglandin $F_{1\alpha}$ levels, thoracic aortae taken for the assessment of their contractile ability and peritoneal macrophages taken for the measurement of in vitro nitrite/nitrate production, mitochondrial respiration, intracellular $NAD^+$ levels and the percentage of single stranded DNA. In control experiments, rats were instrumented as above, sham-shocked for 3 h and then infused with saline ($n = 4$) or mercaptoethylguanidine ($n = 4$) (doses as above) for 3 h.

### 2.2. Measurement of isometric force in vascular rings

Thoracic aortae from rats were cleared of adhering periadventitial fat and cut into rings of 3–4 mm width.

Endothelium was removed from some of the rings by gently rubbing the intimal surface. Lack of an acetylcholine-induced relaxation was taken as evidence that endothelial cells had been removed. The rings were mounted in organ baths (5 ml) filled with warmed (37°C), oxygenated (95% $O_2$/5% $CO_2$) Krebs' solution (pH 7.4) consisting of (mM): NaCl, 118; KCl, 4.7; $KH_2PO_4$, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25 and glucose, 11.7, in the presence of indomethacin (10 μM). Isometric force was measured with isometric transducers (Kent Scientific, Litchfield, CT), digitized using a Maclab A/D converter (AD Instruments) and stored and displayed on a Macintosh personal computer. A tension of 1 g was applied and the rings were equilibrated for 60 min. Fresh Krebs' solution was provided at 15 min intervals.

To investigate changes in contractility, concentration–response curves to noradrenaline ($10^{-9}$–$10^{-5}$ M) were then obtained in endothelium-denuded aortic rings. To investigate changes in the endothelium-dependent relaxant ability of the blood vessels, endothelium-intact vessels were precontracted with noradrenaline (1 μM) and concentration–response curves to acetylcholine (0.01 μM–10 μM) were then obtained.

### 2.3. Nitrotyrosine immunohistochemistry in vascular rings

Aortae were taken from control animals and from animals subjected to haemorrhagic shock in the absence or presence of mercaptoethylguanidine treatment for nitrotyrosine immunohistochemical localization. 8 μm frozen sections were incubated with either anti-nitrotyrosine polyclonal antibody at 2 μg/ml for 3 h, or antigen-competed primary antibody. The antigen-competed antibody was prepared by diluting the primary antibody at 2 μg/ml in 10 mM nitrotyrosine in 0.1 M phosphate-buffered saline (PBS) pH 7.4. The antibody binding on tissue sections was visualized by 1 h incubation with an anti-rabbit IgG conjugated to Texas Red (1:100 dilution in PBS). Under these experimental conditions, antibody binding is inhibited by excess nitrotyrosine during the incubation period (Szabó et al., 1995a). Slides were examined under a Nikon Diaphot-TND epifluorescence inverted microscope.

### 2.4. Nitric oxide synthase activity measurements

At the end of the experiments, in sham-shocked animals and in animals subjected to haemorrhagic shock, lungs were excised and frozen at −70°C for later measurement of inducible and constitutive NO synthase activities. The lungs were first homogenized in HEPES buffer (pH 7.5). Conversion of L-[$^3$H]arginine to L-[$^3$H]citrulline was measured in the homogenates as previously described (Southan et al., 1996). Briefly, 50 μl of tissue homogenate (approximately 100 μg protein per sample and equal for all groups) was incubated in the presence of L-arginine/[$^3$H]arginine (10 μM, 5000 Bq per tube) and NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and $Ca^{2+}$ (2 mM) for 20 min in HEPES buffer. Reactions were stopped by dilution with 1 ml of ice-cold Hepes buffer, pH 5.5/2 mM EGTA/2 mM EDTA. Reaction mixtures were applied to Dowex 50W ($Na^+$ form) columns; L-[$^3$H]citrulline was eluted and measured by scintillation counting. Parallel experiments done in the absence of NADPH determined the extent of [$^3$H]citrulline formation independent of a specific NOS activity (< 3% above background). To measure the $Ca^{2+}$-independent (i.e., inducible) NO synthase activity, incubations were done with NADPH and EGTA (5 mM) and without $Ca^{2+}$. L-[$^3$H]arginine was obtained from DuPont NEN. Protein concentration was measured spectrophotometrically in 96-well plates using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Four animals were used for all experimental groups.

### 2.5. Plasma nitrite / nitrate levels

In plasma samples, nitrate is the major degradation product of NO. Nitrate was converted to nitrite by incubation with 60 mU nitrate reductase and 25 μM NADPH for 180 min. Nitrite was then measured by the Griess reaction (Zingarelli et al., 1996). Nitrite/nitrate concentrations were calculated by comparison with $OD_{550}$ of standard solutions of sodium nitrite and sodium nitrate.

### 2.6. Plasma 6-keto-prostaglandin $F_{1\alpha}$ levels

Plasma samples were diluted 1:10 in a buffer containing 0.1% polyvinylpyrrolidone, 0.9% NaCl, 50 mM Tris base, 1.7 mM $MgSO_4$ and 0.16 mM $CaCl_2$ (pH 7.4) before radioimmunoassay. The stable metabolite of prostacyclin, 6-keto-prostaglandin $F_{1\alpha}$ was determined by radioimmunoassay as described (Zingarelli et al., 1997b).

### 2.7. Preparation of peritoneal macrophages

Peritoneal macrophages from rats were harvested by peritoneal lavage with Dulbecco's modified Eagle's medium containing L-glutamine (3.5 mM), penicillin (50 U/ml), streptomycin (50 μg/ml) and heparin sodium (10 U/ml) (Zingarelli et al., 1996). The cells were plated on 12 well plastic plates at 1 million cells/ml and incubated for 2 h at 37°C in a humidified 5% $CO_2$ incubator. After incubation, supernatant was collected for the measurement of nitrite/nitrate (as above) and non-adherent cells were removed by rinsing the plates three times with 5% dextrose water. Adherent macrophages were then used for the measurement of DNA strand breaks, cellular $NAD^+$ content and mitochondrial respiration as described below.

### 2.8. Measurement of mitochondrial respiration

Cell respiration was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Zingarelli

et al., 1996). Cells in 96-well plates were incubated at 37°C with MTT (0.2 mg/ml) for 1 h. Culture medium was removed by aspiration and the cells were solubilized in dimethylsulfoxide (100 μl). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of $OD_{550}$.

### 2.9. Determination of DNA single strand breaks

The formation of strand breaks in double-stranded DNA was determined by the alkaline unwinding method as previously described (Zingarelli et al., 1996). Cells in 12-well plates were scraped into 0.2 ml of solution A buffer (myoinositol 250 mM, $NaH_2PO_3$ 10 mM, $MgCl_2$ 1 mM, pH 7.2). The cell lysate was then transferred into plastic tubes designated T (maximum fluorescence), P (fluorescence in sample used to estimate extent of DNA unwinding), or B (background fluorescence). To each tube, 0.2 ml of solution B (alkaline lysis solution: NaOH 10 mM, urea 9 M, ethylenediaminetetraacetic acid 2.5 mM, sodium dodecyl sulfate 0.1%) was added and incubated at 4°C for 10 min to allow cell lysis and chromatin disruption. 0.1 ml each of solutions C (0.45 volume solution B in 0.2 M NaOH) and D (0.4 volume solution B in 0.2 M NaOH) was then added to the P and B tubes. 0.1 ml of solution E (neutralizing solution: glucose 1 M, mercaptoethanol 14 mM) was added to the T tubes before solutions C and D were added. From this point incubations were carried out in the dark. A 30 min incubation period at 0°C was then allowed during which the alkali diffused into the viscous lysate. Since the neutralizing solution, solution E, was added to the T tubes before addition of the alkaline solutions C and D, the DNA in the T tubes was never exposed to a denaturing pH. At the end of the 30 min incubation, the contents of the B tubes were sonicated for 30 seconds to ensure rapid denaturation of DNA in the alkaline solution. All tubes were then incubated at 15°C for 10 min. Denaturation was stopped by chilling to 0°C and adding 0.4 ml of solution E to the P and B tubes. 1.5 ml of solution F (ethidium bromide 6.7 μg/ml in 13.3 mM NaOH) was added to all the tubes and fluorescence (excitation: 520 nm, emission: 590 nm) was measured by a Perkin-Elmer fluorimeter. Under the conditions used, in which ethidium bromide binds preferentially to double stranded DNA, the percentage of double stranded DNA ($D$) may be determined using the equation: $\%D = 100 \times [F(P) - F(B)]/[F(T) - F(B)]$; where $F(P)$ is the fluorescence of the sample, $F(B)$ the background fluorescence, i.e., fluorescence due to all cell components other than double stranded DNA and $F(T)$ the maximum fluorescence.

### 2.10. Measurement of cellular $NAD^+$ levels

Cells in 12-well plates were extracted in 0.25 ml of 0.5 M $HClO_4$, scraped, neutralized with 3 M KOH and centrifuged for 2 min at $10\,000 \times g$. The supernatant was assayed for $NAD^+$ using a modification of the colorimetric method (Zingarelli et al., 1996), in which NADH, produced by enzymatic cycling with alcohol dehydrogenase, reduces MTT to formazan through the intermediation of phenazine methosulfate. The rate of MTT reduction is proportional to the concentration of the co-enzyme. The reaction mixture consisted of 10 μl of a solution of 2.5 mg/ml MTT, 20 μl of a solution of 4 mg/ml phenazine methosulfate, 10 μl of a solution of 0.6 mg/ml alcohol dehydrogenase (300 U/ml) and 190 μl of a 0.065 M glycyl–glycine buffer (pH 7.4) containing 0.1 M nicotinamide and 0.5 M ethanol. The mixture was warmed to 37°C for 10 min and the reaction started by addition of 20 μl of the sample. The rate of increase in the absorbance was read immediately after addition of the $NAD^+$ samples and after 10 and 20 min incubation at 37°C against a blank at 560 nm in the Spectramax spectrophotometer.

### 2.11. Materials

Mercaptoethylguanidine was prepared as previously described (Southan et al., 1996). Alcohol dehydrogenase and $NAD^+$ were obtained from Boehringer-Mannheim (Indianapolis, IN, USA). All other chemicals were obtained as specified. Where not specified, chemicals were from Sigma/Aldrich (St. Louis, MO, USA).

### 2.12. Data analysis

All values in Figs. 1–7 and text are expressed as the mean ± S.E.M. of $n$ observations, where $n$ represents the number of animals or vascular rings studied. Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with the Student's unpaired $t$-test. A $P$-value less than 0.05 was considered significant.

## 3. Results

### 3.1. Plasma nitrite/nitrate and 6-keto-prostaglandin $F_{1\alpha}$ concentration, tissue NO synthase activities and vascular nitrotyrosine immunoreactivity

Low nitrite/nitrate (Fig. 1a) and 6-keto-prostaglandin $F_{1\alpha}$ (Fig. 1b) concentrations were found in the plasma of sham-shocked animals. Moreover, in accordance with previous findings (Szabó et al., 1995a), no remarkable nitrotyrosine staining was found in the thoracic aortae of sham-shocked rats (Fig. 2a). At the end of the haemorrhagic shock protocol, there was a significant increase in plasma nitrite/nitrate and 6-keto-prostaglandin $F_{1\alpha}$ levels (Fig. 1), as well as an increase in nitrotyrosine immunoreactivity in the thoracic aorta, with somewhat more marked staining in the vicinity of the vascular endothelium (Fig. 2b). The

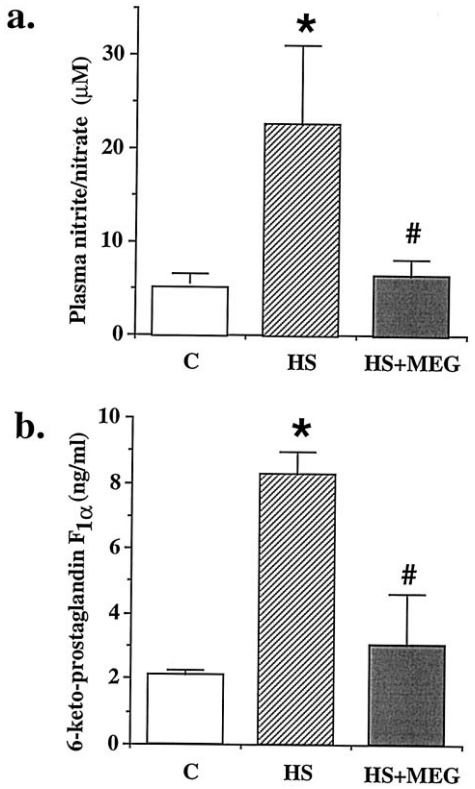


Fig. 1. Effect of administration of mercaptoethylguanidine (MEG) in vivo on plasma nitrite/nitrate (a) and 6-keto-prostaglandin $F_{1\alpha}$ (b) levels in rats subjected to haemorrhagic shock. Depicted are values in control rats (C), in rats subjected to haemorrhagic shock (HS) and in rats treated with MEG during haemorrhagic shock (HS + MEG). *A significant increase in nitrite/nitrate or 6-keto-prostaglandin $F_{1\alpha}$ in response to haemorrhagic shock ($P < 0.05$), #significant protective effects of MEG in haemorrhaged rats when compared to haemorrhage alone ($P < 0.05$); $n = 4–6$.

increase in plasma nitrite/nitrate and 6-keto-prostaglandin $F_{1\alpha}$ as well as the increase in nitrotyrosine was abolished by treatment with mercaptoethylguanidine during haemorrhagic shock (Fig. 1 and Fig. 2c).

Since the increase in plasma nitrite/nitrate, in theory, may derive from up-regulated constitutive NO synthase activity, or from inducible NO synthase activity, or, in the case of bacterial translocation in haemorrhagic shock, even from the endogenous bacterial flora, direct measurements of constitutive and inducible NO synthase activity were also performed in the lung ex vivo. The results have confirmed our previous observation (Thiemermann et al., 1993) and recent data (Kelly et al., 1997), demonstrating the expression of the inducible NO synthase in haemorrhagic shock (Fig. 3). In addition, evidence for up-regulation of constitutive NO synthase was also found (Fig. 3).

### 3.2. Haemodynamic decompensation

After resuscitation with Ringers-lactate, there was a gradual decrease in mean arterial blood pressure in the vehicle-treated animals subjected to haemorrhagic shock (Fig. 4). This delayed fall in mean arterial blood pressure was significantly ameliorated by mercaptoethylguanidine treatment (Fig. 4). There was no significant change in heart rate throughout the experiment and there was no difference between the vehicle-treated and the mercaptoethylguanidine treated groups. Heart rate in the vehicle-treated group was $397 \pm 8$ beats/min (bpm) before the start of the bleeding and $408 \pm 6$ bpm at the end of the experiment, 6 h after the start of the bleeding ($n = 6$).

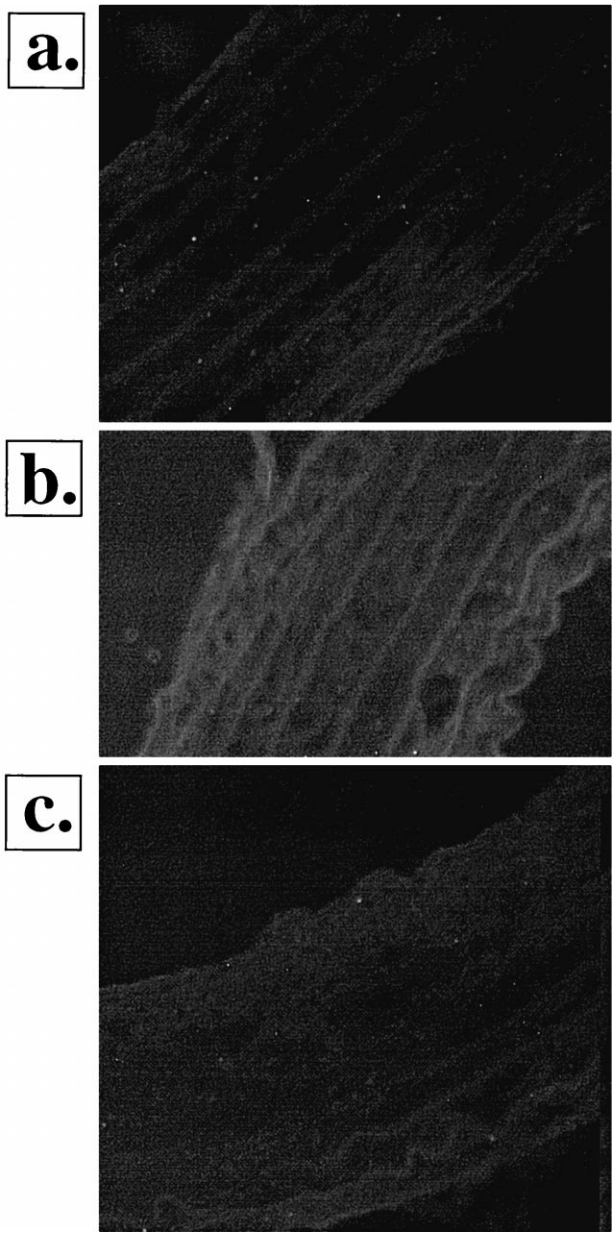


Fig. 2. Representative nitrotyrosine immunofluorescence photomicrographs of rat thoracic aortae. (a) Control rats; (b) rats subjected to haemorrhagic shock; (c) rats subjected to haemorrhagic shock in with mercaptoethylguanidine treatment. Similar results were observed in 3–4 different aortae in each experimental group.

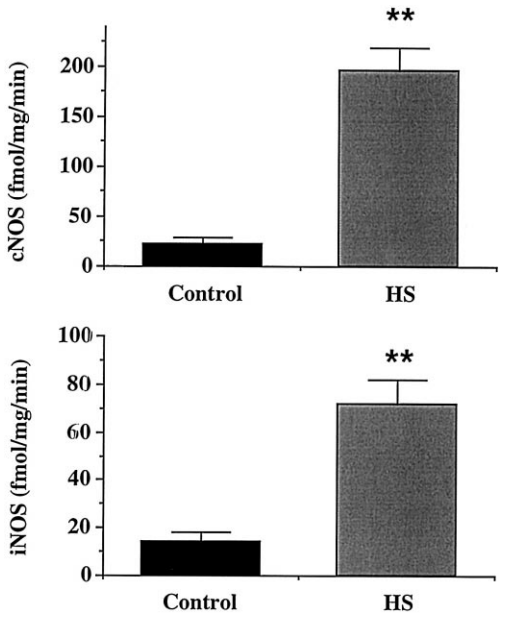


Fig. 3. Constitutive and inducible NO synthase activities (cNOS and iNOS, respectively) in lungs of sham-treated animals, animals subjected to hemorrhagic shock, when compared to sham animals. * *A significant increase in NO synthase activity in response to haemorrhagic shock ($P < 0.01$); $n = 4$.

Similar values in the mercaptoethylguanidine-treated group were $370 \pm 17$ and $386 \pm 11$ bpm, respectively ($n = 4$).

The same infusion of mercaptoethylguanidine did not alter mean arterial blood pressure in control animals, nor was there a significant change in mean arterial blood pressure in vehicle-treated control animals (not subjected to haemorrhage) over 3 h. Mean arterial blood pressure at the beginning of the mercaptoethylguanidine administration was $119 \pm 10$ mmHg and was $123 \pm 6$ mmHg at 3 h after mercaptoethylguanidine administration (i.e., at 6 h after the beginning of the experiments ($n = 4$). The respective values in sham-shocked and vehicle-treated controls were $101 \pm 6$ and $109 \pm 2$ mmHg ($n = 4$), showing no decline in mean arterial blood pressure over the 6 h period.

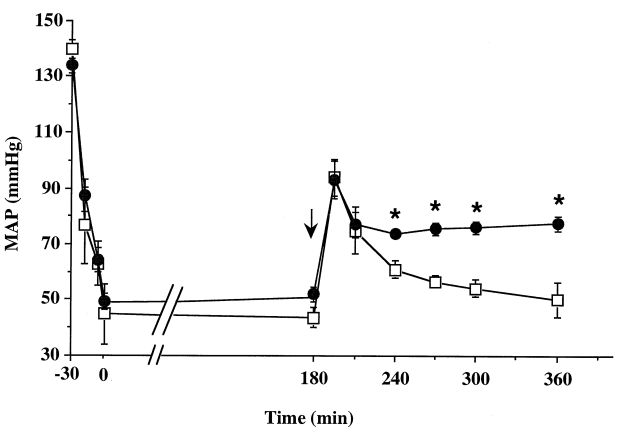


Fig. 4. Mean arterial blood pressure in anesthetized rats subjected to haemorrhagic shock in the presence of vehicle ($n = 5$) or mercaptoethylguanidine (MEG, 10 mg/kg i.v. bolus and 10 mg/kg per h infusion, starting from resuscitation) treatment ($n = 6$). Arrow indicates the time of resuscitation. *A significant difference in the mean arterial blood pressure between vehicle-treated and MEG-treated rats ($P < 0.05$).

### 3.3. Ex vivo vascular contractility

As demonstrated previously (Thiemermann et al., 1993; Zingarelli et al., 1994), haemorrhagic shock resulted in a marked suppression of the contractility of the thoracic aorta ex vivo (Fig. 5). The suppression of the contractility was prevented by in vivo treatment with mercaptoethylguanidine (Fig. 5).

Treatment with mercaptoethylguanidine during haemorrhagic shock did not affect the endothelium-dependent relaxant ability of the thoracic aorta ex vivo (Fig. 6). This finding, and the fact that mercaptoethylguanidine, at the dose used in the present study, failed to elicit a pressor response (see above), indicate that in the experiments described in the current study, mercaptoethylguanidine did not interfere with the activity of the constitutive, endothelial NO synthase (see also: Southan et al., 1996).

### 3.4. Cellular energetic status of peritoneal macrophages

Similar to our recent findings in endotoxic shock (Zingarelli et al., 1996, 1997a), haemorrhagic shock caused a significant increase in the production of nitrite/nitrate

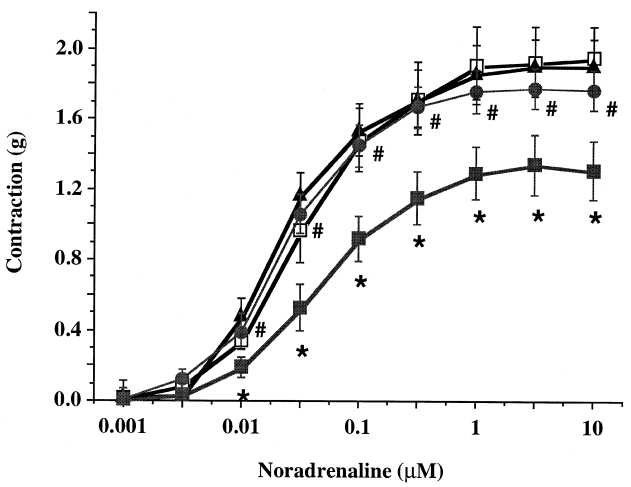


Fig. 5. Effect of haemorrhagic shock on the contractile activity to noradrenaline (1 nM–10 μM) in thoracic aortic rings ex vivo (closed squares) when compared to sham-operated controls (open squares), and the effect of in vivo treatment with mercaptoethylguanidine in sham-shocked animals (closed triangles) and in animals subjected to haemorrhagic shock (closed circles). Haemorrhagic shock significantly decreased contractility at 0.01–10 μM ($^{*}P < 0.05$). $^{\#}P < 0.05$ represents significant protective effect of mercaptoethylguanidine against the haemorrhagic shock-induced vascular hyporeactivity. Data are expressed as means ± S.E.M. of $n = 6$–8 vascular rings.

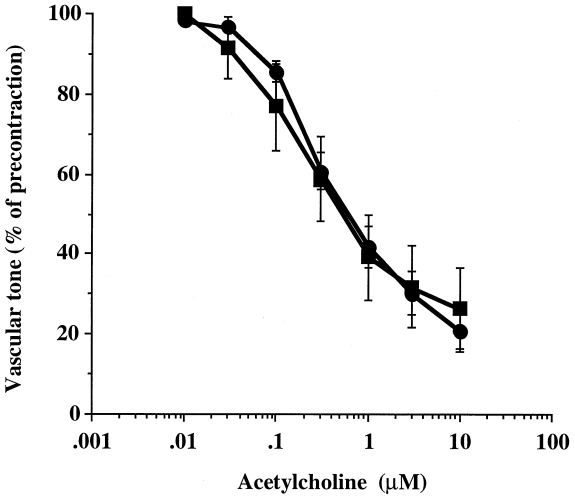


Fig. 6. Lack of effect of mercaptoethylguanidine on the endothelium-dependent relaxations in haemorrhagic shock. Depicted are endothelium-dependent relaxant responses to acetylcholine (0.1 mM–10 μM) in thoracic aortic rings precontracted with noradrenaline (1 μM). Responses in animals subjected to haemorrhagic shock in the presence of vehicle are shown with closed squares, responses in animals subjected to haemorrhagic shock in the presence of in vivo treatment with mercaptoethylguanidine are shown with closed circles. Mercaptoethylguanidine did not affect the endothelium-dependent relaxations in haemorrhagic shock. Data are expressed as means ± S.E.M. of $n = 6–8$ vascular rings.

(Fig. 7a), a significant suppression of the mitochondrial respiration (Fig. 7b) and the intracellular $NAD^+$ content (Fig. 7c) and an increase in the DNA strand breakage (Fig. 7d) in peritoneal macrophages ex vivo. Nitrite/nitrate production was inhibited by in vivo mercaptoethylguanidine treatment (Fig. 7a). In addition, mercaptoethylguanidine caused a significant amelioration of the haemorrhagic shock-induced decrease in the mitochondrial respiration (Fig. 7b), prevented the decrease in the intracellular $NAD^+$ levels (Fig. 7c) and the haemorrhagic shock-induced DNA single strand breakage (Fig. 7d). mercaptoethylguanidine treatment in the absence of haemorrhagic shock, did not significantly alter any of the above parameters ($n = 4$, not shown).

## 4. Discussion

The main findings of the present study are that (i) prolonged periods of haemorrhagic shock induce the expression of the inducible NO synthase, as demonstrated by direct enzyme measurements, increased plasma nitrite/nitrate levels and increased NO production by peritoneal macrophages ex vivo; (ii) haemorrhagic shock induces the formation of peroxynitrite and (iii) mercaptoethylguanidine, a novel NO synthase inhibitor with selectivity towards the inducible isoform (Southan et al., 1996), a scavenger of peroxynitrite (Szabó et al., 1997) and an inhibitor of cyclooxygenase (Zingarelli et al., 1997b), exerts beneficial effects on the haemodynamic alterations, vascular contractility and the cellular energetic status in haemorrhagic shock.

The increase in plasma nitrite/nitrate levels (Fig. 1)

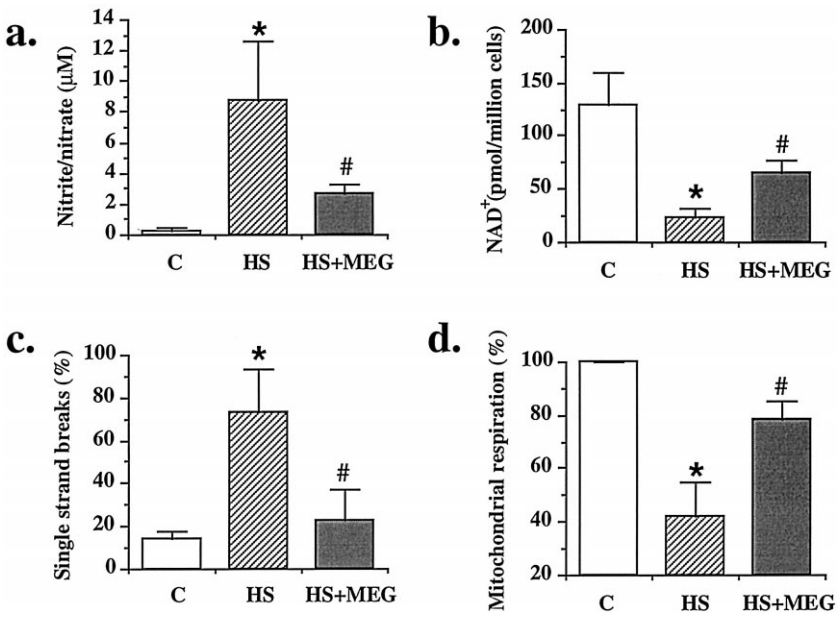


Fig. 7. Effect of administration of mercaptoethylguanidine (MEG) in vivo on cellular energetics in peritoneal macrophages harvested from rats subjected to haemorrhagic shock (HS). Depicted are nitrite/nitrate production (a), intracellular $NAD^+$ content (b) DNA single strand breaks (c) and mitochondrial respiration (d) in peritoneal macrophages obtained from control rats (C), from rats subjected to haemorrhagic shock (HS) and from rats treated with MEG during haemorrhagic shock (HS + MEG). *A significant increase in nitrite/nitrate or single strand breakage or decrease in the respiration or $NAD^+$ content in response to haemorrhagic shock ($P < 0.05$), #significant protective effects of MEG in macrophages from haemorrhaged rats when compared to haemorrhage alone ($P < 0.05$); $n = 5–6$.

was approximately 10–20% of what can be observed, in the same animal species, after intravenous injection of bacterial LPS for 3–6 h (Zingarelli et al., 1996). Thus, it may be somewhat surprising that lower amounts of NO, produced due to a relatively low expression of the inducible NO synthase (Thiemermann et al., 1993), were sufficient to substantially contribute to the observed vascular and cellular energetic changes. It is important to mention in this respect that, in addition to up-regulation of inducible NO synthase, we have also found evidence for an increase in $Ca^{2+}$-dependent (constitutive, presumably endothelial) NO synthase in the tissue homogenates.

The site of the inducible NO synthase induction and NO production is crucial in determining the changes in vascular reactivity. It is possible that in haemorrhagic shock, the circulating nitrite/nitrate does not reflect the level of the up-regulation of NO synthase in all tissues. It is conceivable that expression of NO synthase within the vascular endothelium and the smooth muscle (as demonstrated by functional studies: Thiemermann et al., 1993; Zingarelli et al., 1994 and direct measurements in the current study) importantly contributes to the progressive vasodilatation and vascular decompensation which is characteristic to circulatory shock, without causing a major elevation in circulating nitrite/nitrate levels.

Recent data have questioned the prevailing view that NO is independently cytotoxic. Current studies indicate that the cytotoxic and oxidant potency of NO is, to a large extent, due to peroxynitrite, a cytotoxic oxidant produced by NO and superoxide (Beckman et al., 1990; Crow and Beckman, 1995). In agreement with previous results demonstrating increased peroxynitrite-mediated oxidation of the fluorescent dye dihydrorhodamine 123 in haemorrhagic shock (Szabó et al., 1995b) our present results indeed have confirmed the production of peroxynitrite in haemorrhagic shock (Fig. 2). Similar to the situation in endotoxin shock, where recent data suggest a role of peroxynitrite in the vascular hypocontractility (Szabó et al., 1996a; Zingarelli et al., 1997a), it is conceivable that in the delayed phase of haemorrhagic shock, NO from the inducible NO synthase combines with superoxide to produce peroxynitrite in the vicinity of the blood vessels, which, in turn, contributes to the development of vascular hypocontractility. In line with this proposal, we demonstrated that both the hyporeactivity of the thoracic aorta and the increase in nitrotyrosine immunoreactivity in haemorrhagic shock are prevented by mercaptoethylguanidine.

In endotoxic shock, current data demonstrate that an important pathway of vascular failure and cellular energetic failure is related to peroxynitrite-induced DNA single strand breakage and subsequent activation of the nuclear enzyme poly (ADP-ribose) synthetase, with eventual depletion of cellular energetic stores (Szabó, 1996; Szabó et al., 1996a,b; Zingarelli et al., 1996). The role of poly (ADP-ribose) synthetase in the pathophysiology of haemorrhagic shock remains a subject of further investigation. However, it is of importance in this respect that (i) DNA single strand breakage is an obligatory trigger of poly (ADP-ribose) synthetase activation (for review, see Szabó, 1996) (ii) previous studies have demonstrated the occurrence of DNA injury in tissues obtained from experimental animals subjected to haemorrhagic shock (Lazarus et al., 1984) and (iii) also in our current study, we have observed a marked increase in the percentage of single stranded DNA in the peritoneal macrophages of animals subjected to haemorrhagic shock.

Various forms of shock, including haemorrhagic shock, have previously been shown to be associated with a cellular energetic suppression and a depletion of intracellular energy stores (Wang et al., 1991). We have recently demonstrated that energy depletion and increased DNA single strand breakage in peritoneal macrophages obtained from animals subjected to endotoxic shock (Zingarelli et al., 1996). Similar to these studies, we now present evidence for depletion of $NAD^+$ and increase in DNA single strand breakage in peritoneal macrophages obtained from animals subjected to haemorrhagic shock. Moreover, the finding that the increase in DNA strand breakage and the decrease in cellular $NAD^+$ is prevented by mercaptoethylguanidine treatment during haemorrhagic shock suggests that NO or peroxynitrite is involved in these changes. These data would be in line with a mechanism which involves poly (ADP-ribose) synthetase activation, triggered by peroxynitrite-induced DNA strand breakage, and subsequent $NAD^+$ depletion. Further experiments will address this hypothesis.

Although the haemodynamic and ex vivo functional data reported in the present investigation are largely related to the vascular smooth muscle, we have used peritoneal macrophages, rather than vascular tissues, to investigate energetic alterations and DNA injury. We have recently reported the importance of peroxynitrite formation (Zingarelli et al., 1997a) and poly (ADP-ribose) synthetase activation (Zingarelli et al., 1996) in the energetic changes in peritoneal macrophages during endotoxic shock. In this respect, the current studies in macrophages provide useful direct comparisons with these previous investigations. On the other hand, we acknowledge the fact that energetic changes in the macrophages may not be directly related to the shock-induced changes in vascular function, or in the cellular energetic status of various organs, in general. Clearly, further studies investigating metabolic alterations in various organs are necessary. Our group, indeed, has recently performed such studies in large animal models of haemorrhagic shock, after treatment with the potent NO synthase inhibitor isopropyl-isothiourea (Vromen et al., 1996) or with the ATP-activated potassium channel inhibitor glibenclamide (Salzman et al., 1997). Large animal studies with mercaptoethylguanidine in haemorrhagic shock are currently on-going in our laboratory.

Although the degree of the expression of the inducible

NO synthase in haemorrhagic shock is lower than that observed in endotoxic shock (Szabó and Thiemermann, 1994), the metabolic changes observed in the peritoneal macrophages and the degree of DNA injury are comparable. In this respect, it is noteworthy that oxidants other than NO or peroxynitrite (hydroxyl radical, for example) also contribute to the cellular metabolic derangements associated with shock (Hamano et al., 1993; Tan et al., 1993; Kapoor and Prasad, 1994) and there may be synergistic or additive interactions between various oxidants and free radicals during shock. Moreover, although the expression of the inducible NO synthase is lower, the production of peroxynitrite in haemorrhagic shock is comparable with that in endotoxin shock (Szabó et al., 1995a,b; see also Fig. 2 of the current study). In the presence of superoxide, NO from constitutive NO synthase isoforms can also combine to form peroxynitrite (Szabó, 1996). As discussed previously mercaptoethylguanidine has a free thiol group and has been shown to be a scavenger of oxyradicals and peroxynitrite (Southan et al., 1996; Szabó et al., 1997). Such action may also contribute to the beneficial effect of the compound in the present model of shock. Furthermore, acting as a selective inhibitor of the inducible NO synthase, mercaptoethylguanidine limits the cytotoxicity of peroxynitrite while maintaining the physiological purpose of the constitutive production of NO.

Guanidine compounds, such as mercaptoethylguanidine, also have a third pharmacological action in addition to inhibition of the inducible NO synthase and peroxynitrite scavenging. Mercaptoethylguanidine is a moderately effective direct inhibitor of cyclooxygenase activity, as shown in recent in vitro studies (Zingarelli et al., 1997b). The inhibition by mercaptoethylguanidine of 6-keto-prostaglandin $F_{1\alpha}$ production in haemorrhagic shock can be due to two mechanisms, a direct and an indirect effect. Mercaptoethylguanidine may inhibit cyclooxygenase activity directly (Zingarelli et al., 1997b). In addition, it has been proposed that NO directly increases the catalytic activity of cyclooxygenase as demonstrated in endotoxic shock (Sautebin and Di Rosa, 1994; Seibert and Masferrer, 1994). While there is some indication that inhibition of cyclooxygenase activity is of benefit in haemorrhagic shock (Leffler and Passmore, 1994), we cannot assess the relative contribution of a direct or indirect inhibition of cyclooxygenase activity to the beneficial effects of mercaptoethylguanidine in the current study.

Pretreatment with relatively large concentrations of aminoguanidine, and aminoethyl-isothiourea (a precursor of mercaptoethylguanidine) have recently been shown to inhibit the expression of the inducible isoform of NO synthase in vitro and in vivo (Joshi et al., 1996; Ruetten and Thiemermann, 1996; Sun et al., 1997). We do not believe, however, that an inhibition of the expression of iNOS substantially contributed to the anti-shock effects of mercaptoethylguanidine in the present study for the following reasons: (1) In the present study, the concentration of mercaptoethylguanidine used was substantially lower than in the studies mentioned above. (2) In order to obtain a substantial inhibition of the expression of the inducible isoform of NO synthase, the pharmacological agents generally need to be administered before the onset of the induction process (for example, see Szabó, 1995; Kilbourn et al., 1997). In our current study, however, the start of mercaptoalkylguanidine administration coincided with the beginning of the induction of the inducible NO synthase (Thiemermann et al., 1993), and so it is less likely that it may have affected this process.

There is some evidence that NO itself can inhibit the process of the expression of the inducible isoform of NO synthase (Colasanti et al., 1995). In this regard, inhibition of the activity of NO synthesis in haemorrhagic shock may have resulted in a paradoxical up-regulation of this enzyme. Thus, taken together, we cannot fully exclude the possibility that mercaptoethylguanidine, in addition to inhibiting the activity of the inducible NO synthase, may also have affected the process of the expression of inducible NO synthase. On the other hand, there are no in vitro studies to suggest that mercaptoethylguanidine may also affect the expression of the inducible isoform of cyclooxygenase (Zingarelli et al., 1997b).

Taken together, the present results demonstrate that mercaptoethylguanidine has beneficial effects in haemorrhagic shock. It is noteworthy that in our protocol the agent was given as a post-treatment, rather than pretreatment. The effectiveness of mercaptoethylguanidine, under these conditions, is likely related to the fact that the agent targets late, rather than early mediators of inflammation. The relative contribution of the individual modes of action by mercaptoethylguanidine to the protection observed remains to be dissected in additional studies. The results of the present study support the view that the development of anti-inflammatory agents with multiple modes of action represents a novel, promising approach of experimental therapy.

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