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# Adrenocorticotropin normalizes the blood levels of nitric oxide in hemorrhage-shocked rats

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**Abstract**

Anesthetized rats were subjected to volume-controlled hemorrhagic shock by stepwise bleeding. Besides cardiovascular and respiratory functions, nitric oxide (NO)-hemoglobin formation in arterial blood was directly evaluated by means of electron spin resonance spectroscopy. During hemorrhagic shock there was a massive increase in NO-hemoglobin, associated with a fall in mean arterial pressure, pulse pressure, respiratory rate and heart rate, and there was a further increase in NO-hemoglobin 15 min after intravenous (i.v.) treatment with saline. All rats died within 30 min. The reversal of the shock condition induced by the i.v. injection of the adrenocorticotropin (ACTH) fragment 1–24 (160 μg/kg, 5 min after bleeding termination) was associated with a prompt disappearance of NO-hemoglobin. Also *S*-methylisothiourea (3 mg/kg i.v.), a selective inhibitor of inducible NO synthase, provoked a disappearance of NO-hemoglobin and reversal of the shock condition. The present results provide a direct demonstration that volume-controlled hemorrhagic shock is associated with highly increased blood levels of NO, as indicated by increased NO-hemoglobin, and indicate that ACTH-induced reversal of the shock condition is associated with the normalization of NO blood levels, and a parallel improvement of cardiovascular and respiratory functions. This occurs probably through the inhibition of inducible NO synthase, as suggested by the fact that *S*-methylisothiourea, a selective inhibitor of this NO synthase isoform, produced the same results. © 1997 Elsevier Science B.V.

*Keywords:* Hemorrhagic shock; Nitric oxide (NO); Electron spin resonance; ACTH (adrenocorticotropin); Nitric oxide (NO) synthase, inducible

## 1. Introduction

A large body of experimental data has accumulated (for a review see Bertolini, 1995) showing that, under conditions of volume-controlled hemorrhagic shock in rats and dogs, which invariably causes the death of all untreated animals within 20–30 min, the intravenous (i.v.) bolus injection of a melanocortin peptide (adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), and several other fragments and fragment analogs of the ACTH molecule, including ACTH-(4-10)) in nanomolar amounts (7–54 nmol/kg) dose dependently restores cardiac output, total peripheral resistance, arterial pressure, pulse amplitude, and tissue blood flow (Bertolini et al., 1986a,b,c; Bertolini, 1995). Survival time is greatly prolonged, and the time limit for blood reinfusion still to be effective and afford a definitive cure is greatly extended (Bertolini et al., 1986a, 1989; Guarini et al., 1990b). The temporary but impressive reversal of hemorrhagic shock induced by these peptides is associated with a massive increase in the volume of circulating blood, as a consequence of the mobilization of peripherally pooled residual blood (Guarini et al., 1987, 1988a, 1989; Bertolini et al., 1989). This peculiar effect of melanocortins has not only been observed in rat and dog models of hemorrhagic shock, but has also been confirmed in a condition of hypovolemic shock produced in rabbits by graded occlusion of the inferior vena cava (Ludbrook and Ventura, 1995), as well as in human conditions of hemorrhagic or cardiogenic shock (Bertolini et al., 1987; Pinelli et al., 1989; Noera et al., 1989, 1991). The complex mechanism of action (Guarini et al., 1986, 1990a, 1992, 1993; Bertolini et al., 1989; Bazzani et al., 1992, 1994) does not involve

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the adrenal glands, because shock reversal is also induced by ACTH fragments practically devoid of corticotropic activity and is obtained in adrenalectomized animals as well (Bertolini et al., 1986a, 1989); however, sympathetic activity is apparently involved and can not be ruled out (Guarini et al., 1988b).

Increasing experimental evidence indicates that nitric oxide (NO) overproduction plays an important role in the pathophysiology of shock (Kilbourn and Griffith, 1992; Zingarelli et al., 1992). NO has been said to be responsible for the hemodynamic decompensation and the vascular hyporeactivity to vasoconstrictor agents that occur after massive hemorrhage and during septic shock (Zingarelli et al., 1992; Thiemermann et al., 1993). However, therapeutic approaches designed to modulate the biosynthesis of NO with the aim to improve hemodynamics and survival in animal models have yielded contradictory results (Thiemermann, 1995).

So far, evidence concerning the role of NO in hemorrhagic shock is scant. NO formation was demonstrated in the brain cortex of rats subjected to bilateral carotid occlusion combined with hemorrhagic hypotension (Sato et al., 1993) and in a less severe model of pressure-controlled hemorrhagic shock (Westenberger et al., 1990).

A method for NO measurement in blood that takes advantage of the specific reactivity of NO with heme has been developed (Murphy and Noack, 1994). NO binds strongly to hemoglobin, and if the NO-hemoglobin complex is subjected to a magnetic field and microwave radiation, a characteristic electron spin resonance (ESR) spectrum is obtained. The spectrum is highly specific and its amplitude can be used for quantitative determination (Vanin et al., 1975). The method has been further developed and used to measure NO-hemoglobin in the blood. The rapid reaction of NO with hemoglobin may also give rise to methemoglobin, which can also be detected by ESR spectroscopy. Both reactions have been exploited for the measurement of NO formation in vivo (Kohno et al., 1995; Kozlov et al., 1996). The method is highly sensitive and does not require any sample manipulation (Kozlov et al., 1996).

In this paper we investigated (i) the effect of hemorrhagic shock on NO production, measured as NO-hemoglobin, and (ii) the effect of ACTH treatment on NO blood levels during hemorrhagic shock.

## 2. Methods

### 2.1. Animals and surgery

Adult Wistar rats of either sex (Morini, S. Polo d'Enza, Reggio nell'Emilia, Italy), weighing 250–280 g, were used. Food and water were continuously available, and the rats were kept in temperature- and humidity-controlled colony rooms on a natural light–dark cycle. Housing conditions and experiments were in strict accordance with the European Community regulations.

The experiments were performed under general anesthesia (urethane, 1.25 g/kg, intraperitoneally). Urethane (Fluka, Buchs, Switzerland) was used because it produces long-lasting and stable general anesthesia with only minor interference with cardiovascular regulatory functions (Maggi and Meli, 1986).

After heparinization (heparin sodium, 600 i.u./kg i.v.; Prodotti Gianni, Milan, Italy) and clean dissection, polyethylene catheters were inserted into a common carotid artery and into an iliac vein. Systemic arterial pressure and pulse pressure were recorded by means of a pressure transducer (P23 Db, Statham, Oxnard, CA, USA) coupled to a polygraph (Battaglia-Rangoni, Bologna, Italy). Heart rate was automatically calculated from the pulse wave by the same polygraph. The respiratory rate was recorded by means of three electrodes subcutaneously implanted in the chest and connected to the polygraph through an ARI A380 preamplifier (Battaglia-Rangoni). Volume-controlled hemorrhagic shock was produced by stepwise bleeding from the venous catheter over a period of 25–30 min until mean arterial pressure, which was automatically calculated and continuously digitally displayed by the polygraph, decreased to, and stabilized at, 21–23 mmHg. The total bleeding volume was $2.19 \pm 0.21$ ml/100 g body weight ($n = 95$; mean values $\pm$ S.E.M.).

### 2.2. Drugs and treatments

ACTH-(1–24) (Ciba-Geigy, Basel, Switzerland), chosen as being the most effective melanocortin in the treatment of hemorrhagic shock (Bertolini et al., 1986c), and *S*-methylisothiourea sulfate (Sigma, St. Louis, MO, USA), chosen as being a selective inhibitor of inducible NO synthase (Szabò et al., 1994; Southan et al., 1995) as well as being effective in reversing hemorrhagic shock (Bazzani et al., 1997), were freshly dissolved in saline. They were i.v. bolus-injected five minutes after bleeding termination, when mean arterial pressure was stabilized at 21–23 mmHg, at the maximally effective anti-shock dose of 160 μg/kg (Bertolini et al., 1989) and 3 mg/kg (Bazzani et al., 1997), respectively, in a volume of 1 ml/kg. Control rats received an equal volume saline by the same route. Animals were continuously monitored for 2 h after treatment, or until death.

### 2.3. Blood sampling and ESR measurement of NO

Each animal had 1 ml of blood rapidly withdrawn via the arterial catheter; only one sample was taken from each rat. Blood was withdrawn at the following times: before starting bleeding; 5 min after bleeding termination; 15, 30, 60 and 120 min after treatment or until death. The samples were immediately centrifuged ($1680 \times g$ for 10 min) and the supernatant was discarded. 300 μl of red blood cells

were pipetted into a purpose built Teflon tube (3 mm internal diameter, 20 mm long) and frozen in liquid nitrogen. The frozen cylinder of packed red blood cells was gently driven out of the Teflon tube and stored in liquid nitrogen.

A X-band ESR Bruker 300 ESP (Bruker Spectrospin, Karlsruhe, Germany) was used. The instrument settings were: microwave power 2.5 mW; modulation amplitude 0.43 mT; scan width 100 mT. The spectrum was accumulated electronically in order to obtain a low noise-to-signal ratio and to perform double integration for the quantitative estimation of the species (nmol NO-hemoglobin). Readings were taken at the temperature of liquid nitrogen. Both NO-hemoglobin and methemoglobin show characteristic features which have been described in detail in the literature (Bennet et al., 1955; Rein et al., 1972).

### 2.4. NO-hemoglobin calibration curve

Oxygen was completely removed from ultrapure water by using oxygen-free nitrogen. The solution was then bubbled with NO gas for 10 min and kept at 2°C. Taking into account that at 760 mmHg 0.00618 g of NO dissolves in 100 g of water, NO-saturated water was taken up into a syringe, from which oxygen was removed, and diluted to the desired NO concentration by mixing with oxygen-free water. The NO solution was added to oxygen-free 3 mM hemoglobin in Ringer phosphate pH 6. The obtained spectra were double integrated, the concentration ranging from 0 to 250 nmol nitrosyl-hemoglobin/ml. The limit of detection was below 5 nmol/ml (Kozlov et al., 1996).

### 2.5. Statistical analysis

Mean arterial pressure, pulse pressure, heart rate and respiratory rate were compared by means of Student's *t*-test for paired or unpaired data. Survival rates were analyzed by Fisher's exact probability test. ESR spectra of NO-hemoglobin adduct in arterial blood were subjected to a first integration to obtain a signal in absorbance, and to a second integration to obtain the area below the curve. The values were expressed in nmol NO-hemoglobin on the basis of the calibration curve, and normalized to a fixed sample volume of 1 ml of red cells. Because of the results obtained, statistical assessment was not meaningful.

## 3. Results

As repeatedly previously described (Bertolini et al., 1986a,b,c, 1989; Guarini et al., 1990b; Bertolini, 1995), in rats bled to a pre-terminal condition of hemorrhagic shock, the i.v. bolus injection of ACTH-(1–24) at the dose of 160 μg/kg, 5 min after bleeding termination, produced an almost complete restoration of cardiovascular and respiratory functions (Fig. 1). Fifteen min after treatment, mean

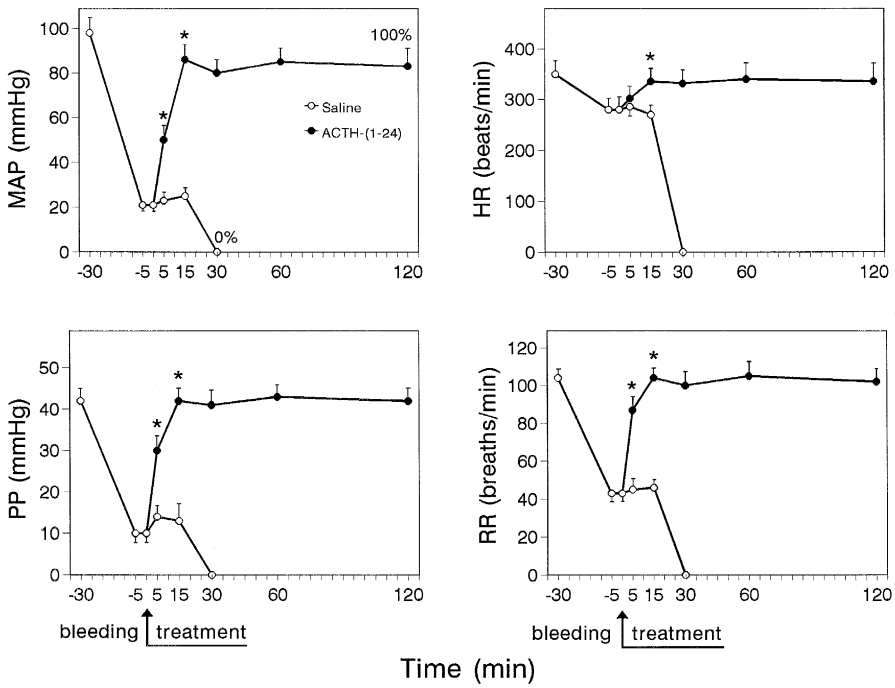


Fig. 1. Influence of the i.v. injection of ACTH-(1–24) (160 μg/kg) or saline (1 ml/kg) on mean arterial pressure (MAP), pulse pressure (PP), heart rate (HR) and respiratory rate (RR) in hemorrhage-shocked rats. Mean values ± S.E.M. for eight animals per group. Treatment = bolus injection of ACTH-(1–24) or saline. * $P < 0.001$ versus the corresponding value of saline-treated rats (Student's *t*-test for unpaired data). The percentage of animals surviving (at the end of lines) 2 h after treatment with ACTH-(1-24) was statistically different from that of saline-treated controls ($P < 0.005$, Fisher's test).

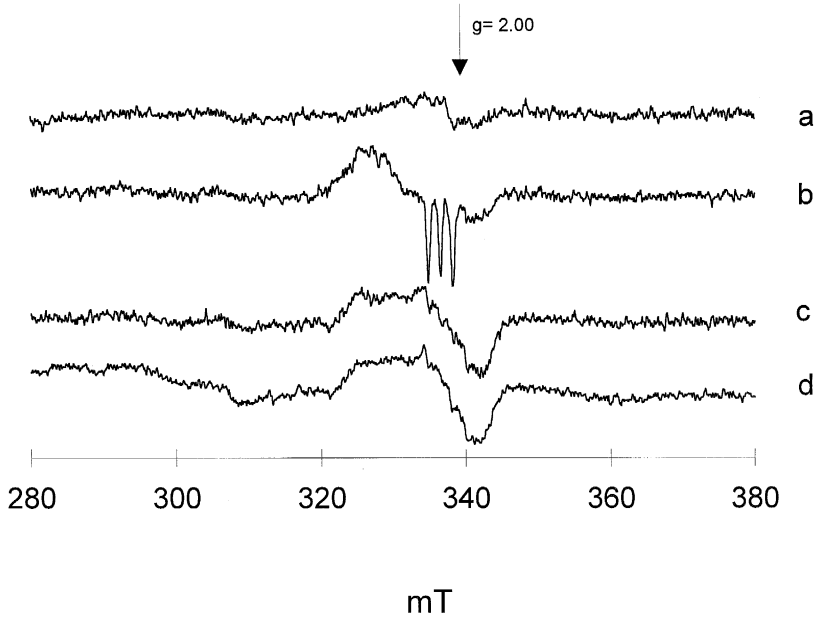


Fig. 2. Representative ESR spectra of NO-hemoglobin adduct in rat blood. *g* is a dimensionless constant denominated electron *g* factor, and mT (milliTesla) indicates the magnetic field intensity. (a) basal, pre-bleeding condition: the spectrum shows no significant absorption but a small peak at *g* about 2.00 (see arrow). This feature can be assigned to naturally occurring free radicals present in the sample; a *g* value around 2 is typical of organic free radicals, such as flavin or ubiquinone. (b) after bleeding, shock condition: the spectrum is characterized by a well-defined triplet, which has been assigned to NO-hemoglobin. (c) 15 min after i.v. treatment with ACTH-(1–24) (160 μg/kg): the broad and featureless absorption spectrum may be assigned to NO-hemoglobin below the detection limit. (d) 15 min after i.v. treatment with *S*-methylisothiourea sulfate (3 mg/kg): the broad and featureless absorption spectrum may be assigned to NO-hemoglobin below the detection limit.

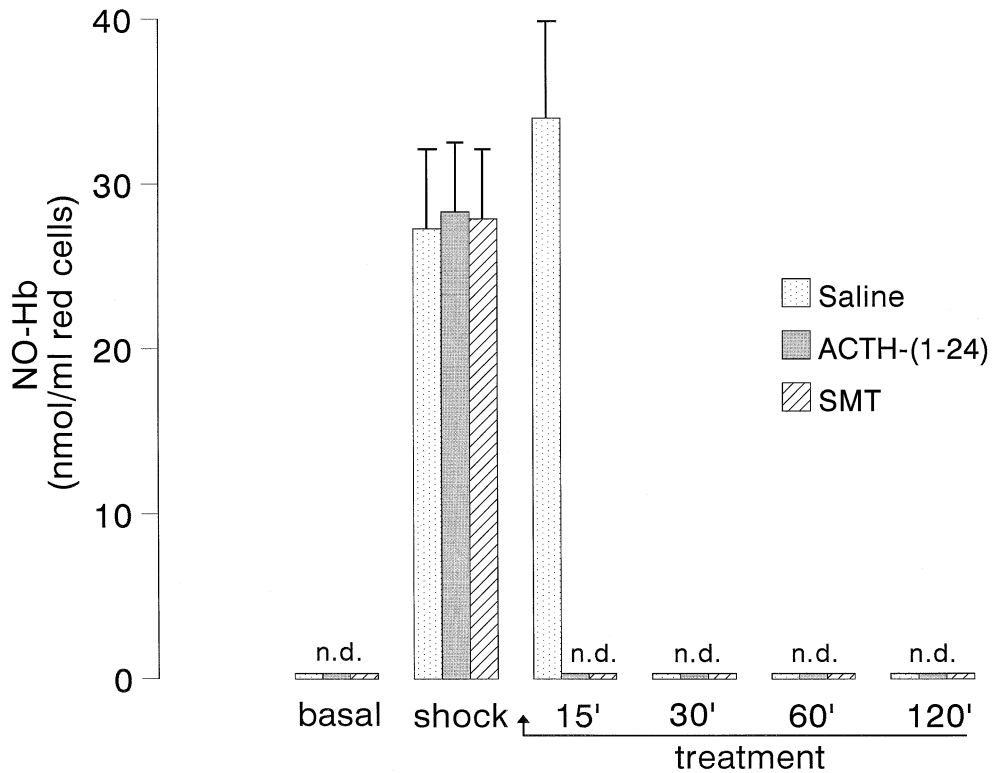


Fig. 3. Time-course of the changes in NO-hemoglobin adduct (NO–Hb) concentration in the blood of hemorrhage-shocked rats before and after i.v. treatment with saline (1 ml/kg), ACTH-(1–24) (160 μg/kg) or *S*-methylisothiourea sulfate (SMT, 3 mg/kg). Mean values ± S.E.M. for 6–8 animals per group. n.d. = not detectable, either because below the lower limit of method sensitivity or because all saline-treated rats died within 30 min. Statistical assessment was obviously not meaningful.

arterial pressure, pulse pressure, heart rate and respiratory rate were not significantly different from baseline, pre-bleeding values and this condition remained quite stable throughout the 2 h observation period ($P > 0.05$, Student's *t*-test for paired data). All hemorrhage-shocked controls died within 30 min after saline injection.

Blood obtained from control, not-bled rats did not show any detectable NO-hemoglobin signal (Fig. 2a). The small signal in the $g = 2.00$ region (arrow) is attributed to physiological concentration of free radical species. The NO-hemoglobin signal rapidly and massively increased during hemorrhagic shock (Fig. 2b), the spectrum showing a characteristic triplet feature, whose hyperfine structure is well evident. No signal was recorded in the methemoglobin absorption area (data not shown).

The reversal of the shock condition induced by the i.v. injection of ACTH-(1–24) was associated with the disappearance of NO in circulating blood. With our method and under our experimental conditions, NO was undetectable 15 min after ACTH injection (Fig. 2c) and remained undetectable throughout the 2 h observation period (Fig. 3).

Treatment with *S*-methylisothiourea, a selective inhibitor of the inducible isoform of NO synthase (Szabò et al., 1994; Southan et al., 1995), at the dose maximally effective in reversing septic and hemorrhagic shock in rats (Szabò et al., 1994; Bazzani et al., 1997), reduced NO blood concentrations to undetectable levels throughout the observation period (Fig. 2d and Fig. 3) and improved survival, which was 100% at the end of the 2 h observation period.

## 4. Discussion

It has been repeatedly suggested that NO plays an important role in the pathophysiology and evolution of shock (Harbrecht et al., 1992; Zingarelli et al., 1992; Thiemermann et al., 1993). This is indirectly supported, for example, by the finding that *N*-nitro-L-arginine methyl ester (L-NAME) — an inhibitor of NO production from L-arginine — injected i.v. into rats subjected to experimental hemorrhagic shock increases survival rate and time, improves blood pressure and protects against the gastric lesions induced by shock (Zingarelli et al., 1992). The central nervous system production of NO may also play a role in hemorrhagic shock, since NO production has been shown to increase in the brain of rats following hypotensive hemorrhage, and this effect can be inhibited by i.v. administration of L-NAME (Sato et al., 1993). Moreover, intracerebroventricular but not i.v. administration of L-NAME is able to prevent the onset of the decompensatory phase of acute hypovolemia in conscious rabbits (Ludbrook and Ventura, 1995). This suggests the involvement of central rather than peripheral nitrergic mechanisms in this shock model.

Our ex vivo data demonstrate the formation of a massive amount of NO, as detected by its reaction product NO-hemoglobin, during the early onset of hemorrhagic shock. NO-hemoglobin faithfully reflects the level of circulating NO. Nitric oxide has a short life-time ($t_{1/2} = 3.3$ s in a biological environment) and cannot diffuse far from the place where it was formed. In contrast, nitrosyl complexes have a long life time and are present for a long time in circulating blood ($t_{1/2} = 3$–$20$ h) (Wennmalm et al., 1990; Henry et al., 1993). The reaction between hemoglobin and NO, yielding either methemoglobin (and nitrate) or nitrosyl–hemoglobin, is diffusion limited, the different yield of the two products strictly depending on the oxygen concentration (Doyle and Hoekstra, 1981). We did not find evidence of an increase in methemoglobin in our experiments, probably because of the high activity of methemoglobin reductase, and/or the hypoxic conditions, which favor NO-hemoglobin formation.

The cardiovascular response to acute hemorrhage consists of two phases. Initially, systemic vascular conductance falls as blood volume and cardiac output fall, so that arterial pressure is well maintained (Schadt and Gaddis, 1986). When the acute blood loss exceeds 30% of blood volume, the compensatory vasoconstriction fails and blood pressure falls abruptly (Ludbrook and Rutter, 1988; Schadt, 1989), and there is a major production of tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (Ertel et al., 1991; Zingarelli et al., 1994). This massive NO production is most likely stimulated by TNF-$\alpha$.

The mechanism of action of ACTH (and of melanocortins in general) in reversing a condition of otherwise rapidly fatal hemorrhagic shock is complex and involves central and peripheral components and steps (Bertolini, 1995), the latter probably playing a pronounced role. On the basis of our results, it is possible that an important component of the anti-shock effect of ACTH (and hence, in all probability of melanocortins in general) consists of the abolition of the overproduction of NO. This would restore the vascular response to endogenous vasoconstrictor agents, including norepinephrine and endothelin.

The ability of *S*-methylisothiourea, a selective inhibitor of the inducible isoform of NO synthase (Szabò et al., 1994; Southan et al., 1995), to improve cardiovascular and respiratory functions and survival (Bazzani et al., 1997), as well as to reduce dramatically NO blood levels in hemorrhage-shocked rats, suggests that this isoform is involved in our model of volume-controlled hemorrhagic shock.

In conclusion, these results provide a direct demonstration that volume-controlled hemorrhagic shock is associated with greatly increased blood levels of NO, and indicate that ACTH-induced reversal of the shock condition is associated with normalization of NO blood levels. On the basis of our previous (Bazzani et al., 1997) and present data, we hypothesize that inhibition of inducible NO synthase is involved.

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