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Influence of ACTH-(1-24) on free radical levels in the blood of haemorrhage-shocked rats: direct *ex vivo* detection by electron spin resonance spectrometry

[1]Salvatore Guarini, Carla Bazzani, Guido Mattera Ricigliano, *Anna Bini, *Aldo Tomasi & Alfio Bertolini

Department of Biomedical Sciences, Sections of Pharmacology and *General Pathology, University of Modena, v.G.Campi 287, 41100 Modena, Italy

1 The influence of ACTH-(1-24) on the blood levels of highly reactive free radicals in haemorrhagic shock was studied in rats.

2 Volume-controlled haemorrhagic shock was produced in adult rats under general anaesthesia (urethane, 1.25 g kg^{-1} intraperitoneally) by stepwise bleeding until mean arterial pressure stabilized at 20-23 mmHg. Rats were intravenously (i.v.) treated with either ACTH-(1-24) (160 $\mu g\ kg^{-1}$ in a volume of 1 ml kg^{-1}) or equivolume saline. Free radicals were measured in arterial blood by electron spin resonance spectrometry using an *ex vivo* method that avoids injection of the spin-trapping agent (α-phenyl-N-tert-butylnitrone).

3 Blood levels of free radicals were 6490 ± 273 [arbitrary units (a.u.) ml^{-1} whole blood, before starting bleeding, and 30762 ± 2650 after bleeding termination (means ± s.e.mean of the values obtained in all experimental groups). All rats treated with saline died within 30 min, their blood levels of free radicals being 35450 ± 5450 a.u. ml^{-1} blood, 15 min after treatment. Treatment with ACTH-(1-24) produced a rapid and sustained restoration of arterial pressure, pulse pressure, heart rate and respiratory function, with 100% survival at the end of the observation period (2 h); this was associated with an impressive reduction in the blood levels of free radicals, that were 12807 ± 2995, 10462 ± 2850, 12294 ± 4120, and 10360 ± 2080 a.u. ml^{-1} blood, 15, 30, 60 and 120 min after ACTH-(1-24) administration, respectively.

4 These results provide a direct demonstration that (i) in haemorrhagic shock there is a rapid and massive production of highly reactive free radicals, and that (ii) the sustained restoration of cardiovascular and respiratory functions induced by the i.v. injection of ACTH-(1-24) is associated with a substantial reduction of free radical blood levels. It is suggested that ACTH-(1-24) prevents the burst of free radical generation during blood mobilisation and subsequent tissue reperfusion, and this may be an important component of its mechanism of action in effectively preventing death for haemorrhagic shock.

Keywords: Haemorrhagic shock; free radicals; ACTH; spin-trapping

Introduction

In an experimental model of volume-controlled haemorrhagic shock in rats and dogs, which causes death of all untreated animals within 20-30 min (Bertolini *et al.*, 1986a, b, c), the intravenous (i.v.) bolus injection of very small amounts of a melanocortin peptide [adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), and other fragments or fragment analogues of the ACTH molecule] (7-54 nmol kg^{-1}, i.e. 20-160 $\mu g\ kg^{-1}$) induces within a few minutes a dose-dependent restoration of cardiac output, total peripheral vascular resistance index, arterial pressure, pulse amplitude, and tissue blood flow, with gradual normalisation of arterial and venous pH and base excess (BE), as well as of venous tension of O_2 (PO_2) and CO_2 (PCO_2) and venous oxygen saturation (SO_2) and lactate (Bertolini *et al.*, 1986a,b,c; 1989; Guarini *et al.*, 1989; Bazzani *et al.*, 1992; Bertolini, 1995). The effect of melanocortins in haemorrhagic shock is adrenal-independent, as it is also produced by ACTH fragments, which have no, or negligible, corticotropic activity (Bertolini *et al.*, 1986b), as well as in adrenalectomized animals (Bertolini *et al.*, 1986a). The survival time of haemorrhage-shocked animals treated with a melanocortin is greatly increased: with the maximum effective dose (160 $\mu g\ kg^{-1}$) of the most potent melanocortin [ACTH-(1-24)] (Bertolini *et al.*, 1986b; 1989) the mean survival time is extended to 44 ± 18 h (range 15 to 312 h; $n = 18$) (mean survival time in saline-treated animals: 26 ± 1 min; $n = 20$) (Bertolini *et al.*, 1989; Guarini *et al.*, 1990). The temporary but impressive reversal of haemorrhagic shock induced by these peptides is associated with a massive increase in the volume of circulating blood (Guarini *et al.*, 1987; 1989; Bertolini *et al.*, 1989), which seems to be the consequence of a mobilisation of the peripherally-pooled residual blood. Indeed, the anti-shock effect of these peptides is greatly impaired in animals deprived of blood reservoirs (splenectomized animals; animals subjected to ligature of the suprahepatic veins) (Guarini *et al.*, 1987; 1988; Bertolini *et al.*, 1989). The restoration of the blood flow in vital organs (Guarini *et al.*, 1989) greatly extends the time-limit for an effective and curing blood reinfusion. For instance, in our model of haemorrhagic shock, while all rats reinfused with their own shed blood at 15 min after haemorrhage die within 6.6 ± 4.4 h (Bertolini *et al.*, 1989; Guarini *et al.*, 1990), a substantial number of haemorrhage-shocked rats treated with a melanocortin peptide [ACTH-(1-24)] shortly (within 5 min) after bleeding survive, even if blood reinfusion is performed 30, 60 or 120 min later (Bertolini *et al.*, 1989; Guarini *et al.*, 1990). This effect of melanocortins has also been confirmed in a model of hypovolaemic shock produced in rabbits by the graded occlusion of the inferior vena cava (Ludbrook & Ventura, 1995), as well as in human subjects with haemorrhagic or cardiogenic shock (Bertolini *et al.*, 1987; Pinelli *et al.*, 1989; Noera *et al.*, 1989; 1991).

There is good evidence that oxygen-derived free radicals play a major role as mediators of damage to the cell membrane

[1] Author for correspondence.

during haemorrhagic shock, as well as in the pathophysiology of injuries caused by reperfusion of ischaemic tissues (Gardner *et al.*, 1983; McCord, 1985; Granger *et al.*, 1986; Weisiger, 1986; von Ritter *et al.*, 1988; Bitterman *et al.*, 1988; Friedl *et al.*, 1989; Lieners *et al.*, 1989; Marzi *et al.*, 1990; Redl *et al.*, 1993).

The impaired antioxidant capacity of poorly-perfused tissue may contribute to increased cell-membrane permeability and organ dysfunction (Gardner *et al.*, 1983; McCord, 1983; 1985; Weisiger, 1986; Schlag *et al.*, 1991). Moreover, it may be assumed that the restoration of blood flow with consequent reoxygenation of ischaemic tissues produces a burst of free radical discharge, exacerbating the ischaemic injury (Horton & Borman, 1987).

Using a method that directly measures, by electron spin resonance (E.S.R.) spectrometry, the levels of highly reactive free radicals in the blood, we have now investigated (i) the effect of our model of volume-controlled haemorrhagic shock on free radical production, and (ii) whether the reversal of shock by melanocortins is associated with a reduced production of free radicals.

Methods

Animals and surgery

Adult female Wistar rats (Morini, S.Polo d'Enza, Reggio nell'Emilia, Italy), weighing 230–260 g, were used. They were kept five per cage, with food and water available *ad libitum*, in colony rooms (temperature 21±1°C; humidity 60%) on a natural light-dark cycle. Housing conditions and experiments were in strict accordance with the European Community regulations on the care and use of animals for scientific purposes (CEE Council 86/609, and D.L. 27/01/92, no.116). The animals were acclimatized to our housing conditions for at least one week before being used. The experiments were performed under urethane anaesthesia (1.25 g kg^{-1}, i.p.). Urethane (Fluka AG, Buchs, Switzerland) was chosen because it provides long-lasting and stable general anaesthesia with minimal interference with cardiovascular regulatory functions (Maggi & Meli, 1986a,b).

After heparinization (heparin sodium; 600 iu kg^{-1}, i.v.) and dissection, polyethylene catheters were inserted into a common carotid artery and into an iliac vein. Systemic arterial pressure and pulse pressure (PP) were recorded by means of a pressure transducer (P23 Db, Statham, Oxnard, CA, U.S.A.) coupled to a polygraph (Battaglia-Rangoni, Bologna, Italy). Heart rate (HR) was automatically calculated from the pulse wave by the same polygraph. Respiratory rate (RR) was recorded by means of three electrodes subcutaneously implanted on the chest and connected to the polygraph through an ARI A380 preamplifier (Battaglia-Rangoni, Bologna, Italy).

Volume-controlled haemorrhagic shock was induced by stepwise bleeding from the venous catheter over a period of 25–30 min until mean arterial pressure (MAP), automatically calculated and continuously digitally displayed by the polygraph, decreased to and stabilized at 20–23 mmHg. The total bleeding volume was 2.24±0.19 ml 100 g^{-1} body wt (overall mean±s.e. mean from all rats subjected to bleeding; $n=132$; the volume was similar for each experimental group, ranging from 2.19±0.21 to 2.29±0.18, $P>0.05$, ANOVA).

Drug and treatment

ACTH-(1-24) (Ciba-Geigy, Basel, Switzerland) was chosen as the most effective melanocortin in the treatment of haemorrhagic shock, on the basis of previous structure-activity studies (Bertolini *et al.*, 1986b; 1989). It was freshly dissolved in saline and injected as i.v. bolus (160 μg kg^{-1} in 1 ml kg^{-1} of saline) 5 min after the terminations of the haemorrhage, with MAP stabilized at 20–23 mmHg (Bertolini *et al.*, 1989). Control animals received equal volumes of saline. Other animals were reinfused with their own shed blood. Animals were continuously monitored for 120 min after treatment, or until death (e.g. treatment with saline or killing after withdrawal of blood for free radical detection).

Blood sampling

A technique modified from Tortolani *et al.* (1993) was employed in order to avoid the injection of the spin-trapping agent *in vivo*. Each animal had 3–4 ml of whole blood rapidly withdrawn via the arterial catheter into a syringe containing 2 ml of a 0.1 M solution of α-phenyl-N-tert-butylnitrone (PBN; Sigma Chemical Co., St. Louis, MO, U.S.A.) in isotonic saline. Each animal served for a single sample.

Extraction of radical species and E.S.R. spectra determination

The samples were immediately centrifuged (1680 *g* for 10 min) and the plasma/PBN supernatant was added to 12 ml of 2:1 (v/v) chloroform/methanol for radical extraction. The chloroform layer was separated, dried under nitrogen flow, the resulting pellet was resuspended in 250 μl chloroform and the E.S.R. spectrum was taken. E.S.R. spectra were recorded at room temperature using a Bruker 300 E.S.R. spectrometer (Bruker Spectrospin, Karlsruhe, Germany); typical instrumental settings were: microwave power, 20 mW; modulation; amplitude, 1 G field width, 100 G; and microwave frequency, 9.14 GHz. In order to test the possible scavenging activity of ACTH-(1-24), an *in vitro* chemical system producing highly reactive carbon and oxygen centred radicals was prepared, the radicals were trapped by PBN. *Tert*-butyl hydroperoxide (1 mM) was decomposed by ferrous ions (5 μM) and PBN (25 mM) was added as spin trapping agent; the samples were processed as above for E.S.R. determination. The same experiment was carried in the presence of ACTH-(1-24) (13.5 nM) added as hypothetical competitor in the reaction mixture. As the spectral line shape was identical between samples, we measured the E.S.R. peak height of the central absorption, expressed in arbitrary units (a.u.), as a direct function of adduct concentration; for statistical analysis, the values (a.u.) were normalised to a fixed sample volume of 1 ml of whole blood.

Experimental protocol for free radical assessment

Animals were randomly assigned to one of the following experimental protocols: (1) anaesthesia, bleeding to haemorrhagic shock, withdrawal of the blood sample for free radical measurement; (2) anaesthesia, bleeding to haemorrhagic shock, treatment with ACTH-(1-24) 5 min after bleeding termination, withdrawal of the blood sample for free radical measurement 15 min after treatment; (3) as for group 2, but withdrawal of the blood sample 30 min after treatment; (4) as for group 2, but withdrawal of the blood sample 60 min after treatment; (5) as for group 2, but withdrawal of the blood sample 120 min after treatment; (6) anaesthesia, bleeding to haemorrhagic shock, treatment with saline 5 min after bleeding termination, withdrawal of the blood sample 15 min after treatment (all rats of this group die within 30 min after bleeding termination, so that no blood sampling at other times is possible); (7) anaesthesia, bleeding to haemorrhagic shock, gradual (during 10 min) reinfusion of the shed blood starting 5 min after bleeding termination, withdrawal of the blood sample 15 min after reinfusion termination; (8) as for group 7, but withdrawal of the blood sample 30 min after reinfusion; (9) as for group 7, but withdrawal of the blood sample 60 min after reinfusion; (10) as for group 7, but withdrawal of the blood sample 120 min after reinfusion; (11) anaesthesia, no bleeding, withdrawal of the blood sample 60 min after anaesthesia.

Statistics

MAP, PP, HR and RR values, total bleeding volume as well as free radical levels in arterial blood were analysed by means of ANOVA followed by Student-Newman-Keuls test. When necessary, Student's *t* test for paired data was also used. Survival rates were analysed by Fisher's exact probability test.

Results

The baseline values of the recorded parameters (MAP, PP, HR, RR) were not significantly different in any of the experimental groups. The acute and severe hypovolaemia induced in our model of volume-controlled haemorrhagic shock in anaesthetized rats was incompatible with survival, and, hence, all saline-treated animals died within 30 min after saline injection. The i.v. bolus injection of ACTH-(1-24), at 5 min after the termination of haemorrhage (160 $\mu g\ kg^{-1}$) produced, within a few minutes, an almost complete restoration of cardiovascular and respiratory functions: indeed, 15 min after treatment, MAP and RR values were not significantly different from baseline (Figure 1); PP and HR values (not shown) were also reversed: 43 ± 5 mmHg and 380 ± 16 beats min^{-1} before bleeding, 10 ± 1 mmHg and 316 ± 10 beats min^{-1} after bleeding, 44 ± 4 mmHg and 362 ± 12 beats min^{-1} 15 min after treatment, respectively ($P > 0.05$; Student's *t* test for paired data). Although no other treatment was given, all ACTH-treated rats were still surviving at the end of the observation period (2 h after treatment) with values for cardiovascular and respiratory parameters not significantly different from baseline (e.g., pre-haemorrhage) ($P > 0.05$; Student's *t* test for paired data). Similar results were obtained with the reinfusion of the shed blood, performed 5 min after bleeding termination.

Using an *ex vivo* spin-trapping technique to measure radicals *in vivo*, we have demonstrated here that haemorrhagic shock results in a substantial increase in the blood levels of free radicals (PBN-adduct signal intensity about five fold greater than that of basal, pre-bleeding condition) (Figure 2, Table 1). The representative E.S.R. spectra depicted had splitting constants $a_N = 14.84$ and $a_H = 3.32$ which are consistent with the trapping of a carbon-centred radical adduct (PBN-RC·) (Buettner, 1987). However, E.S.R. features displayed a relatively motionally-restricted spectrum, indicating the trapping of a bulky radical such as a primary or secondary protein radical, arising from β-scission or an intramolecular rearrangement of alkoxy radicals (Davies *et al.*, 1991).

Treatment with ACTH-(1-24), 5 min after induction of shock, produced an impressive reduction of free radical levels measured 15 min after treatment (Figure 2, Table 1); the levels remained quite stable and were not significantly different from baseline, throughout the 2 h observation period. Similar results were obtained in rats reinfused with their own shed blood 5 min after shock induction (Figure 1, Table 1). In shocked rats treated with saline, on the other hand, there was a further increase in free radical levels, as assessed 15 min after treatment (Table 1).

Finally, ACTH-(1-24) itself, did not show any radical scavenging ability (data not shown).

Discussion

Severe haemorrhagic shock may be viewed as whole body ischaemia, and is characterized by inadequate perfusion of tissues, altered cell membrane permeability and subsequent cellular dysfunction (Baue *et al.*, 1971; 1973; Horton & Tuggle, 1983). A large body of indirect evidence suggests that an increased production of oxygen-derived free radicals mediates damage to the cell membrane during haemorrhagic shock (Horton & Borman, 1987; Lee *et al.*, 1987; Bond *et al.*, 1988; Vedder *et al.*, 1988; von Ritter *et al.*, 1988; Lieners *et al.*, 1989; Sanan *et al.*, 1989; Marzi *et al.*, 1990; Fleckenstein *et al.*, 1991;

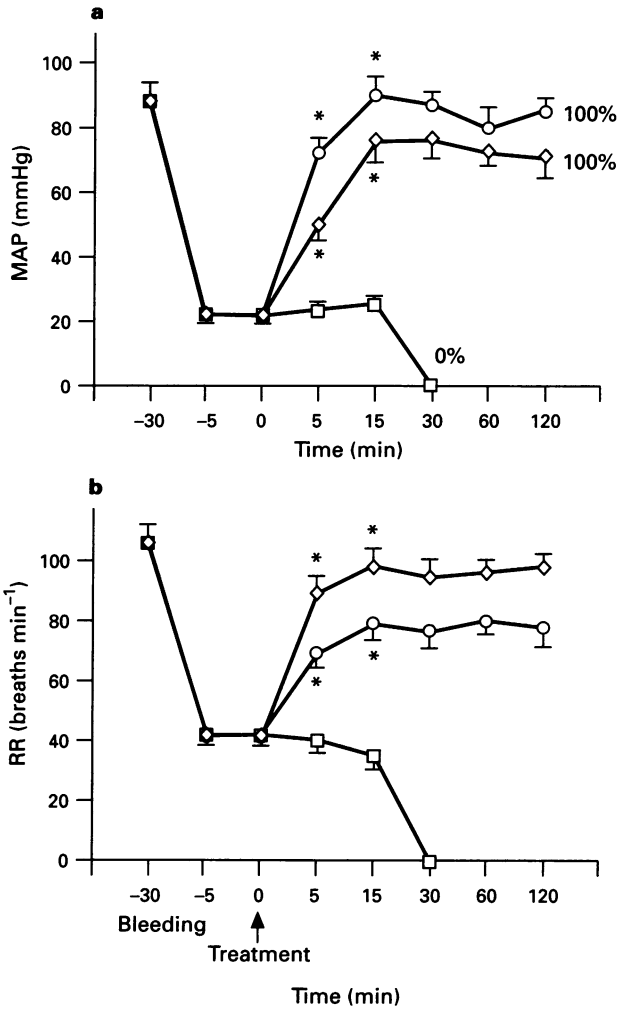

Figure 1 Influence of the i.v. injection of ACTH-(1-24) (◇, $160\ \mu g\ kg^{-1}$), saline (□, $1\ ml\ kg^{-1}$) or of the gradual reinfusion of the shed blood (○) on mean arterial pressure (MAP) and respiratory rate (RR) in haemorrhage-shocked rats. Mean values ± s.e. mean for 8 animals per group. Treatment = bolus injection of ACTH-(1-24), saline or termination of blood reinfusion. *$P < 0.001$ versus the corresponding value of saline-treated rats (Student's *t* test). Percentage of surviving animals (at the end of lines) 2 h after treatment with ACTH-(1-24) or after blood reinfusion was statistically different from that of saline-treated controls ($P < 0.005$; Fisher's test).

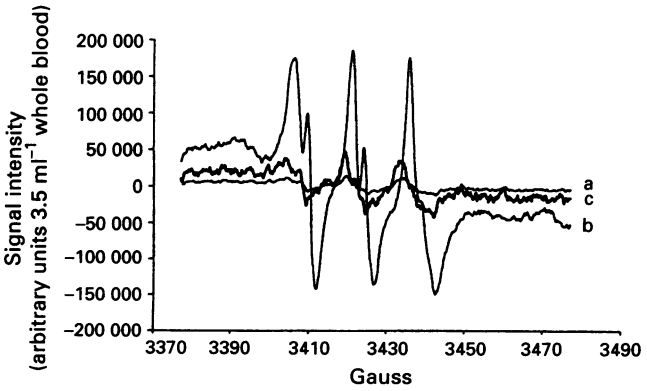

Figure 2 Representative electron spin resonance spectra of α-phenyl-N-tert-butylnitrone-adduct in rats' blood: (a) basal, pre-bleeding condition; (b) after bleeding, shock condition; (c) 30 min after i.v. treatment with ACTH-(1-24) ($160\ \mu g\ kg^{-1}$).

Table 1 Summary of α-phenyl-N-tert-butylnitrone-adduct signal (electron spin resonance) intensity (arbitrary units ml^{-1} whole blood) in haemorrhage-shocked rats before and after i.v treatment with saline ($1 ml kg^{-1}$), ACTH-(1–24) ($160 \mu g kg^{-1}$) or after gradual reinfusion of the shed blood

Treatment	*Before bleeding*	*5 min after bleeding termination*	*At the following times (min) after treatment: 15*	*30*	*60*	*120*
Saline	6240±606	31250±4750	35450±5450	ND	ND	ND
ACTH-(1–24)	6380±503	30916±5001	12807±2995*	10462±2850	12294±4120	10360±2080
Blood reinfusion	6850±470	30120±4705	9520±1650*	7515±1905	8410±2350	6150±955

Values represent the mean±s.e. mean for 8–10 animals per group.
ND = not detected: all rats die within 30 min. In blood reinfused rats, the after treatment time started at reinfusion termination.
*$P < 0.01$ versus the corresponding value of saline-teated rats (Student-Newmann-Keuls test).

Schlag *et al.*, 1991; Redl *et al.*, 1993). Oxygen radicals also, play a major role in the pathophysiology of injuries caused by reperfusion of ischaemic tissues following resuscitation (Stewart *et al.*, 1982; Parks *et al.*, 1983; McCord, 1985; Mitsos *et al.*, 1986): restoration of blood volume with consequent reoxygenation of ischaemic tissues may produce a sudden further increase in free radical discharge and exacerbate the ischaemic damage. Oxygen radical production in conditions associated with ischaemia-reperfusion is though to be in part due to xanthine oxidase, and/or phagocyte activation (Redl *et al.*, 1988; Barroso-Aranda & Schmid-Schönbein, 1989; Schlag *et al.*, 1991).

Ischaemic injury to the cell promotes metabolism of adenosine triphosphate to hypoxanthine and irreversible conversion of xanthine dehydrogenase to xanthine oxidase (Redl *et al.*, 1988). This aberrant metabolic process damages the cell either through the generation of cytotoxic radicals (O_2^- and H_2O_2) from xanthine oxidase, or through a depletion of cellular purine stores, which impairs the ability of the cells to maintain sufficient phosphate compounds for cell membrane integrity. Allopurinol, a xanthine oxidase inhibitor, has been repeatedly shown to increase the survival period in haemorrhagic shock in dogs (Crowell *et al.*, 1969; Cunningham & Keaveny, 1978; Chambers *et al.*, 1985) and rats (Cederna *et al.*, 1990), and superoxide dismutase has been found to ameliorate splanchnic artery occlusion shock in rats (Bitterman *et al.*, 1988). Favourable results have also been obtained with the spin-trapping agent PBN in a traumatic model of shock in rats (Novelli, 1992).

The importance of phagocyte activation for shock-related tissue damage is suggested by several observations. Neutrophils accumulate in tissues and blood during shock and retransfusion (Redl *et al.*, 1984; Guarini *et al.*, 1987; Schlag *et al.*, 1991). Rats with a large number of nitroblue tetrazolium-positive (= oxygen radical producing) neutrophils do not survive haemorrhage (Barroso-Aranda & Schmid-Schönbein, 1989), whereas neutropenia offers protection in shock models (Johnson & Malik, 1980) and monoclonal antibodies against the leukocyte adhesion complex CD11/CD18 attenuate organ damage (Vedder *et al.*, 1988).

In addition to increased production, an impaired antioxidant capacity of poorly-perfused tissues may contribute to increased levels of oxygen radicals (Horton & Borman, 1987). In a primate model of haemorrhagic-traumatic shock, α-tocopherol plasma levels fall significantly during the shock period, before reinfusion of shed blood; similar data have been obtained in human conditions of haemorrhagic shock, with plasma tocopherol levels of 5–6 $\mu g\ ml^{-1}$, comparable to those seen in congenital vitamin E deficiency (Redl *et al.*, 1993).

However, as most free radicals have a short half-life and are therefore difficult to measure, there is no direct evidence of their role in shock. E.S.R. spectroscopy coupled to the spin-trapping technique is the most widely used method for the detection of free radicals. It involves an addition reaction between a reactive free radical with a diamagnetic compound, generally a nitrone or a nitroso compound (spin trap) (methyl nitroso propane, PBN, pyridyl-N-oxide-*t*-butyl-nitrone, dimethyl pyrrolidine-N-oxide, etc.) (De Gray & Mason, 1994), the resulting nitroxide is a stable radical and can be detected easily by E.S.R. The radical species usually cannot be unambiguously assigned, however in our experiments the relative anisotropy of the nitroxide clearly refers to a bulky carbon centred radical, as previously described by Davies *et al.* (1991). Most studies on free radical formation have been performed on *in vitro* model systems, which however cannot reproduce the complexity of the *in vivo* systems. On the other hand, techniques of *in vivo* spin-trapping, usually with PBN, have several shortcomings: first of all, there is concern over PBN toxicity at high concentrations *in vivo* (Cova *et al.*, 1992); secondly, if a model is to illustrate the role of free radicals during a pathological insult, then the presence of an agent that traps these radicals would act as a protecting agent to the system (Novelli, 1992; Hartell *et al.*, 1994).

We therefore used an *ex vivo* technique, developed as an alternative approach to direct infusion of a spin trap *in vivo* (Mergner *et al.*, 1991; Tortolani *et al.*, 1993; Hartell *et al.*, 1994). These results provide a direct proof that in a severe condition of haemorrhagic shock there is a rapid and massive production of highly reactive free radicals, with a five fold increase in arterial blood concentration. Furthermore, our present results show that the rapid and sustained shock reversal induced by the i.v injection of ACTH-(1-24) is associated with a reversal of the elevated blood levels of the radicals. This effect of ACTH-(1-24) is prompt (reaching its maximum 15 min after treatment) and long-lasting (the free radical blood level remains quite stable throughout the 2 h observation period). It parallels the effects of ACTH-(1-24) on arterial pressure and tissue blood flow (Bertolini *et al.*, 1989; Guarini *et al.*, 1989). This effect cannot be attributed to an ACTH-(1-24) radical scavenging activity, as demonstrated by the competition experiment.

As the beneficial effects of ACTH in haemorrhagic shock are associated with the mobilization of peripherally-pooled residual blood (Guarini *et al.*, 1987; 1989), one might expect a transient further increase in free radical levels, due to the reoxygenation of ischaemic tissues. Indeed, venous pH and arterial pH, P_{CO_2}, HCO_3^- and BE are significantly decreased during haemorrhagic shock and continue to decrease during the first 15 min after ACTH-(1-24) injection, while venous lactate, which is significantly increased during haemorrhagic shock, continues to increase. Complete recovery of these parameters are observed only 60 min after treatment (Bazzani *et al.*, 1992).

On the other hand, in a canine model of haemorrhagic shock, the administration of free radical scavengers during shock or during reperfusion is not able to improve haemodynamic function (Horton & Borman, 1987), and it has been suggested that prevention of free radical generation rather than scavenging them during ischaemia and reperfusion may be required (Horton & Borman, 1987).

Thus it is possible that ACTH-(1-24) prevents the burst of free radical generation during blood mobilisation and tissue reperfusion, and this may be an important component of its mechanism of action in effectively reversing haemorrhagic

shock. It may be important that in haemorrhage-shocked animals, ACTH-(1-24) causes a gradual restoration of circulating blood volume and of tissue reperfusion (Guarini *et al.*, 1989): indeed, our present data indicate that similar effects on free radical levels are obtained with the gradual reinfusion of the shed blood.

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