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RESEARCH PAPER

# Selective melanocortin $MC_4$ receptor agonists reverse haemorrhagic shock and prevent multiple organ damage

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**Background and purpose:** In circulatory shock, melanocortins have life-saving effects likely to be mediated by $MC_4$ receptors. To gain direct insight into the role of melanocortin $MC_4$ receptors in haemorrhagic shock, we investigated the effects of two novel selective $MC_4$ receptor agonists.
**Experimental approach:** Severe haemorrhagic shock was produced in rats under general anaesthesia. Rats were then treated with either the non-selective agonist [Nle$^4$, D-Phe$^7$]α-melanocyte-stimulating hormone (NDP-α-MSH) or with the selective $MC_4$ agonists RO27-3225 and PG-931. Cardiovascular and respiratory functions were continuously monitored for 2 h; survival rate was recorded up to 24 h. Free radicals in blood were measured using electron spin resonance spectrometry; tissue damage was evaluated histologically 25 min or 24 h after treatment.
**Key results:** All shocked rats treated with saline died within 30-35 min. Treatment with NDP-α-MSH, RO27-3225 and PG-931 produced a dose-dependent (13-108 nmol kg$^{-1}$ i.v.) restoration of cardiovascular and respiratory functions, and improved survival. The three melanocortin agonists also markedly reduced circulating free radicals relative to saline-treated shocked rats. All these effects were prevented by i.p. pretreatment with the selective $MC_4$ receptor antagonist HS024. Moreover, treatment with RO27-3225 prevented morphological and immunocytochemical changes in heart, lung, liver, and kidney, at both early (25 min) and late (24 h) intervals.
**Conclusions and Implications:** Stimulation of $MC_4$ receptors reversed haemorrhagic shock, reduced multiple organ damage and improved survival. Our findings suggest that selective $MC_4$ receptor agonists could have a protective role against multiple organ failure following circulatory shock.
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**Keywords:** haemorrhagic shock; melanocortin peptides; melanocortin $MC_4$ receptors; free radicals; organ damage

**Abbreviations:** ACTH, adrenocorticotropin; ESR, electron spin resonance; IL-10, interleukin-10; MAP, mean arterial pressure; NDP-α-MSH, [Nle$^4$,D-Phe$^7$]α-melanocyte-stimulating hormone; PBN, α-phenyl-*N*-*tert*-butylnitrone; PP, pulse pressure; RR, respiratory rate

## Introduction

Melanocortin peptides and in particular the adrenocorticotropin/α-melanocyte-stimulating hormone (ACTH/α-MSH) sequences, as well as shorter fragments, have a life-saving effect in animals and humans in conditions of circulatory shock (Bertolini *et al*., 1986a, b; Guarini *et al*., 1987, 1990, 1999, 2004; Squadrito *et al*., 1999; Noera *et al*., 2001). These neuropeptides, including ACTH-(1–24), α-MSH and synthetic analogs, are likewise protective in other severe hypoxic

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conditions, such as prolonged respiratory arrest (Guarini *et al*., 1997a), myocardial ischaemia (Bazzani *et al*., 2001; Guarini *et al*., 2002; Mioni *et al*., 2003, 2005; Vecsernyes *et al*., 2003) and ischaemic stroke (Giuliani *et al*., 2006a, b), as well as in experimental heart transplantation (Gatti *et al*., 2002).

Circulatory shock, including haemorrhagic shock, initiates an inflammatory cascade (systemic inflammatory response) with production of cytokines and recruitment of neutrophils (Schlag *et al*., 1991; Baue, 1997; Le Tulzo *et al*., 1997; Wheeler and Bernard, 1999; McDonald *et al*., 2001; Tracey, 2002). The multiple organ injury that often occurs after haemorrhagic shock is believed to be caused by ischaemia (during haemorrhage) and reperfusion (during resuscitation) of target organs (Schlag *et al*., 1991; Baue, 1997; Cockerill *et al*., 2001; McDonald *et al*., 2001; Cui *et al*., 2005). Moreover, the pathophysiologic processes involved in multiple organ injury are greatly influenced by reactive oxygen species (ROS), released in large amounts during haemorrhagic shock (McCord, 1985; Horton and Borman, 1987; Schlag *et al*., 1991; Redl *et al*., 1993; Guarini *et al*., 1996; Cockerill *et al*., 2001; Victor *et al*., 2005).

Recent research indicates that during shock due to endotoxin, haemorrhage or splanchnic artery occlusion, the central nervous system (CNS) modulates the systemic inflammatory response through the rapid activation of efferent vagal nerve fibers (the 'brain cholinergic anti-inflammatory pathway') (Borovikova *et al*., 2000; Tracey, 2002; Guarini *et al*., 2003, 2004; Pavlov and Tracey, 2005; Altavilla *et al*., 2006). It has been demonstrated that the antishock effect of melanocortins, which is independent of effects on the adrenal gland (Bertolini *et al*., 1986a, b), is due to the activation of the brain cholinergic anti-inflammatory pathway and is associated with a marked reduction in the plasma levels of inflammatory mediators including free radicals (Guarini *et al*., 1996, 1997b, 2004; Bertuglia and Giusti, 2004). Indirect evidence from our studies suggests that the cholinergic anti-inflammatory pathway is activated by stimulation of melanocortin $MC_4$ receptors within the CNS (Guarini *et al*., 1999, 2004).

To gain direct insight into the role of $MC_4$ receptors in haemorrhagic shock-induced cardiovascular and respiratory failure and organ injury, we determined the effects of two novel melanocortin agonists, highly selective at $MC_4$ receptors, in a rat model of severe haemorrhagic shock.

## Methods

*Animals, surgery and haemorrhagic shock induction*

Wistar rats of both sexes (270–300 g body weight) (Harlan, Milan, Italy) were used. They were kept in air conditioned colony rooms (temperature $21 \pm 1^{\circ}C$; humidity 60%) on a natural light/dark cycle, with food in pellets and tap water available *ad libitum*. Housing conditions and experimental procedures were in strict accordance with the European Community regulations on the use and care of animals for scientific purposes (CEE Council 89/609; Italian D.L.22–1–92 No. 116), and were approved by the Committee on Animal Health and Care of Modena and Reggio Emilia University. Under general anaesthesia (urethane, $1.25\,g\,kg^{-1}$ intraperitoneal (i.p.) and after heparinization (heparin sodium, $600\,IU\,kg^{-1}$ intravenous (i.v.), rats were instrumented with indwelling polyethylene catheters in a common carotid artery, to record arterial blood pressure, and into an iliac vein to inject drugs and to bleed. The arterial catheter was connected to a pressure transducer coupled to a polygraph (Mortara-Rangoni, Bologna, Italy). Arterial blood pressure was reported as mean arterial pressure (MAP) and pulse pressure (PP). Respiratory rate (RR) was recorded by means of three electrodes implanted subcutaneous (s.c.) on the chest and connected through an ARI A380 preamplifier (Mortara–Rangoni) to the above polygraph. Acute haemorrhagic shock was induced by a stepwise (within 20–25 min) withdrawal of about 50% of the circulating blood (a total of 2–2.5 ml $100\,g^{-1}$ body weight) until MAP, automatically calculated and continuously digitally displayed on the polygraph, fell to and stabilized at 20–25 mm Hg (Bertolini *et al*., 1986a, b; Guarini *et al*., 1990, 1996, 1999, 2004). Sham shocked rats were subjected to all surgical procedures experienced by haemorrhage-shocked animals, but were not bled.

*Drug treatments*

All drugs were dissolved in saline immediately before use, and injections were given in a volume of $1\,ml\,kg^{-1}$. Melanocortin peptides were administered as i.v. bolus 5 min after termination of bleeding, when MAP was stabilized at 20–25 mm Hg. Pretreatment with HS024, a cyclic MSH analog, potent and selective $MC_4$ receptor antagonist ($K_i$ values (nM, mean $\pm$ s.e.m.) obtained from competition curves on $MC_1$, $MC_3$, $MC_4$ and $MC_5$ receptors: $18.6 \pm 3.3$, $5.45 \pm 2.06$, $0.29 \pm 0.14$ and $3.29 \pm 1.15$, respectively (Kask *et al*., 1998)) was performed i.p. 2 min before starting bleeding. The dose of HS024 and the pretreatment time were chosen on the basis of previous studies (Kask *et al*., 1998; Getting *et al*., 2003; Ling *et al*., 2004; Giuliani *et al*., 2006a, b). Control rats (shocked or sham-shocked) received equal volumes of saline. Cardiovascular and respiratory parameters were continuously monitored for 2 h after treatment, or until death or killing at the scheduled intervals for histological and blood studies. The surviving rats were killed at 24 h; some of these animals were used to perform a late histological assessment.

*Blood sampling, extraction of radical species and electron spin resonance measurement*

A technique modified from (Tortolani *et al*., 1993; Guarini *et al*., 1996; Mioni *et al*., 2003, 2005) was employed in order to avoid the injection of the spin-trapping agent *in vivo*. Fifteen minutes after treatment with a melanocortin agonist or saline, each animal had 2–3 ml of whole blood rapidly withdrawn via the arterial catheter into a syringe containing 2 ml of a 0.1 M solution of $\alpha$-phenyl-*N*-tert-butylnitrone (PBN; Sigma Chemical Co., St Louis, MO, USA) in isotonic saline. Each animal served for a single sample. The samples were immediately centrifuged (1680 *g* for 10 min) and the supernatant (containing plasma and PBN) was added to 12 ml of 2:1 (v/v) chloroform/methanol for extraction of free

radicals. The chloroform layer was separated, dried under nitrogen flow, the resulting pellet was resuspended in 250 $\mu$l chloroform and the ESR spectrum was taken. ESR spectra were recorded at room temperature using a Bruker EMX spectrometer (Bruker BioSpin, Billerica, MA, USA). Typical instrumental settings were microwave power, 20 mW; modulation amplitude, 1 Gauss; field width, 60 Gauss; microwave frequency, 9.14 GHz. The electron spin resonance (ESR) peak height of the central absorption was measured, and expressed in arbitrary units, as a direct function of adduct concentration. For statistical analysis, the values were normalized to a fixed sample volume of 1 ml of whole blood.

In order to test the possible scavenging activity of NDP-α-MSH, RO27-3225 and PG-931, an *in vitro* chemical system producing highly reactive carbon and oxygen centered radicals was prepared; radicals were trapped by PBN. Tert-butyl hydroperoxide (1 mM) was decomposed by ferrous ions (5 $\mu$M), and PBN (25 mM) was added as spin-trapping agent; the samples were processed as above for ESR determination. The same experiment was carried out in the presence of NDP-α-MSH, RO27-3225 or PG-931 (13.5 nM) added as hypothetical competitors in the reaction mixture.

*Histology*

In shocked and sham-shocked rats, hearts, lungs, livers and kidneys were removed 25 min after treatment with a melanocortin agonist or saline or 24 h thereafter, and processed as described previously (Bazzani *et al*., 2001; Mioni *et al*., 2005). Histology (Hematoxylin-Eosin; Fluka Chemie, Buchs SG, Switzerland) and immunocytochemistry (Streptavidin-Biotin complex; Dako, Glostrup, USA) of organ sections (7 μm thick) were evaluated using an Axiophot photomicroscope (Carl Zeiss, Jena, Germany). The antiapoptotic reaction of organs was evaluated using a monoclonal antibody to bcl2 (Dako), whereas a monoclonal antibody to actin (Dako) was used to evaluate the shock effect on myocardial tissue. Slides were incubated overnight at 4°C with the antibodies, in a moist and darkened chamber; slides were then incubated with 1:200 Streptavidin Biotinylated Complex (Dako) for 60 min, developed in diaminobenzidine (Fluka) and counterstained in Harris Hematoxylin. Histometry was performed by means of an image analyser (VIDAS, Carl Zeiss) in each of five seriated slices for each organ. Morphological signs and immunocytochemical indexes were classified by appropriate score scale of values, according to the percentage of the organ volume involved.

*Statistics*

Data are expressed as means ± s.e.m. Comparison of MAP, PP and RR values, as well as free radical blood levels, was performed using one-way analysis of variance (ANOVA) followed by a multiple comparison test (Student–Newman–Keuls). Survival rates were analysed by means of Fischer's exact probability test. Histological parameters were analysed by means of Kruskal–Wallis test, followed by Mann–Whitney *U*-test. A probability value $<0.05$ was considered significant.

*Materials*

Butir-His-D-Phe-Arg-Trp-Sar-$NH_2$ (RO27-3225), Ac-Nle-c[Asp-Pro-D-Phe-Arg-Trp-Lys]-Pro-Val-$NH_2$ (PG-931) and [$Nle^4$, D-$Phe^7$]α-MSH (NDP-α-MSH) were synthesized in our laboratory by conventional solid phase chemistry, purified by reverse phase-high performance liquid chromatography (RP-HPLC) and checked for proper molecular weight by mass-spectroscopy, as reported previously (Grieco *et al*., 2002, 2003). HS024 was purchased from Neosystem (Strasbourg, France).

## Results

*NDP-α-MSH, RO27-3225 and PG-931 improve MAP, PP, RR and survival rate*

The acute hypovolaemia induced in our model of haemorrhagic shock was incompatible with survival and, consistent with previous observations, all saline-treated rats died within 30–35 min (Figure 1). The i.v. bolus injection of NDP-α-MSH – agonist at $MC_1$, $MC_3$, $MC_4$ and $MC_5$ receptors (Wikberg *et al*., 2000; Catania *et al*., 2004) – produced a dose-dependent restoration of cardiovascular and respiratory functions within a few minutes. The maximal restoration of MAP, PP and RR occurred 10–15 min after treatment, and remained stable during the 2 h observation period (Figure 1). Treatment with the highest dose of NDP-α-MSH was associated with a 100% survival rate at the end of the 24 h observation period. Treatment of haemorrhage-shocked rats with the selective $MC_4$ receptor agonists RO27-3225 and PG-931 (Benoit *et al*., 2000; Grieco *et al*., 2003) also restored in a dose-related manner cardiovascular and respiratory parameters (Figures 2 and 3). When the highest doses of RO27-3225 and PG-931 were administered, restoration of MAP, PP and RR was associated with a 100% survival rate at the end of the 24 h observation period. Moreover, RO27-3225 was slightly more potent than NDP-α-MSH and PG-931 in reversing the shock condition.

Pretreatment with the selective $MC_4$ receptor antagonist HS024 totally prevented the protective effects of NDP-α-MSH, RO27-3225 and PG-931 on MAP, PP, RR and survival rate (Figures 1–3). When pretreatment with HS024 was given without a melanocortin agonist, that is, to shocked animals given i.v. saline at time 0, the time course of haemodynamic and respiratory parameters and the survival rate were similar to those observed in saline-pretreated, saline-treated shocked rats (data not shown).

Administration of the highest dose of NDP-α-MSH, RO27-3225, and PG-931 in sham shocked rats had no effect on arterial blood pressure (data not shown).

*NDP-α-MSH, RO27-3225 and PG-931 prevent free radical discharge*

Consistent with our previous observations, haemorrhage caused a large increase in blood levels of free radicals, as detected 15 min after treatment with saline (Figure 4). The representative ESR spectra (data not shown) featured a well-defined three line signal consistent with the trapping of multiple radical species, lipids and proteins arising from

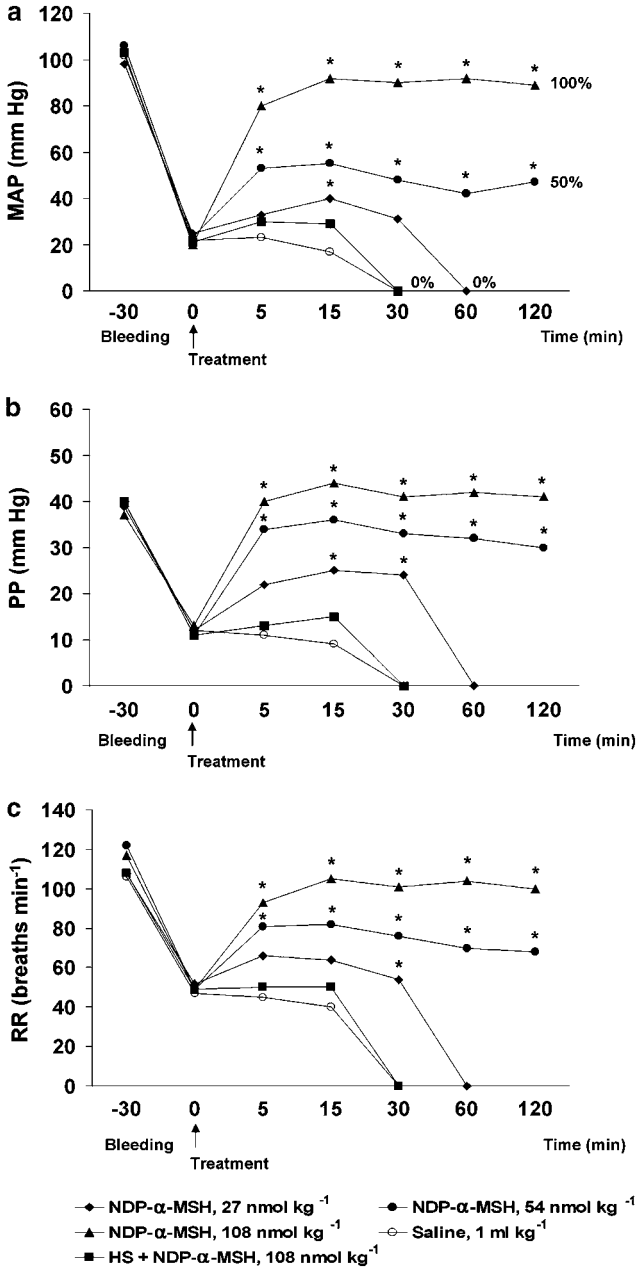


**Figure 1** In rats subjected to haemorrhagic shock, i.v. treatment with NDP-α-MSH improved MAP (**a**), PP (**b**), RR (**c**) and survival rate (values at the end of lines, panel a). Pretreatment with the $MC_4$ receptor antagonist HS024 (HS, 130 $\mu g\,kg^{-1}$ i.p.) prevented the effect of NDP-α-MSH. Values are means for 8–10 rats per group; s.e.m. (within ±10%) was omitted for the sake of clarity. *$P<0.05$, at least, vs the corresponding value in saline-treated rats (Student–Newman–Keuls test); percentage of surviving animals 120 min after treatment (value at the end of lines) gave $P$-values from $<0.025$ (50% survival) to $<0.005$ (100% survival) (Fisher's test).

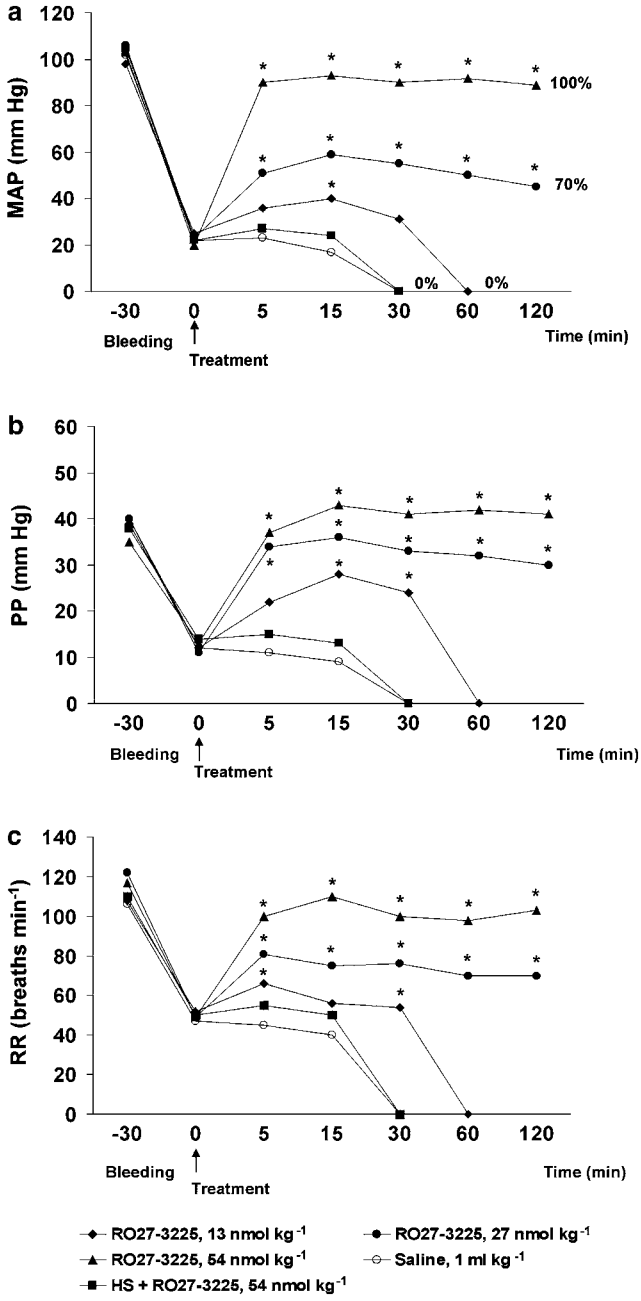


**Figure 2** Treatment i.v. with RO27-3225 improved MAP (**a**), PP (**b**), RR (**c**) and survival rate (values at the end of lines, panel a) in rats subjected to haemorrhagic shock. Pretreatment with the $MC_4$ receptor antagonist HS024 (HS, 130 $\mu g\,kg^{-1}$ i.p.) prevented the effect of RO27-3225. Values are means for 8–10 rats per group; s.e.m. (within ±10%) was omitted for the sake of clarity. *$P<0.05$, at least, vs the corresponding value in saline-treated rats (Student—Newman–Keuls test); percentage of surviving animals 120 min after treatment (value at the end of lines) gave $P$-values $<0.005$ (70 and 100% survival) (Fisher's test).

$\beta$-scission or intramolecular rearrangement of alkoxy radicals (Buettner, 1987; Davies *et al*., 1991; Guarini *et al*., 1996). Treatment with either the non specific melanocortin receptor agonist NDP-α-MSH or the selective $MC_4$ receptor agonists RO27-3225 and PG-931, at the dose that caused the maximal protective effect on shock parameters, also produced a marked reduction in free radical blood levels (Figure 4). In melanocortin-treated animals, the ESR signal was virtually absent, similar to that of sham shocked rats (not shown).

Pretreatment with the selective $MC_4$ receptor antagonist HS024 totally prevented the effects of melanocortin agonists on free radical blood concentrations (Figure 4). Pretreatment

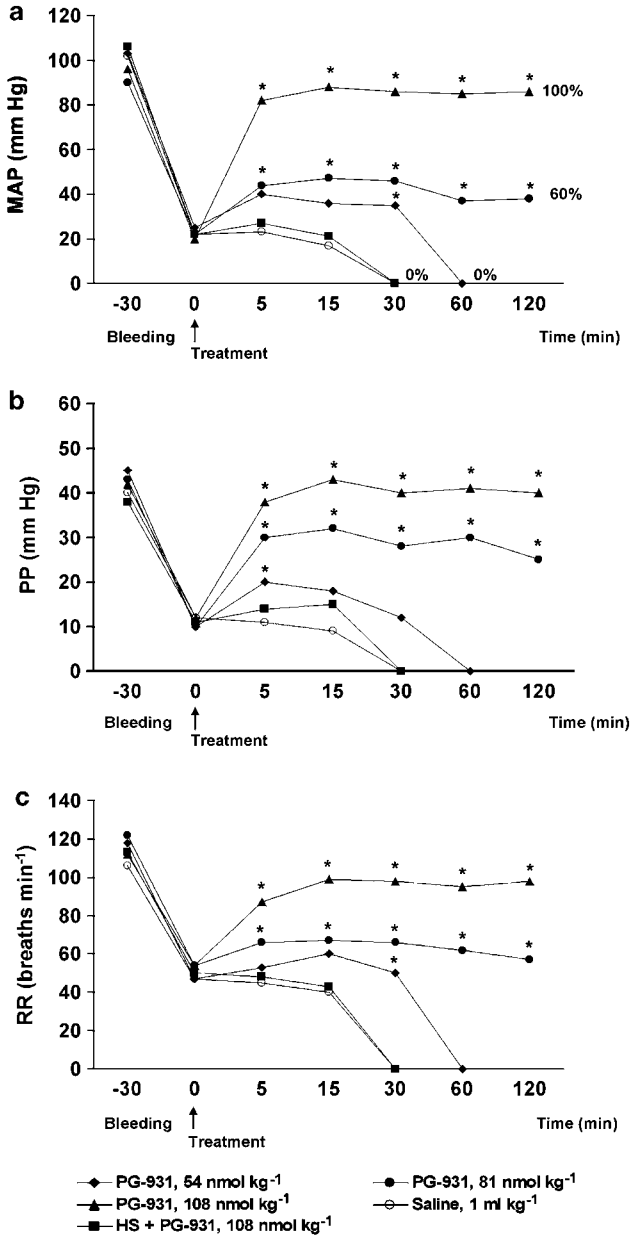


**Figure 3** Treatment i.v. with PG-931 improved MAP (**a**), PP (**b**), RR (**c**) and survival rate (values at the end of lines, panel a) in rats subjected to haemorrhagic shock. Pretreatment with the $MC_4$ receptor antagonist HS024 (HS, 130 $\mu$g kg$^{-1}$ i.p.) prevented the effect of PG-931. Values are means for 8–10 rats per group; s.e.m. (within $\pm$10%) was omitted for the sake of clarity. *$P<0.05$, at least, vs the corresponding value in saline-treated rats (Student–Newman–Keuls test); percentage of surviving animals 120 min after treatment (value at the end of lines) gave *P*-values from $<0.01$ (60% survival) to $<0.005$ (100% survival) (Fisher's test).

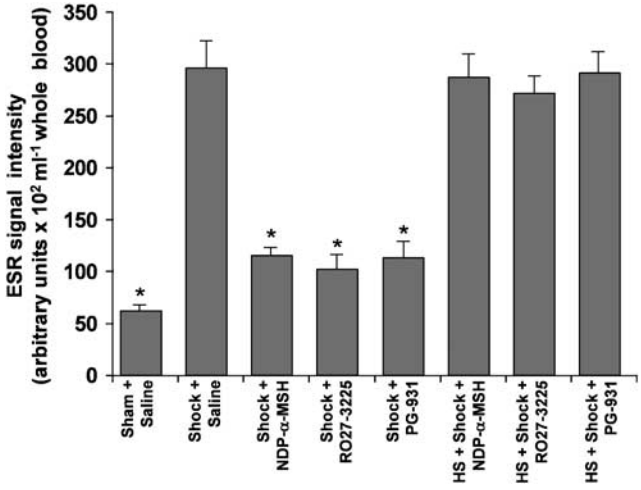


**Figure 4** NDP-$\alpha$-MSH, RO27-3225 and PG-931 reduced electron spin resonance (ESR) signal intensity in the blood of rats subjected to haemorrhagic shock. Pretreatment with the $MC_4$ receptor antagonist HS024 (HS, 130 $\mu$g kg$^{-1}$ i.p.) prevented the effect of NDP-$\alpha$-MSH, RO27-3225 and PG-931. Height of histograms indicates mean values $\pm$ s.e.m. for 6–8 rats per group, 15 min after i.v. treatment with saline, NDP-$\alpha$-MSH (108 nmol kg$^{-1}$), RO27-3225 (54 nmol kg$^{-1}$) or PG-931 (108 nmol kg$^{-1}$). Sham = sham shock. *$P<0.005$ vs shocked rats treated with saline (Student–Newman–Keuls test).

of shocked animals with HS024 in the absence of melanocortin agonists did not alter free radical discharge. Indeed, when saline was i.v. given after shock induction (time 0), circulating free radicals were similar in HS024 or saline-pretreated animals, as detected 15 min after treatment with saline (data not shown).

NDP-$\alpha$-MSH, RO27-3225, and PG-931 did not exert any direct radical scavenging activity, as assessed by a competition experiment in an *in vitro* chemical system producing highly reactive carbon and oxygen centred radicals (data not shown).

*RO27-3225 reduces histologic damage*

As reported above, in our experimental model of haemorrhagic shock, all saline-treated rats died within 30–35 min after treatment. Based on this survival time in control animals, hearts (left ventricle), lungs, livers and kidneys were analysed for histologic and histometric changes in animals killed 25 min after saline or melanocortin treatment. This short time between shock induction and sampling did not allow development of severe morphological and immunocytochemical changes in the tissues that would have permitted more precise quantitative evaluations. However, statistically significant modifications were detected by means of a score scale.

In saline-treated shocked rats there were some substantial organ alterations (Figure 5). In the heart, the left ventricle showed zones of swelling and vacuolar degeneration, focal necrotic sites in subendocardial locations and contraction band necrosis with low expression of actin filaments (Figure 5a and d). Lungs had scattered, low-level emphysema. Livers showed small, scattered areas of necrotic hepatocytes (Figure 5b and e). Enhanced expression of the bcl2 protein was detected in kidney proximal and distal tubule cells; immunoreactivity was particularly evident in the outer end of the renal parenchyma, inside the renal papillae (Figure 5c and f).

In shocked rats, treated with the $MC_4$ receptor agonist RO27-3225 at the dose that caused the maximal protective effect on cardiovascular shock parameters, the histological

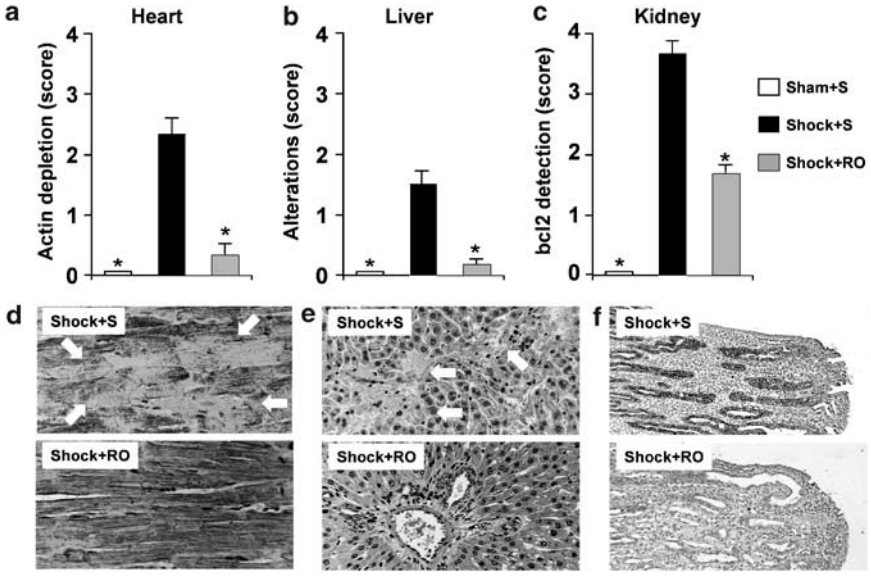


**Figure 5** RO27-3225 prevented organ damage in rats subjected to haemorrhagic shock. Height of histograms indicates mean values ± s.e.m. for six rats per group, 25 min after i.v. treatment with saline (S) or RO27-3225 (RO, 54 nmol kg$^{-1}$). (**a**) evaluation of antiactin immunoreaction; (**b**) evaluation of morphological alterations (hematoxylin–eosin); (**c**) evaluation of anti-bcl2 immunoreaction; (**d**) antiactin immunoreaction in the heart: the arrows point to the contraction band necrosis of a shocked rat treated with saline; (**e**) hematoxylin–eosin staining: the arrows point to necrotic zones of the liver of a shocked rat treated with saline; (**f**) anti-bcl2 immunoreaction of renal pyramids: the cells of almost all collecting tubules and papillar ducts of the shocked rat treated with saline are bcl2 positive (dark brown). Sham = sham shock. Actin score scale: 0 = no depletion, 1 = focal depletion, 2 = depletion under 0.5%, 3 = 0.5–2% depletion, 4 = 2–10% depletion. Hematoxylin–eosin score scale: 0 = no alterations, 1 = focal alterations, 2 = alterations under 0.5%, 3 = 0.5–2% alterations, 4 = 2–10% alterations. *bcl2* score scale: 0 = no detection, 1 = focal detection, 2 = detection under 0.5%, 3 = 0.5–2% detection, 4 = detection over 2%. *$P<0.001$ vs shocked rats treated with saline (Mann–Whitney *U*-test). Field width: d = 264 $\mu$m; e = 408 $\mu$m; f = 1054 $\mu$m.

picture showed no or sporadic signs of low levels of myocardial degeneration, without actin filament depletion and no necrosis (Figure 5a and d). No substantial differences between saline and RO27-3225-treated animals were found in the lung. No morphological signs of degeneration were detected in livers of melanocortin-treated rats (Figure 5b and e). In peptide-treated rats, lower levels of bcl2 were detected in kidneys (Figure 5c and f).

In a group of shocked rats treated with the same dose of RO27-3225 – 54 nmol kg$^{-1}$ i.v., a treatment that afforded prolonged survival – histological evaluation of heart left ventricle, lung, liver and kidney was performed 24 h after treatment: no appreciable differences were found relative to evaluations carried out in animals killed at 25 min ($n = 6$; data not shown).

There were no substantial morphological and immunocytochemical differences in sham saline-treated rats relative to shocked animals treated with RO27-3225, at both 25 min (Figure 5) and 24 h ($n = 6$; data not shown), except for a lesser extent of bcl2 immunoreactivity in the saline group.

## Discussion

Circulatory shock, including haemorrhagic shock, is a severe dysfunction causing decreased tissue oxygenation that leads to multiple organ failure if untreated at a proper phase. Activation of the systemic inflammatory response, including the increase in oxidative and nitrosative stress and tissue injury, are important pathophysiologic components of shock (Redl *et al*., 1993; Guarini *et al*., 1996, 2004; Baue, 1997; Le Tulzo *et al*., 1997; Wheeler and Bernard, 1999; Cockerill *et al*., 2001; Tracey, 2002). Mortality rate closely depends on the number of failing organs (Schlag *et al*., 1991; Baue, 1997; Wheeler and Bernard, 1999; Cockerill *et al*., 2001; McDonald *et al*., 2001; Cui *et al*., 2005). Our previous research indicated that melanocortin peptides improve outcome in circulatory shock (Bertolini *et al*., 1986a, b; Guarini *et al*., 1990, 1999, 2004; Squadrito *et al*., 1999; Noera *et al*., 2001). The present data confirm and expand previous observations and indicate that selective stimulation of the $MC_4$ receptor subytpe can help in the treatment of circulatory shock. Five melanocortin receptor subtypes ($MC_1$–$MC_5$) are recognized (for reviews see Tatro, 2000; Wikberg *et al*., 2000; Catania *et al*., 2004; Getting, 2006). $MC_1$ is expressed in melanocytes, melanoma cells and cells involved in the immune/inflammatory response including monocytes, dendritic cells and lymphocytes; $MC_2$, the ACTH receptor, is mainly expressed in the adrenal glands but also in white adipose tissue and in the skin; $MC_3$ is expressed in the brain, placenta, gut and heart; $MC_4$ occurs in various brain areas but has been recently recognized also in peripheral organs in the rat (Mountjoy *et al*., 2003); the $MC_5$ receptor, initially recognized in the brain, was subsequently found to be ubiquitous, but mainly in the periphery.

The present research on haemorrhage-shocked rats shows that the non selective melanocortin agonist NDP-α-MSH, which activates the $MC_1$, $MC_3$, $MC_4$ and $MC_5$ receptor subtypes (Wikberg *et al*., 2000; Catania *et al*., 2004), and the selective $MC_4$ receptor agonists RO27-3225 and PG-931

(Benoit *et al*., 2000; Grieco *et al*., 2003), produced a rapid and dose-dependent restoration of cardiovascular and respiratory functions and increased survival. This antishock effect occurred at nanomolar doses and was mediated by melanocortin $MC_4$ receptors. Indeed, the selective $MC_4$ receptor antagonist HS024 (Kask *et al*., 1998; Getting *et al*., 2003; Ling *et al*., 2004; Giuliani *et al*., 2006a, b) totally prevented effects of NDP-α-MSH, RO27-3225 and PG-931.

Experimental evidence indicates that melanocortins reach the CNS after systemic injection (Guarini *et al*., 1987, 1999, 2004; Wilson, 1988; Banks and Kastin, 1995; Wikberg *et al*., 2000; Catania *et al*., 2004; Mioni *et al*., 2005; Getting, 2006; Giuliani *et al*., 2006a, b). We have demonstrated previously that ACTH-(1–24) – which activates all MC receptor subtypes – reverses haemorrhagic shock through the rapid activation of an efferent vagal anti-inflammatory pathway through stimulation of MC receptors within the brain (Guarini *et al*., 2004). The antishock effect of ACTH-(1–24) was exerted at lower concentrations when the peptide was administered i.c.v., and effectiveness of peripheral administration was prevented by pretreatment (i.v. or i.c.v.) with the $MC_4$ receptor antagonist HS014 (Guarini *et al*., 1987, 1999, 2004). Consistent with the idea that the antishock effects of central melanocortins occur through selective stimulation of the $MC_4$ receptor, administration of ACTH-(1–24) was effective also in adrenalectomized rats (strongly arguing against an involvement of $MC_2$ receptors) and administration of $\gamma_1$-MSH, a selective agonist at $MC_3$ receptors – the other melanocortin receptor subtype predominant within the brain (Tatro, 2000; Wikberg *et al*., 2000; Catania *et al*., 2004; Getting, 2006) – was ineffective (Guarini *et al*., 1999). Moreover, permeability of the blood–brain barrier significantly increases during haemorrhagic shock (Krizbai *et al*., 2005). Although $MC_1$ receptors expressed by cells involved in the immune/inflammatory response seem to be involved in the anti-inflammatory effect of melanocortins (a peripheral mechanism: Catania *et al*., 2004), there is no evidence that these receptors play a crucial role in haemorrhagic shock. The fact that the MC receptor antagonist HS024 has an approximately 65-fold higher affinity for the $MC_4$ receptors relative to $MC_1$ receptors (Kask *et al*., 1998) argues against an involvement of $MC_1$ receptors in the here reported protective effects of melanocortins. Finally, the very low affinity of HS024, as well as of the agonists NDP-α-MSH, RO27-3225 and PG-931, for $MC_5$ receptors (Kask *et al*., 1998; Benoit *et al*., 2000; Grieco *et al*., 2003) – which are expressed mainly in the periphery – argues against a role for these receptors in the antishock effect of melanocortins. Collectively, these data support the hypothesis that the antishock effects of NDP-α-MSH, RO27-3225 and PG-931 occur through the activation of $MC_4$ receptors located within the brain.

Another interesting observation was the marked reduction in circulating free radicals (multiple radical species arising from oxidative and nitrosative stress) associated with NDP-α-MSH, RO27-3225 and PG-931 treatments. Based on our previous (Guarini *et al*., 1996) and present data, the antiradical effect of melanocortins does not appear to depend on a direct radical scavenging activity but, rather, on a reduction in tissue damage. In other words, $MC_4$ receptor stimulation might inhibit radical formation at a very early phase, through prevention of tissue injury. This effect is particularly relevant as oxygen participates in control of gene expression; there is evidence, indeed, that hypoxia induces the transcription of a large array of genes, including those involved in free radical control (for a review see Giordano, 2005).

The present data also showed that the selective $MC_4$ receptor agonist RO27-3225 inhibited progression of haemorrhagic shock to organ damage, the prelude to multiple organ failure, which is the consequence of shock in the clinical setting (Schlag *et al*., 1991; Baue, 1997; Wheeler and Bernard, 1999; Cockerill *et al*., 2001; McDonald *et al*., 2001; Cui *et al*., 2005). RO27-3225 significantly reduced myocardial damage, including actin depletion, hepatic necrosis and the marked expression of the bcl2 protein in the kidney, even when histological examination was performed 24 h after treatment. In saline-treated rats, the short time between the induction of shock and the removal of the organs sampled did not allow development of severe morphological and immunocytochemical alterations. This severe experimental model of haemorrhagic shock is associated, in fact, with a 30–35 min survival after shock induction and precludes, therefore, a late evaluation of organ damage in the control group. With regard to the marked bcl2 expression found in the kidney of saline-treated shocked rats, the finding should be regarded as a negative index in this experimental condition of oxygen deprivation consequent to haemorrhage. Overexpression of bcl2 observed in saline-treated rats is more likely to represent an adaptative mechanism in the renal tubule cells in response to ischaemic injury (Gobe *et al*., 2000). The reduced expression of this antiapoptotic protein in RO27-3225-treated rats is consistent with a protective effect of melanocortin treatment against ischaemic damage.

Although the exact mechanism leading to multiple organ failure is not clear, the rapid increase in cytokine and adhesion molecule production early after trauma and haemorrhage and the rapid decrease in interleukin-10 (IL-10) levels in patients who develop multiple organ failure suggest a defect in endogenous antiinflammatory mechanisms (Simons *et al*., 1996; Seekamp *et al*., 1998). Indeed, IL-10 is an important antiinflammatory cytokine, and an inadequate IL-10 response after systemic injury can have detrimental consequences. Consistent with this idea, an increase in IL-10 levels exerts protective influences in haemorrhagic shock (Cui *et al*., 2005). Reduced concentrations of circulating α-MSH was likewise associated with a worse outcome in patients with septic shock (Catania *et al*., 2000) or brain injury (Magnoni *et al*., 2003). Interestingly, melanocortins inhibit nuclear factor-κB activation (NF-κ*B*) and, consequently, reduce proinflammatory cytokine and adhesion molecule production and upregulate IL-10 modulating the inflammatory cascade (Altavilla *et al*., 1998; Squadrito *et al*., 1999; Wikberg *et al*., 2000; Catania *et al*., 2004; Guarini *et al*., 2004; Getting, 2006). Reduction of free radical discharge may likewise contribute to prevent organ damage (Schlag *et al*., 1991; Redl *et al*., 1993; Cockerill *et al*., 2001). Further, the protective actions of melanocortins in pathological conditions of ischaemia and reperfusion are not restricted

to anti-inflammatory effects. Recently, through a gene expression profiling study, we found multiple protective influences of NDP-α-MSH in experimental heart transplantation that extend beyond inflammatory mediator production to include several pathways (Colombo *et al*., 2005).

In conclusion, the present data give direct evidence for the first time of the antishock and life-saving effects of melanocortin $MC_4$ receptor agonists. Moreover, our data suggest for the first time that selective $MC_4$ agonists, in addition to their effects on systemic inflammatory responses and free radical discharge, could also prevent or significantly reduce late organ damage following a shock condition. In fact, whereas shock either resolves rapidly or is fatal, serious abnormalities in multiple organs occur hours to days after the onset of shock (Wheeler and Bernard, 1999). Using the present experimental model of haemorrhagic shock, which rapidly (within 30–35 min) progresses to irreversibility and death, we have been able to detect only initial signs of organ damage in shocked control rats; further, a proportion of melanocortin-treated shocked rats were still alive 24 h after shock and did not show appreciable signs of organ damage. Therefore, investigations designed at determining the early pathophysiological molecular mechanisms of organ damage following haemorrhagic shock – and the molecular targets of selective $MC_4$ agonists – may help to better assess the protective effect of melanocortins against multiple organ failure. Such findings could be of clinical relevance for the management of circulatory shock.

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## Conflict of Interest

The authors state no conflict of interest.

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