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SPECIAL REPORT

Tumour necrosis factor-α as a target of melanocortins in haemorrhagic shock, in the anaesthetized rat

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The cytokine tumour necrosis factor-α (TNF-α) is involved (mostly through the activation of inducible nitric oxide synthase) in the pathogenesis of circulatory shock. On the other hand, melanocortin peptides are potent and effective in reversing haemorrhagic shock, both in animals (rat, dog) and in humans. This prompted us to study the influence of the melanocortin peptide ACTH-(1–24) on the blood levels of TNF-α in haemorrhage-shocked rats and on the *in vitro* production of TNF-α by lipopolysaccharide (LPS)-activated macrophages. Plasma levels of TNF-α were undetectable before starting bleeding and greatly increased 20 min after bleeding termination in saline-treated rats. In rats treated with ACTH-(1–24) the almost complete restoration of cardiovascular function was associated with markedly reduced levels of TNF-α 20 min after bleeding termination. On the other hand, ACTH-(1–24) did not influence TNF-α plasma levels in sham-operated, unbled rats. *In vitro*, ACTH-(1–24) (25–100 nM) dose-dependently reduced the LPS-stimulated production of TNF-α by peritoneal macrophages harvested from untreated, unbled rats. These results indicate that inhibition of TNF-α overproduction may be an important component of the mechanism of action of melanocortins in reversing haemorrhagic shock.

Keywords: Adrenocorticotropin; haemorrhagic shock; tumour necrosis factor-α; macrophages; rat

Introduction The cardiovascular response to acute haemorrhage occurs in two phases. Initially, sistemic vascular conductance falls as blood volume and cardiac output fall, owing to a sharp increase in plasma noradrenaline levels, so that arterial pressure is well maintained (non-hypotensive, compensated haemorrhage). When the acute blood loss exceeds 30% of blood volume there is no further increase in plasma noradrenaline, the compensatory vasoconstriction fails and blood pressure falls abruptly (hypotensive, decompensated haemorrhage) (Schadt, 1989). This is associated with a massive release of endogenous opioids, an overproduction of tumour necrosis factor-α (TNF-α) and an overproduction of nitric oxide (NO), most likely through the stimulation of the inducible isoform of NO synthase (iNOS) by TNF-α (for reviews see: Bernton *et al*., 1985; Squadrito *et al*., 1994; Zingarelli *et al*., 1994; Bertolini, 1995).

We have recently confirmed, with direct *ex vivo* measurements performed by means of electron spin resonance spectroscopy, that NO is overproduced during severe haemorrhagic shock (Guarini *et al*., 1997). We have shown, moreover, that the haemorrhagic shock reversal produced by the intravenous (i.v.) bolus injection of a melanocortin peptide (for review see: Bertolini, 1995) is associated with the normalization of NO blood levels (Guarini *et al*., 1997), and that inhibition of iNOS enhances the effect of melanocortins in haemorrhagic shock (Bazzani *et al*., 1997).

In order to further define the mechanisms that underlie the effect of melanocortins in haemorrhagic shock, we have now studied their influence on the haemorrhage-induced systemic overproduction of TNF-α.

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Methods Wistar rats of both sexes weighing 210–280 g, were used, 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 86/609; Italian D.L.: 27-1-92, No. 116).

Our model of volume-controlled haemorrhagic shock has been repeatedly described in detail elsewhere (Bertolini, 1995; Bazzani *et al*., 1997; Guarini *et al*., 1997). In brief, under general anaesthesia (urethane, 1.25 g kg^{-1} intraperitoneally) and after heparinization (heparin sodium, 600 iu kg^{-1} i.v.) rats were instrumented with indwelling polyethylene catheters in a common carotid artery and an iliac vein. Systemic arterial pressure and pulse pressure (PP) were recorded by means of a pressure transducer coupled to a polygraph. 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 a preamplifier. Blood (2–2.5 ml 100 g^{-1} body wt) was withdrawn stepwise from the venous catheter over a period of 25–30 min until a condition of volume-controlled haemorrhagic shock was produced, with mean arterial pressure (MAP) reduced to, and stabilized at, 21–23 mmHg.

Twenty minutes after the last bleeding (i.e., 15 min after treatment) 18 rats [nine treated with ACTH-(1–24) and nine treated with equivolume saline] were subjected to venous blood sample withdrawal for evaluation of TNF-α, by means of a biological assay. Blood samples were also collected for the same purpose from 18 sham-operated rats [i.e., anaesthetized with urethane, heparinized, instrumented with arterial and venous catheters, treated with either ACTH-(1–24) or saline, but not bled], 15 min after treatment. For the *in vitro* studies, macrophages were collected from untreated normal rats by peritoneal lavage with RPMI 1640 medium and were incubated for 4 h with RPMI 1640 either alone or with several

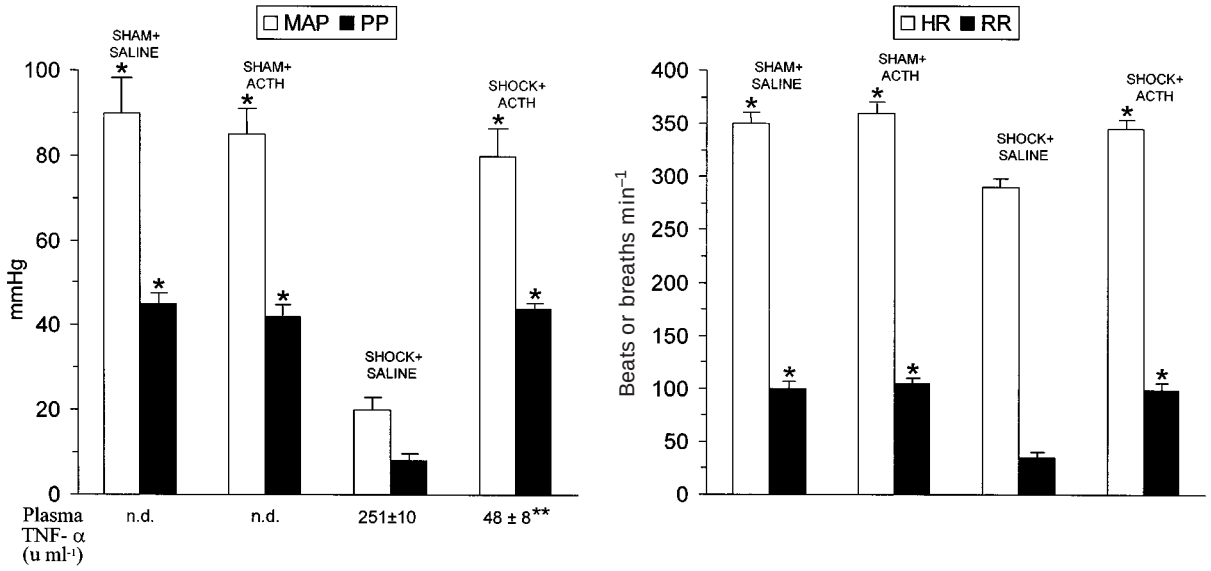

Figure 1 Influence of i.v. treatment with ACTH-(1–24) (ACTH, 160 μg kg^{-1}) or saline (1 ml kg^{-1}) on mean arterial pressure (MAP), pulse pressure (PP), heart rate (HR), respiratory rate (RR) and TNF-α plasma levels in haemorrhage-shocked or sham-shocked rats. Histograms' height and the below values indicate mean values ± s.e.mean obtained 15 min after treatment from nine animals per group; n.d. = not detectable. *$P<0.05$, at least and **$P<0.001$ *versus* the corresponding value of shock + saline (Student-Newman-Keuls' test and Student's *t*-test respectively).

concentrations of ACTH-(1–24) (25, 50 and 100 nM). Cytokine production was evaluated before and after lipopolysaccharide (LPS, *E.coli* 026: B6; Sigma) stimulation (50 μg ml^{-1} for 4 h). Killing of L929 mouse tumour cells was used to measure TNF-α levels in plasma and in peritoneal macrophage supernatants on the basis of a standard assay, as previously described (Squadrito *et al*., 1994).

The adrenocorticotropin fragment 1–24 [ACTH-(1–24), Ciba Geigy], chosen as the most effective melanocortin in the treatment of haemorrhagic shock (for review see: Bertolini, 1995), was freshly dissolved in saline and i.v. bolus injected 5 min after bleeding termination, when MAP was stabilized at 21–23 mmHg, at the maximally effective anti-shock dose of 160 μg kg^{-1} (Bertolini, 1995), in a volume of 1 ml kg^{-1}. Control rats were treated with equivolume saline by the same route and at the same time after bleeding termination. MAP, PP, HR, RR and macrophage TNF-α values were analysed by means of ANOVA followed by Student-Newman-Keuls' test. Plasma TNF-α levels were analysed by means of Student's *t*-test for unpaired data.

Results Figure 1 shows that under this condition of haemorrhagic shock there was an overproduction of TNF-α, whose plasma levels increased from an undetectable pre-bleeding basal value to a value of 251 ± 10 u ml^{-1} 20 min after bleeding termination in saline-treated rats. In rats treated with the melanocortin peptide ACTH-(1–24), at the dose of 160 μg kg^{-1} i.v., the almost complete restoration of cardiovascular and respiratory functions was associated with markedly reduced plasma levels of TNF-α, which were 48 ± 8 u ml^{-1} 20 min after bleeding termination (i.e., 15 min after treatment). On the other hand, ACTH-(1–24) did not affect TNF-α levels, MAP, PP, HR and RR in sham-operated, unbled rats.

In vitro, ACTH-(1–24) (25–100 nM) dose-dependently reduced the LPS-stimulated production of TNF-α in macrophages harvested from untreated, unbled rats (Table 1).

Table 1 *In vitro* dose-effect relationship of ACTH-(1–24) (ACTH: 25, 50 or 100 nM) on TNF-α production by peritoneal macrophages collected from untreated normal rats, before and after LPS stimulation (50 μg ml^{-1})

Treatment	*TNF-α* (u ml^{-1})
Control + RPMI	n.d.
LPS + RPMI	170 ± 8
LPS + ACTH 25	161 ± 5
LPS + ACTH 50	117 ± 5*
LPS + ACTH 100	68 ± 6*

Data, presented as means ± s.e.mean ($n=6$), were obtained 4 h after incubation. RPMI: 1 ml; n.d. = not detectable. *$P<0.001$ *versus* LPS + RPMI (Student-Newman-Keuls' test).

Discussion Our present results confirm that haemorrhagic shock is associated with an overproduction of TNF-α and show that the prompt and sustained, almost complete reversal of the shock condition caused by the i.v. injection of a melanocortin peptide is associated with a markedly curtailed production of TNF-α. Moreover, they show that the *in vitro* production of TNF-α by LPS-stimulated macrophages is also dose-dependently reduced in the presence of a melanocortin peptide.

Available experimental data indicate that several factors may contribute to the decompensation of pressure homeostasis that occurs in haemorrhagic shock. In such a condition there is an activation of endogenous opioid systems and an opioid-mediated inhibition of sympathetic outflow and noradrenaline release (for reviews see: Bernton *et al*., 1985; Bertolini, 1995).

Another line of evidence involves the pleiotropic cytokine TNF-α in the pathophysiology of hypotensive, decompensated haemorrhage. High levels of TNF-α are found in the plasma/serum and macrophages of haemorrhage-shocked rats, as also confirmed by our present data, while being almost undetectable in the plasma/serum of sham-operated animals (Zingarelli *et al*., 1994). However, some reports are at variance with the

above-quoted and our present findings (Stylianos *et al*., 1991). Additional experimental data have suggested a role for TNF-α in other low-flow states: elevated plasma/serum levels of TNF-α have been found in splanchnic artery occlusion shock, in endotoxin-injected animals and human volunteers, in humans with septic shock and in hepatic and myocardial ischaemia-reperfusion injury. Moreover: (i) passive immunization against TNF-α reduces lethality in endotoxic shock, in splanchnic artery occlusion shock, in myocardial infarction and in acute haemorrhagic shock and (ii) systemic administration of human recombinant TNF-α produces a severe hypotension in dogs and in rats (for reviews see: Squadrito *et al*., 1994; Zingarelli *et al*., 1994). It has been recently reported that TNF-α may act on the sympathetic nerve terminals to inhibit noradrenaline release (Foucart & Abadie, 1996). Moreover, TNF-α induces a marked vascular hyporeactivity to alpha$_1$-adrenoceptor stimulating agents, whereas the administration of specific antibodies against TNF-α significantly improves vascular responsiveness to alpha$_1$-adrenoceptor agonists in haemorrhage-shocked rats (for reviews see: Squadrito *et al*., 1994; Zingarelli *et al*., 1994). Finally, *in vitro* studies have shown that TNF-α exerts a transient myocardial depressant effect (Murray & Freeman, 1996).

Other experimental evidences indicate that NO overproduction plays an important role in the pathophysiology and evolution of shock (Thiemermann *et al*., 1993). And we have recently shown that during haemorrhagic shock, in rats, there is an increase in NO arterial blood levels (measured as NO-haemoglobin by means of electron spin resonance spectroscopy). The inducible isoform of NO synthase seems to be involved, since the i.v. administration of S-methylisothiourea, a selective inhibitor of iNOS, provokes the disappearance of NO-haemoglobin in blood and the reversal of the shock condition (Guarini *et al*., 1997). Finally, enhanced formation of NO causes vascular hyporeactivity to noradrenaline (Thiemermann *et al*., 1993) and inhibits noradrenaline release from sympathetic terminals (Schwarz *et al*., 1995).

Close links exist between TNF-α and NO synthases. The transition from compensated, non-hypotensive haemorrhage to decompensated, hypotensive haemorrhage is characterized by a large increase in TNF-α blood levels and an overproduction of NO takes place at this moment, most likely through the stimulation of iNOS by TNF-α (Squadrito *et al*., 1994; Zingarelli *et al*., 1994).

A large number of experimental data indicates that the anti-shock effect of melanocortins may be, at least in part, the consequence of a functional antagonism towards endogenous opioids, which are released in shock conditions (for review see: Bertolini, 1995). On the other hand, melanocortins are also potent inhibitors of NO overproduction, both *in vitro*, in cultured macrophages stimulated with bacterial LPS and interferon gamma (Star *et al*., 1995) and *in vivo*, in experimental conditions of haemorrhagic and endotoxic shock in rats (Abou-Mohamed *et al*., 1995; Guarini *et al*., 1997). *In vitro*, ACTH-(1–24), when applied 6 h after LPS, does not significantly modify nitrite overproduction by stimulated macrophages; in contrast, when it is applied together with LPS, significantly prevents nitrite overproduction (Squadrito, personal communication): these data suggest that ACTH inhibits iNOS induction rather than its activity. Moreover, the effect of melanocortins in haemorrhagic shock is prevented by L-arginine and enhanced by NOS inhibitors (Bazzani *et al*., 1997).

Finally, α-melanocyte stimulating hormone antagonizes the pyrogenic action of TNF-α in rabbits (Martin *et al*., 1991) and would partially block macrophage-mediated tumour cytotoxicity, probably as a result of the inhibition of TNF-α with respect to both secretion by macrophages and effect on melanoma cells (Eberle, 1988).

Our present data show that melanocortin peptides not only inhibit the haemorrhage-induced overproduction of TNF-α *in vivo*, but also the production of TNF-α *in vitro* by LPS-stimulated macrophages. This latter finding seems to rule out the possibility that the *in vivo* effect may merely be the consequence (and not one of the possible mechanisms) of the melanocortin-induced shock reversal.

Macrophages, as well as other immune cells, synthesize the precursor molecule of melanocortin peptides (pro-opiomelanocortin, POMC) and process it to corticotropin [ACTH-(1–39)] as well as to shorter ACTH fragments, including ACTH-(1–24) (Blalock, 1989). Moreover, they have receptors for melanocortins and it has been suggested that in stressful situations these neuropeptides may play a role as transmitters of signals from the neuroendocrine system to the immune system (and *vice versa*) (Blalock, 1989). Direct effects of malenocortin peptides on specific functions of macrophages have indeed been observed in *in vitro* experiments (Genedani *et al*., 1992).

In conclusion, our present data show that the melanocortin peptide ACTH-(1–24) inhibits the overproduction of TNF-α induced by severe bleeding in rats and suggest that this effect may play an important role in the complex mechanism of action of melanocortin-induced shock reversal. Overall, functional antagonism towards endogenous opioids (Bertolini, 1995), and inhibited overproduction of TNF-α (present data) and NO (Guarini *et al*., 1997) (which are probably related) would remove the main causes of haemorrhage-induced circulatory decompensation, namely, the blunted release of noradrenaline from sympathetic terminals and the reduced responsivity of resistance vessels to noradrenaline.

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