

Adrenocorticotropin reverses hemorrhagic shock in anesthetized rats through the rapid activation of a vagal anti-inflammatory pathway

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Abstract

Objective: Several melanocortin peptides have a prompt and sustained resuscitating effect in conditions of hemorrhagic shock. The transcription nuclear factor κ B (NF- κ B) triggers a potentially lethal systemic inflammatory response, with marked production of tumor necrosis factor- α (TNF- α), in hemorrhagic shock. Here we investigated whether the hemorrhagic shock reversal produced by the melanocortin ACTH-(1–24) (adrenocorticotropin) depends on the activation of the recently recognized, vagus nerve-mediated, brain “cholinergic anti-inflammatory pathway”. **Methods and results:** Anesthetized rats were stepwise bled until mean arterial pressure (MAP) stabilized at 20–25 mm Hg. The severe hypovolemia was incompatible with survival, and all saline-treated animals died within 30 min. In rats intravenously (i.v.) treated with ACTH-(1–24), neural efferent activity along vagus nerve (monitored by means of a standard system for extracellular recordings) was markedly increased, and the restoration of cardiovascular and respiratory functions was associated with blunted NF- κ B activity and with decreased TNF- α mRNA liver content and TNF- α plasma levels. Bilateral cervical vagotomy, pretreatment with the melanocortin MC₄ receptor antagonist HS014, atropine sulfate or chlorisondamine, but not with atropine methylbromide, prevented the life-saving effect of ACTH-(1–24) and the associated effects on NF- κ B activity and TNF- α levels. HS014 and atropine sulfate prevented, too, the ACTH-(1–24)-induced increase in neural efferent vagal activity, and accelerated the evolution of shock in saline-treated rats. **Conclusions:** The present data show, for the first time, that the melanocortin ACTH-(1–24) suppresses the NF- κ B-dependent systemic inflammatory response triggered by hemorrhage, and reverses shock condition, by brain activation (in real-time) of the “cholinergic anti-inflammatory pathway”, this pathway seeming to be melanocortin-dependent.

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1. Introduction

In conditions of circulatory shock, systemic inflammatory response plays a fundamental pathogenetic role, with activation of transcription nuclear factors (mainly NF- κ B) and markedly increased production of cytokines (mainly TNF- α) [1–4]. The brain has an important modulatory role

in such response, through both well-known humoral mechanisms [5–7] and the rapid activation (in “real-time”) of efferent vagus nerve fibres (the recently recognized “brain cholinergic anti-inflammatory pathway”) [4,8,9].

Several melanocortin peptides—namely, those of the adrenocorticotropin/ α -melanocyte-stimulating hormone (ACTH/ α -MSH) group—such as ACTH-(1–24), α -MSH, shorter fragments and synthetic analogues, have a life-saving effect in animal and human conditions of hemorrhagic shock, as well as in other animal models of shock and

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in other severe hypoxic conditions [10–17]. The antishock effect is adrenal-independent, dose-dependent, can be obtained with intracerebroventricular (i.c.v.) injection of doses much lower than those needed by the i.v. route, and is mediated by melanocortin MC₄ receptors located in the central nervous system (CNS) [10,13,18]. Such effect is associated with a marked reduction in the plasma levels of inflammatory mediators, such as TNF- α , oxygen free radicals and nitric oxide, and in intercellular adhesion molecule expression by vascular endothelium [2,14,19,20]. This is in agreement with the notion that melanocortins have a peculiar anti-inflammatory activity [7,21]. The antishock effect of melanocortins is prevented by bilateral cervical vagotomy, by the i.c.v. injection of the acetylcholine-depleting agent hemicholinium-3, or of the N-calcium channel blocker ω -conotoxin, and by the muscarinic antagonist atropine sulfate, but not by atropine methylbromide, that cannot cross the blood–brain barrier (for review, see Ref. [13]). These data suggest an important role of CNS cholinergic pathways involving muscarinic receptors; and, indeed, cholinomimetic agents able to cross the blood–brain barrier reverse hemorrhagic shock in rats and rabbits [22–24].

Overall, these observations prompted us to investigate whether, in hemorrhage-shocked rats, the melanocortin ACTH-(1–24), through CNS MC₄ receptors, triggers the activation of the recently identified cholinergic anti-inflammatory pathway [4,8,9], with consequent suppression of NF- κ B and inflammatory cascade activation.

2. Materials and methods

2.1. Acute hemorrhagic shock model

Wistar rats of both sexes (270–300 g body wt) (Harlan, Italy) were used; food in pellets and tap water were available ad libitum. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Under general anesthesia [urethane, 1.25 g/kg intraperitoneally (i.p.)] and after heparinization (heparin sodium, 600 iu/kg 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, Italy). Arterial blood pressure was reported as MAP and pulse pressure (PP). Respiratory rate (RR) was recorded by means of three electrodes subcutaneously (s.c.) implanted on the chest and connected through a preamplifier to the above polygraph. In vagotomized rats RR was not recorded, because of no meaning. Acute hemorrhagic 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 body wt) until MAP, automatically calculated and continuously digitally displayed on the polygraph, fell to and

stabilized at 20–25 mm Hg [2,10,11,18–20,22]. Sham shocked rats were subjected to all surgical procedures experienced by hemorrhage-shocked animals, but were not bled. A group of rats was prepared for i.c.v. treatment as previously described [18], 1 week before the experiment, by stereotaxically implanting, under ketamine plus xylazine anesthesia (115 + 2 mg/kg i.p.), stainless-steel guide cannulae into a brain lateral ventricle.

2.2. Vagotomy and recording of efferent activity along vagus nerve

In some experimental groups, bilateral cervical vagotomy was performed 2 min before starting bleeding, as previously described [8,25]. Shocked or sham shocked rats are to be considered also sham vagotomized. Neural efferent activity along vagus nerve was monitored by means of a standard system for extracellular recordings [26]. Briefly, left rostral vagal trunk was placed on a silver wire electrode (recording electrode), and an indifferent electrode was positioned nearby in the wound. Vagal action potentials were fed into an AC amplifier (A-M Systems, model 1800) and displayed on an oscilloscope screen. Signals were passed through a band-pass filter (100–1000 Hz), and filtered nerve activity was recorded and analyzed using a Pentium III computer equipped with Digidata 1322A data acquisition system and Axoscope 8.1 software (Axon Instruments, USA). The efferent impulses were integrated at constant times with Clampfit software so to evaluate total efferent vagal activity.

2.3. Drugs and treatments

All drugs were dissolved in saline (1 ml/kg for i.v., i.p. and s.c. injections; 5 μ l/rat for i.c.v. injections). ACTH-(1–24) (160 μ g/kg; Sigma, USA) or saline were injected as i.v. bolus 5 min after bleeding termination, when MAP was stabilized at 20–25 mm Hg [10,11,18–20]. The dose of ACTH-(1–24) had proven to be maximally effective in restoring cardiovascular and respiratory functions, and in increasing survival rate, in hemorrhage-shocked rats [27]. Atropine sulfate and atropine methylbromide (Sigma) were administered i.p. (2 mg/kg) [25,28], chlorisondamine diiodide (Tocris, UK) was administered s.c. (0.25 mg/kg) [8] and HS014 (Sigma) was injected i.c.v. (5 μ g/rat) [18], 2 min before starting bleeding. Control animals (shocked or sham shocked) received equal volumes of saline by the same routes. Cardiovascular and respiratory parameters were continuously monitored for 3 h after treatment, or until prior death. Efferent vagus nerve activity was recorded for 30 min following treatment.

2.4. Isolation of nuclear and cytoplasmatic proteins

As previously described [3,8], 70 mg of pulverized liver samples, obtained from shocked or sham shocked rats 10–15 min after treatment with ACTH-(1–24) or with saline, were

homogenized in 0.8 ml ice cold hypotonic buffer [10 mM HEPES pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DDT); protease inhibitors: 0.5 mM phenyl methylsulfonyl fluoride, aprotinin, pepstatin, leupeptin (10 µg/ml each); and phosphatase inhibitors: 50 mM NAF, 30 mM β-glycerophosphate, 1 mM Na₃VO₄ and 20 mM ρ-nitrophenyl phosphate]. The homogenates were centrifuged (2000 rpm) for 30 s at 4 °C to eliminate any unbroken tissues. The supernatants were incubated on ice for 20 min, vortexed for 30 s after addition of 50 µl of 10% Nonidet P-40 and then centrifuged for 1 min at 4 °C in an Eppendorf centrifuge. Supernatants containing cytoplasmatic protein were collected and stored at –80 °C. The pellets, after a single wash with the hypotonic buffer without Nonidet P-40, were suspended in an ice-cold hypertonic salt buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitors, and phosphatase inhibitors), incubated on ice for 30 min, mixed frequently, and centrifuged for 15 min at 4 °C. The supernatants were collected as nuclear extracts and stored at –80 °C. The concentration of total proteins in the samples was determined by a commercially available protein assay reagent (Microbca, protein assay kit, USA).

2.5. Electrophoretic mobility shift assay (EMSA)

NF-κB binding activity was performed [3,8] in a 15-µl binding reaction mixture containing 1% binding buffer [50 µg/ml of double-stranded poly(dI-dC), 10 mM Tris HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM MgCl₂, and 10% glycerol], 15 µg of nuclear proteins, and 35 fmol (50000 cpm, Cherenkov counting) of double-stranded NF-κB consensus oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') which was end-labeled with [³²P]ATP (3000 Ci/mmol at 10 mCi/ml; Amersham Life Sciences, USA) using T4 polynucleotide kinase. The binding reaction mixture was incubated at room temperature for 20 min and analyzed by electrophoresis on 5% non-denaturing polyacrylamide gels. After electrophoresis, the gels were dried using a gel-drier and exposed to Kodak X-ray films at –70 °C. The binding bands were quantified by scanning densitometry of a bio-image analysis system (Bio-Profil, Italy). The results for each group were expressed as relative integrated intensity compared with the sham shock group liver measured in the same batch, because the integrated intensity of group samples from different electrophoretic mobility shift assay (EMSA) batches would be affected by the half-life of the isotope, exposure time and background levels.

2.6. Western blot analysis of IκBα in cytoplasm

Cytoplasmatic proteins (40 µg) from each liver sample were mixed with 2 × SDS sample buffer [62 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue], heated at 95 °C for 5 min, and separated by SDS-polyacrylamide gel electrophoresis. After electrophoresis on 12.5% polyacrylamide gels, the separated

proteins were transferred from the gels into Hybond electrochemiluminescence membranes (Amersham) using a Bio-Rad semidry transfer system (Bio-Rad, USA) for 2 h. The membranes were blocked with 5% not-fat dry milk in TBS–0.05% Tween for 1 h at room temperature, washed three times for 10 min each in TBS–0.05% Tween 20, and incubated with a primary IκBα antibody (Santa Cruz Biotechnology, USA) in TBS–0.05% Tween 20 containing 5% not-fat dry milk for 1–2 h at room temperature. After being washed three times for 10 min each in TBS–0.05% Tween 20, the membranes were incubated with a second antibody peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham). The IκBα protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-profil, Italy) [3,8]. The results from each experimental group were expressed as relative integrated intensity compared with sham shocked liver measured with the same batch.

2.7. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted [3,8] from liver sections obtained from shocked or sham shocked rats 10–15 min after treatment with ACTH-(1–24) or with saline. In brief, approximately 100 mg of liver was homogenized with 800 µl RNAzol STAT (Teltest, USA) in a microfuge tube, after which 80 µl chloroform was added. After vortexing and centrifugation, the aqueous phase was transferred to a new microfuge tube containing an equal volume of cold isopropanol and the RNA recovered by precipitation by chilling at –80 °C for 15 min. The pellet was washed with cold ethanol 70%, centrifuged, dried in speed vacuum, centrifuged a second time and then dissolved in 20 µl of buffer. A 2 µg portion of total RNA was subjected to first strand cDNA synthesis in a 20-µl reaction mixture containing the AMV reverse transcriptase (Superscript II, BRL, USA), each dNTP, the specific primers, Tris–HCl and MgCl₂. After dilution of the product with distilled water, 5 µl were used for each polymerase chain reaction (PCR) which contained the Taq polymerase (Perkin Elmer, Italy), the buffer as supplied with the enzyme, each dNTP and the specific primers, designed to cross introns and to avoid confusion between mRNA expression and genomic contamination. The following oligonucleotide pairs were used (5' oligo/ 3' oligo), each sequence as 5' to 3':

TNF-α: CACGCTCTTCTGTCTTACTGA/GGACT-CCGTGATGTCTAAGT
 GAPDH: ACCACCATGGAGAAGGTCGG/CTCAGTGTAGCCCAGGATGGC.

The optimal cycle number for TNF-α was 25 and we used a PCR negative and a PCR positive control without

cDNA or with a known cDNA, respectively. A portion of the PCR product was electrophoresed into a gel. The gel was analyzed in a dark-room with a fixed camera. The captured image, sent to an image analysis software (Bio-Profil, Italy) was subjected to densitometric analysis.

2.8. Plasma TNF- α levels

Blood (750 μ l) was drawn from shocked or sham shocked rats 10–15 min after treatment with ACTH-(1–24) or with saline. Blood samples were collected in EDTA tube and centrifuged for 10 minutes at 5000 rpm; plasma was then removed and stored at -20°C until the analysis. The day of the assay, after reconstituting the reagents, the amount of TNF- α was measured with a specific ELISA kit (Genzyme,

USA) and compared with a TNF- α standard curve which demonstrated a direct relationship between optical density and cytokine concentration [3,8]. The assay was then visualized using a streptavidin alkaline phosphatase conjugate and an ensuing chromogenic reaction.

2.9. Statistical analysis

Data are expressed as means \pm SEM and were analyzed by means of analysis of variance (ANOVA) followed by a multiple comparison test (Student–Newman–Keuls). A probability error of less than 0.05 was selected as criterion for statistical significance. For survival rate, statistical analysis was done by Fisher's exact probability test. Data from control rats pretreated with saline instead of the

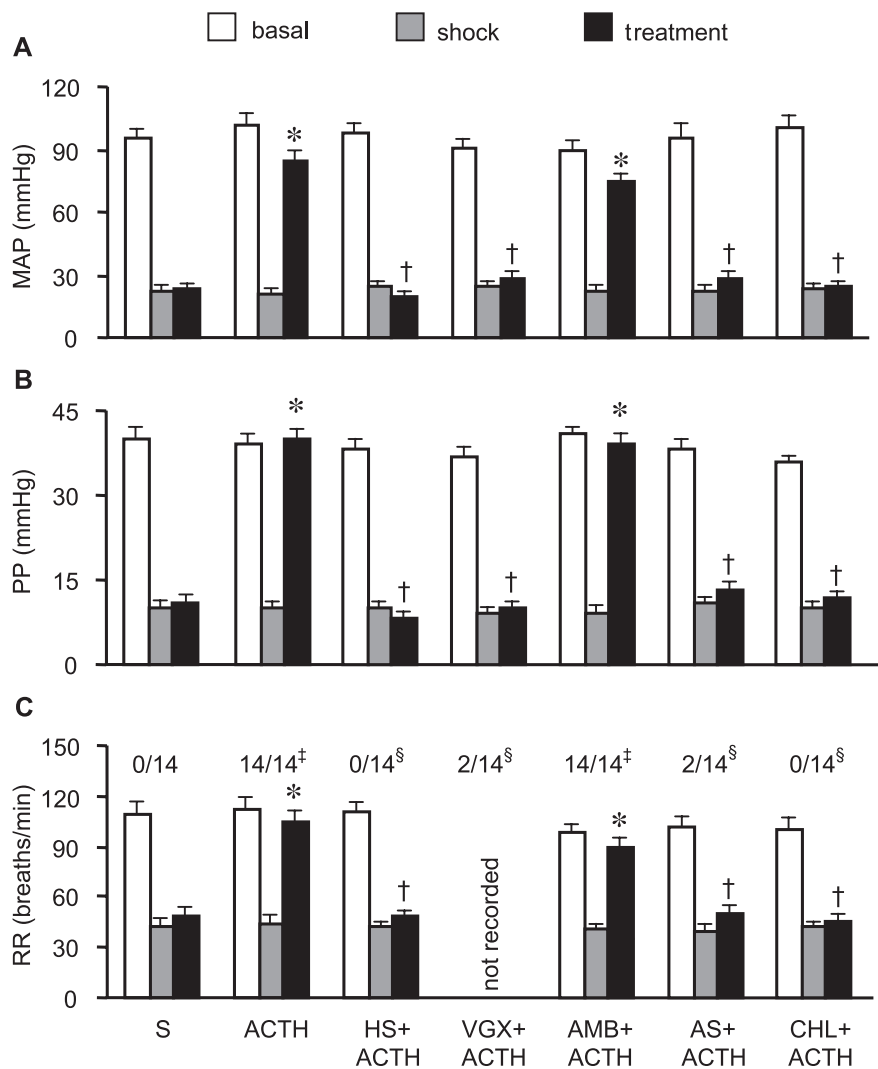


Fig. 1. The melanocortin ACTH-(1–24) improves mean arterial pressure (MAP, panel A), pulse pressure (PP, panel B), respiratory rate (RR) and survival rate (panel C) in rats subjected to hemorrhagic shock. Vagotomy (VGX) or pretreatment with HS014 (HS), or atropine sulfate (AS) and chlorisondamine (CHL), but not with atropine methylbromide (AMB), prevents the antishock effect of ACTH-(1–24). Histograms' height indicates mean values \pm SEM ($n=14$) obtained in basal conditions, after bleeding (shock) and 10–15 min after treatment with saline (S) or ACTH-(1–24) (ACTH). * $P<0.001$ versus saline; † $P<0.001$ versus ACTH alone. Survival rates 3 h after treatment (panel C, above the histograms): ‡ $P<0.001$ versus saline; § $P<0.001$ versus ACTH alone.

various antagonists are not reported in the figures, because of no meaning.

3. Results

3.1. ACTH-(1–24) improves MAP, PP, RR and survival rate

As repeatedly reported [2,3,10,11,18–20,22,25,27,28], the severe and acute hypovolemia induced in our model of volume-controlled hemorrhagic shock was incompatible with survival, and all saline-treated control animals died within 30 min (Fig. 1). The i.v. bolus injection of ACTH-(1–24) at a dose (160 $\mu\text{g}/\text{kg}$) maximally effective in reversing hypovolemic/hemorrhagic shock in rats—but devoid of any cardiovascular effect in the normal animal—[2,10,11,13,14,18–20,27] produced, within a few min, an almost complete restoration of cardiovascular and respiratory functions: 10–15 min after treatment, MAP, PP and RR attained values not significantly different from baseline (Fig. 1), and kept so during the 3-h observation period, with 100% survival rate (Fig. 1).

3.2. ACTH-(1–24) stimulates the activity of efferent vagal fibres through the activation of brain MC_4 receptors

To verify our hypothesis, i.e. that in hemorrhagic shock melanocortins may activate a life-saving, vagus nerve-mediated, cholinergic anti-inflammatory pathway through the stimulation of CNS MC_4 receptors, we recorded the neural

efferent activity along vagus nerve. The action potential recordings at the end of bleeding showed that there was a significant compensatory increase in efferent vagal activity (see below), which in shocked rats treated with saline progressively decreased until animal death (within 30 min). On the contrary, in shocked rats treated with ACTH-(1–24) there was a prompt (within a few min) and marked increase in efferent vagal activity, the maximum effect being observed after 10–15 min (Fig. 2) and keeping quite stable during the 30-min observation period. The same dose of ACTH-(1–24) did not modify efferent vagal activity in normal, non-bled rats (not shown). Either bilateral cervical vagotomy or i.c.v. pretreatment with the selective MC_4 receptor antagonist HS014 (at a dose, 5 $\mu\text{g}/\text{rat}$, maximally effective on the basis of previous dose–response studies) [18] prevented the effects of ACTH-(1–24) on cardiovascular and respiratory functions, and on survival (Fig. 1). HS014 also counteracted the stimulating effect of ACTH-(1–24) on efferent vagal activity (Fig. 2). Moreover, in rats subjected either to bilateral cervical vagotomy or to pretreatment with HS014, the volume of blood to be withdrawn in order to induce shock was significantly less respect to intact (unvagotomized and untreated) rats (intact: 2.21 ± 0.10 ml/100 g body wt, $n=28$; vagotomized: 1.85 ± 0.12 , $n=28$, $P<0.05$; HS014-pretreated: 1.75 ± 0.11 , $n=22$, $P<0.005$). Accordingly, in rats pretreated with HS014 the compensatory, bleeding-induced increase in efferent vagal activity was lacking (unpretreated: $171 \pm 10\%$, $n=10$; HS014-pretreated: $65 \pm 4\%$, $n=8$; $P<0.001$; values at the end of bleeding normalized to sham bleeding = 100%).

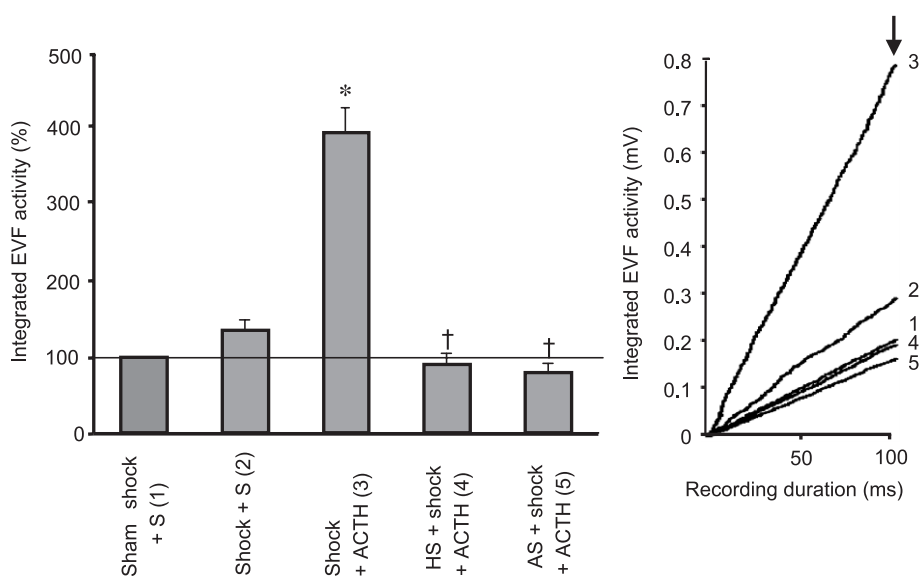


Fig. 2. The melanocortin ACTH-(1–24) stimulates the activity of efferent vagal fibres (EVF) in rats subjected to hemorrhagic shock. Pretreatment with HS014 (HS) or atropine sulfate (AS) prevents the effect of ACTH-(1–24). Histograms' height indicates mean values \pm SEM ($n=8$) of the integrated EVF activity, normalized to sham shock+saline (=100%), obtained in sham shocked or shocked rats 10–15 min after treatment with saline (S) or ACTH-(1–24) (ACTH). The right of the figure shows representative integrated traces of the EVF activity. For data comparison, we used integrated values obtained at the end of a 100-ms period (arrow) for each electrophysiological recording. * $P<0.005$ versus shock+saline; † $P<0.005$ versus shock+ACTH.

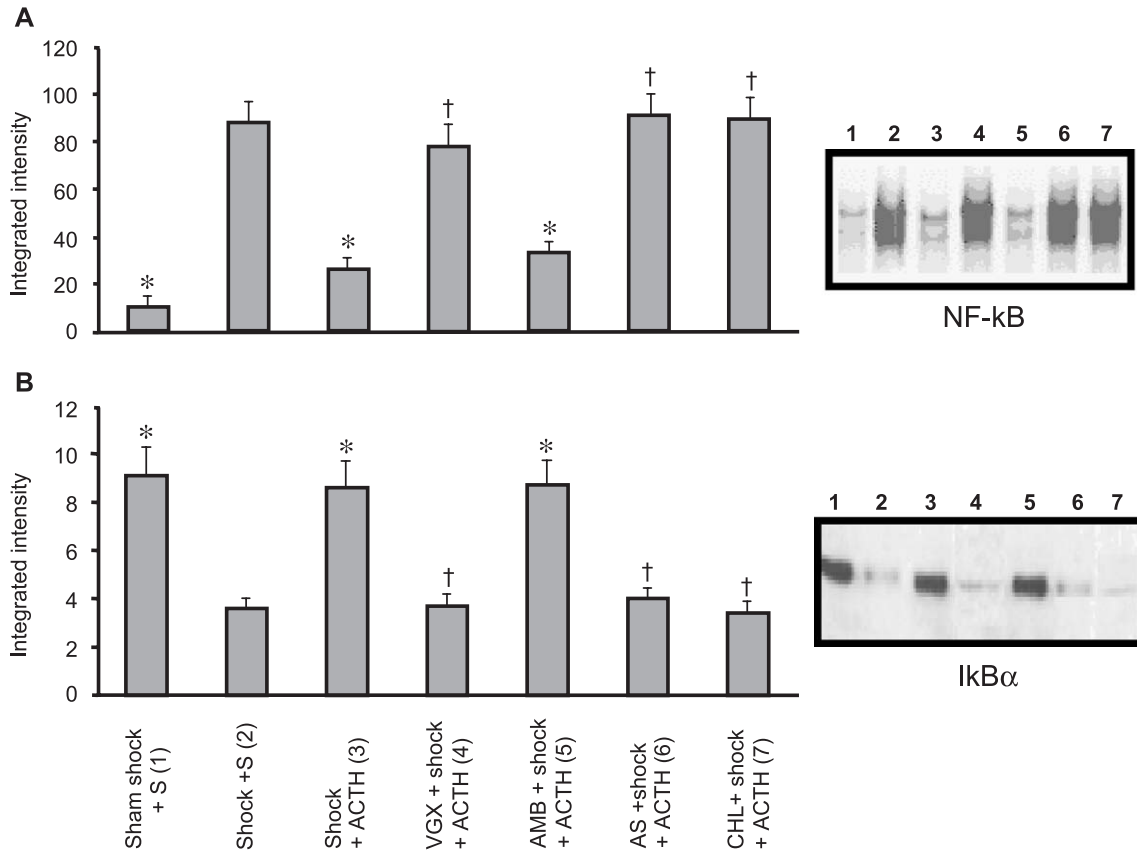


Fig. 3. The melanocortin ACTH-(1–24) inhibits liver NF- κ B activation (panel A) and counteracts the loss of I κ B α protein expression (panel B) in rats subjected to hemorrhagic shock. Vagotomy (VGX) or pretreatment with atropine sulfate (AS) or chlorisondamine (CHL), but not with atropine methylbromide (AMB), prevents the effect of ACTH-(1–24). Histograms' height indicates mean values \pm SEM ($n=10$) obtained in sham shocked or shocked rats 10–15 min after treatment with saline (S) or ACTH-(1–24) (ACTH). The right of panel A shows representative EMSA picture highlighting NF- κ B binding activity, and that of panel B shows representative autoradiograms highlighting I κ B α protein levels. * $P<0.005$ versus shock + saline; † $P<0.005$ versus shock + ACTH.

3.3. ACTH-(1–24) inhibits liver NF- κ B activation, I κ B α reduction, TNF- α mRNA liver content, and TNF- α plasma levels

Massive acute hemorrhage triggers an inflammatory response characterized by the potentially lethal up-regulation of cytokine expression in macrophages [29]. To investigate whether the ACTH-(1–24)-activated parasympathetic pathway suppresses the inflammatory response to acute hemorrhage, we measured liver NF- κ B activation 10–15 min after treatment (when the melanocortin antishock effect attains its maximum). Inactive NF- κ B is present in the cytoplasm, complexed with the inhibitory protein I κ B α [30]; so, we also measured liver I κ B α . Moreover, we measured the liver TNF- α mRNA expression, and TNF- α plasma levels. Liver activation of NF- κ B causes, in fact, an increased hepatic production of TNF- α in shock conditions [8]. NF- κ B activity, which was very low in sham shocked rats, markedly increased in rats subjected to hemorrhagic shock; treatment with ACTH-(1–24) blunted NF- κ B activation (Fig. 3). The inhibitory protein I κ B α was significantly reduced in hemorrhage-shocked rats; treatment with ACTH-(1–24) counteracted such reduction (Fig. 3). Final-

ly, liver TNF- α mRNA and circulating levels of TNF- α greatly increased in hemorrhage-shocked rats (Fig. 4); treatment with ACTH-(1–24) significantly blunted such increase (Fig. 4). Cervical vagotomy, as well as i.c.v. pretreatment with HS014 (not shown), as expected prevented the effect of ACTH-(1–24) on NF- κ B activity, and on I κ B α , circulating TNF- α and TNF- α mRNA levels (Figs. 3 and 4).

3.4. The blockade of brain muscarinic receptors and of peripheral nicotinic receptors prevents the antishock effect of ACTH-(1–24)

All the above-described effects of ACTH-(1–24) in hemorrhage-shocked rats were prevented by atropine sulfate (antagonist of the acetylcholine muscarinic receptors, able to cross the blood–brain barrier), but not by atropine methylbromide (which cannot cross the blood–brain barrier and only blocks the peripheral muscarinic receptors for acetylcholine) (Figs. 1–4). Moreover, pretreatment with atropine sulfate significantly reduced the volume of blood to be withdrawn in order to induce shock [1.78 ± 0.08 ml/100 g body wt, $n=32$, $P<0.002$ versus intact (unvagotomized and

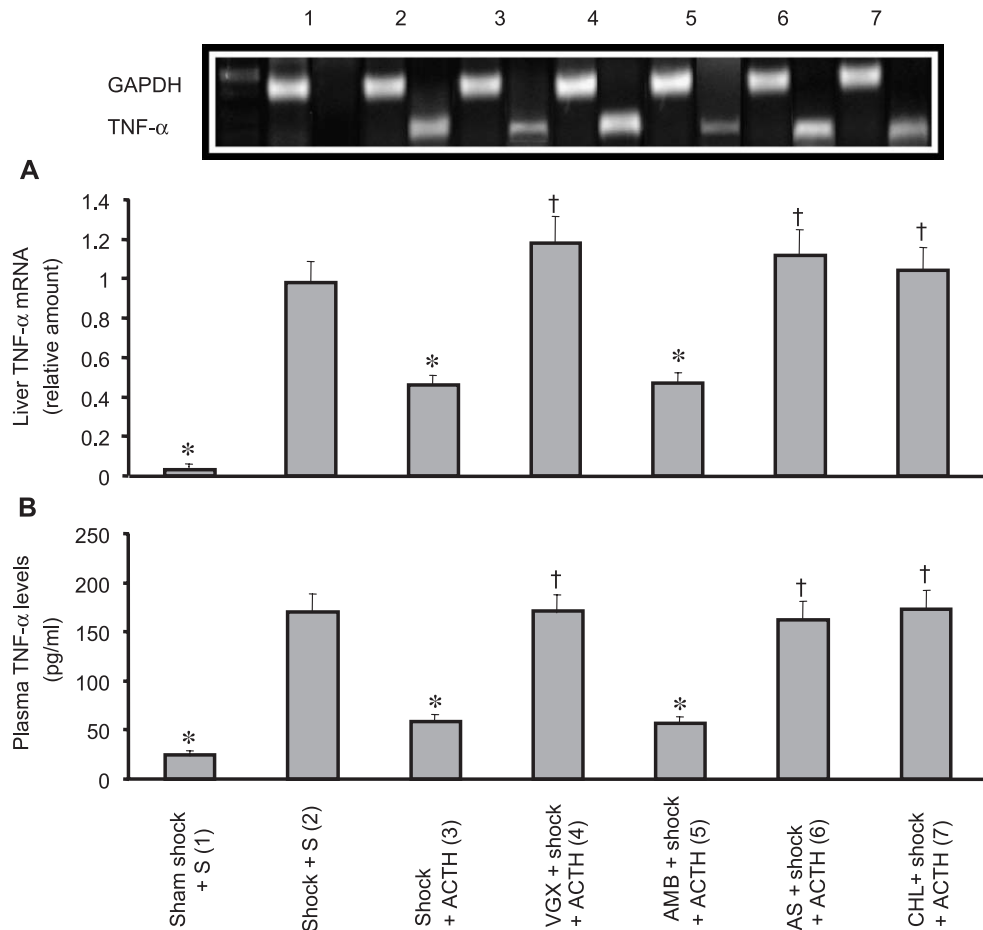


Fig. 4. The melanocortin ACTH(1–24) decreases liver TNF- α mRNA expression (panel A) and TNF- α plasma levels (panel B) in rats subjected to hemorrhagic shock. Vagotomy (VGX) or pretreatment with atropine sulfate (AS) or chlorisondamine (CHL), but not with atropine methylbromide (AMB), prevents the effect of ACTH(1–24). Histograms' height indicates mean values \pm SEM ($n=10$) obtained in sham shocked or shocked rats 10–15 min after treatment with saline (S) or ACTH(1–24) (ACTH). The top of panel A shows representative gel highlighting peak TNF- α mRNA expression. GAPDH = Glyceraldehyde 3-phosphate dehydrogenase. * $P<0.005$ versus shock + saline; † $P<0.005$ versus shock + ACTH.

unpretreated) rats]. Accordingly, the above reported compensatory increase in efferent vagal activity during bleeding was lacking in rats pretreated with atropine sulfate ($75 \pm 4\%$, $n=8$, $P<0.001$ versus unpretreated rats). Also s.c. pretreatment with the irreversible nicotinic receptor antagonist chlorisondamine, at a dose able to block only peripheral receptors [8], prevented the life-saving effect of ACTH(1–24) (Fig. 1) and the associated effect on liver NF- κ B activity, I κ B α and TNF- α mRNA liver content, and TNF- α plasma levels (Figs. 3 and 4). Chlorisondamine caused also a significant reduction in the volume of blood to be withdrawn in order to induce shock (1.80 ± 0.09 ml/100 g body wt, $n=24$, $P<0.005$ versus intact rats).

4. Discussion

Circulatory shock, including hemorrhagic shock, is a severe condition characterized by systemic inflammatory response, with accumulation of inflammatory cells in several tissues, mostly lung and liver, and overproduction of

several cytokines, chemokines, cell adhesion molecules, proteases, oxygen free radicals and other inflammatory mediators [1–4,8,9,14,19,20,29,31]. The ubiquitous transcription factor NF- κ B triggers the inflammatory response by activation of genes expressing cytokines, chemokines and cell adhesion molecules [30]. NF- κ B-triggered inflammatory cascade is early activated also during acute hemorrhage, with consequent overproduction of TNF- α , a pleiotropic cytokine that plays an important role in circulatory failure and consequent organ damage [1,3,8].

CNS modulates the inflammatory response to various stressor agents through humoral mechanisms [5–7,9]. The stimulation of afferent vagus nerve fibres by endotoxin or cytokines, in fact, slowly activates the hypothalamic–pituitary–adrenal anti-inflammatory pathway. Recently, it has been reported that in experimental endotoxic or hemorrhagic shock in rats, electrical stimulation of efferent vagal fibres rapidly reverses hypotension, counteracts hepatic NF- κ B activation, reduces TNF- α plasma levels and improves survival rate [4,8]. This effect seems to be mediated by peripheral nicotinic receptors located on liver resident macrophages, that

play an important role in shock. These observations have led to hypothesize a “cholinergic anti-inflammatory pathway” by which the brain modulates in real-time the systemic inflammatory response in circulatory shock [4,8,9].

Here we demonstrate that, in hemorrhage-shocked rats, the melanocortin ACTH-(1–24), through CNS MC₄ receptors, triggers the activation of the vagus nerve-mediated brain cholinergic anti-inflammatory pathway, with consequent suppression of NF-κB and inflammatory cascade activation, and reversal of the shock condition.

In fact, our results show that ACTH-(1–24) improves cardiovascular and respiratory functions, and survival rate, by increasing efferent vagal activity. On the other hand, either bilateral cervical vagotomy or i.c.v. pretreatment with the selective MC₄ receptor antagonist HS014 prevents the life-saving effect of ACTH-(1–24). HS014 also blunts the stimulating effect of ACTH-(1–24) on efferent vagal activity, reduces, like bilateral cervical vagotomy, the volume of blood to be withdrawn in order to induce shock, and prevents the compensatory increase in efferent vagal activity during bleeding. Overall, these results indicate that the blockade of either brain MC₄ receptors or efferent vagal transmission accelerates the evolution of shock, suggesting that, through these pharmacological and surgical procedures, a physiological antishock pathway becomes blocked.

Moreover, we show that ACTH-(1–24) inhibits liver NF-κB activation, IκBα reduction, TNF-α mRNA liver content and TNF-α plasma levels. These findings give further evidence that melanocortins are endogenous peptides with a peculiar anti-inflammatory activity [7,21]. Previously, it has been reported that the anti-inflammatory effect of centrally administered α-MSH in mouse models of local inflammation is prevented by spinal cord transection, but not by blockade of acetylcholine muscarinic receptors [32]. Here we unequivocally demonstrate that ACTH-(1–24) requires, at least in conditions of systemic inflammatory response as occurs in circulatory shock, vagal acetylcholine to counteract a transcriptional mechanism of inflammatory cytokine production. In fact, not only the blockade of brain acetylcholine muscarinic receptors (also in normal, non-shocked animals melanocortins increase the cholinergic transmission in CNS) [33], but also that of peripheral acetylcholine nicotinic receptors prevents all the above-described effects of ACTH-(1–24) in hemorrhage-shocked rats. Such nicotinic receptors are very likely located on liver resident macrophages [4,8,29] (lipopolysaccharide-activated macrophages express nicotinic receptor α7 subunits, extremely sensitive to acetylcholine and nicotine, whose stimulation inhibits the release of proinflammatory cytokines) [4,34]. Accordingly, nicotine and dimethylphenylpiperazinium reverse hemorrhagic shock through a peripheral, nicotinic receptor-mediated mechanism [25,28]. Quite recent data [35] suggest that also cardiac (but not pulmonary) TNF-α production may be a target of the parasympathetic anti-inflammatory pathway.

Interestingly, independent lines of evidence have demonstrated that melanocortins exert their well-known and pecu-

liar anti-inflammatory activities mainly by a central mechanism, that leads to reduce the production of proinflammatory cytokines and chemokines and to increase the production of the anti-inflammatory cytokine interleukin-10 (IL-10), through inhibition of NF-κB activation (for reviews, see Refs [7,21]). Indeed, according to the recently identified parasympathetic anti-inflammatory pathway [4,8,9], brain modulates the systemic inflammatory response in shock conditions by inhibiting macrophage production and release of proinflammatory cytokines, but not of the anti-inflammatory cytokine IL-10.

The possibility that anti-inflammatory and antishock effects of ACTH-(1–24), including regulation of NF-κB activity, may be an indirect consequence of corticosterone release from adrenal glands can be easily ruled out. In fact, it has been repeatedly reported that these effects are adrenal-independent [7,10,11,13,14,36,37], and we previously showed that methylprednisolone, at high doses, is unable to reverse our experimental condition of hemorrhagic shock in rats [38]. Accordingly, in endotoxin shock, the increase in efferent vagal activity by means of electrical stimulation of efferent vagal fibres does not provoke any increase in corticosterone release [4].

In conclusion, the present results show, for the first time, that ACTH-(1–24) suppresses the NF-κB-dependent systemic inflammatory response triggered by hemorrhage, and reverses shock condition, by activation in the CNS of a cholinergic anti-inflammatory pathway, with involvement of brain muscarinic and, as main final step, of liver nicotinic receptors. A posttranscriptional mechanism of cytokine synthesis, inhibited by acetylcholine, has been described in isolated macrophages of healthy volunteers [4]. The here described transcriptional mechanism, suppressed by the endogenous peptide ACTH-(1–24) through vagal acetylcholine, seems to be specific for shock conditions. The fact that shock evolution is faster after the blockade of either brain MC₄ receptors or efferent vagal transmission, further suggests that this antishock pathway may be physiological and melanocortin-dependent. The discovery of this previously unrecognized, melanocortin-activated, antishock pathway further highlights the notion that these endogenous peptides, devoid of acute toxicity, may represent a class of drugs for a new approach (i.e., the triggering of a physiological mechanism) to the rapid management of a potentially lethal inflammatory response, as occurs during shock states [12,13].

The main advantage of the clinical use of melanocortins in shock would be the prompt availability of a safe, non-toxic treatment able to rapidly improve cardiovascular function and tissue perfusion for some hours. The main, but obvious, shortcoming, is that the melanocortin-induced improvement is transient, and anyway requires the restoration of volemia within 1–3 h [39,40]. It is of fundamental importance, however, that melanocortins can (per se alone) restore cardiovascular and respiratory functions and tissue perfusion for hours, thus considerably extending the time-limit for blood reinfusion to be effective [39,40].

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