Impact of Hydrocortisone and of CRH Infusion on the Hypothalamus-Pituitary-Adrenocortical Axis of Septic Male Mice

Amo Téblick,1 Lauren De Bruyn,1 Tim Van Oudenhove,1 Sarah Vander Perre,1 Lies Pauwels,1 Sarah Derde,1 Lies Langouche,1,* and Greet Van den Berghe1,*

1Clinical Division and Laboratory of Intensive Care Medicine, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium

ORCID numbers: 0000-0001-8829-0855 (A. Téblick); 0000-0002-5036-3403 (L. De Bruyn); 0000-0002-8564-6809 (L. Langouche); 0000-0002-5320-1362 (G. Van den Berghe).

*Contributed equally.

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Abstract

Purpose: Sepsis is hallmarked by high plasma cortisol/corticosterone (CORT), low adrenocorticotropic hormone (ACTH), and high pro-opiomelanocortin (POMC). While corticotropin-releasing hormone-(CRH) and arginine-vasopressin (AVP)-driven pituitary POMC expression remains active, POMC processing into ACTH becomes impaired. Low ACTH is accompanied by loss of adrenocortical structure, although steroidogenic enzymes remain expressed. We hypothesized that treatment of sepsis with hydrocortisone (HC) aggravates this phenotype whereas CRH infusion safeguards ACTH-driven adrenocortical structure.

Methods: In a fluid-resuscitated, antibiotics-treated mouse model of prolonged sepsis, we compared the effects of HC and CRH infusion with placebo on plasma ACTH, POMC, and CORT; on markers of hypothalamic CRH and AVP signaling and pituitary POMC processing; and on the adrenocortical structure and markers of steroidogenesis. In adrenal explants, we studied the steroidogenic capacity of POMC.

Results: During sepsis, HC further suppressed plasma ACTH, but not POMC, predominantly by suppressing sepsis-activated CRH/AVP-signaling pathways. In contrast, in CRH-treated sepsis, plasma ACTH was normalized following restoration of pituitary POMC processing. The sepsis-induced rise in markers of adrenocortical steroidogenesis was unaltered by CRH and suppressed partially by HC, which also increased adrenal markers of inflammation. Ex vivo stimulation of adrenal explants with POMC increased CORT as effectively as an equimolar dose of ACTH.

Conclusions: Treatment of sepsis with HC impaired integrity and function of the hypothalamic-pituitary-adrenal axis at the level of the pituitary and the adrenal cortex while CRH restored pituitary POMC processing without affecting the adrenal cortex.
Sepsis is defined as a life-threatening (multi-)organ dysfunction caused by a dysregulated host response to an infection and is a leading cause of critical illness and mortality worldwide (1,2). Patients with sepsis and septic shock typically present with increased plasma (free) cortisol (corticosterone in rodents; CORT) and suppressed plasma adrenocorticotrophic hormone (ACTH) concentrations (3,4). Beyond the first week(s) of intensive care unit stay, plasma (free) CORT levels gradually decline and plasma ACTH levels become less suppressed (5). Recently, it was documented that from early sepsis onward the mainly peripherally driven increase in systemic CORT availability, via reduced plasma binding and reduced CORT breakdown, suppresses processing of the polypeptide precursor of ACTH, pro-opiomelanocortin (POMC), into mature ACTH via glucocorticoid receptor (GR) ligand binding, at the level of the pituitary (6). Meanwhile, ongoing central stress-induced CRH- and arginine-vasopressin (AVP)-driven expression and signaling results in increased pituitary POMC expression, which in face of suppressed downstream processing into smaller peptides, results in full-length unprocessed POMC leaking into the systemic circulation (6). The biological significance of up to 7- to 10-fold increased POMC concentrations in the systemic circulation during sepsis remains unknown (6,7). At the level of the adrenal cortex, prolonged sepsis-induced critical illness results in loss of zonational structure and a decrease in cholesterol ester content, both possibly resulting from loss of trophic ACTH signaling (6,8).

Glucocorticoids are potent immune modulators and may be administered in septic patients for several indications such as a complicated course of illness with the development of acute respiratory distress syndrome (ARDS) or as part of advanced-stage blood pressure management in those patients with a vasopressor-refractory shock state (9,10). However, the reported mortality and morbidity benefit is not consistent across large-scale randomized controlled trials (RCTs) (11-16), and recent guidelines recommending the use of glucocorticoids in patient with moderate to severe ARDS (17) and vasopressor-refractory shock (18,19) are only conditional and based on low to moderate quality of evidence. Together, this may explain the wide variety in actual clinical practice regarding use of glucocorticoids in the intensive care unit (20). In addition, most studies investigating the use of “stress doses” of glucocorticoids [ie, the recommended 200-400mg of hydrocortisone (HC) per day (18,19)] have not reported the impact of such treatments on plasma hormone levels or on the patient’s endogenous adrenocortical CORT production. However, outside the context of critical care medicine, it is long known that substantially augmenting the systemic CORT availability with glucocorticoid treatment suppresses the hypothalamic-pituitary axis, with time resulting in distorted adrenocortical structure and function (21-25). It is currently unclear to what extent glucocorticoid treatment affects the endocrine function of the hypothalamic-pituitary-adrenal (HPA) axis of patients with sepsis (3-6). Furthermore it is unclear if glucocorticoid therapy alters the expression and/or function of immune cells within the HPA axis (26).

In contrast with glucocorticoid treatment, corticotropin-releasing hormone (CRH) infusion could theoretically prevent or overcome the sepsis-induced central suppression of the HPA axis at the level of the pituitary. Indeed, in CRH knockout mice, peripheral CRH infusion (1 µg/day) resulted in a restoration of pulsatile pituitary ACTH secretion and normalized adrenocortical steroidogenesis (27). Whether CRH infusion could exert a similar effect in the context of sepsis-induced critical illness has not been studied. Moreover, the impact of increasing plasma ACTH on the adrenocortical phenotype and adrenocortical steroidogenesis during sepsis is unknown.

We hypothesized that administration of stress doses of HC may augment negative feedback inhibition at the pituitary level, thereby aggravating the sepsis-induced impaired processing of POMC into ACTH, further lowering plasma ACTH and thereby worsening the adrenocortical phenotype of sepsis. Vice versa, we hypothesized that infusion of CRH may restore pituitary processing of POMC into ACTH, resulting in a normalization of plasma ACTH, in turn preventing to some extent the abnormal adrenocortical phenotype that develops during the course of sepsis-induced critical illness. We also hypothesized that the centrally activated production and leaching of unprocessed POMC into the circulation may stimulate the adrenal cortex to produce some CORT in the context of low ACTH during sepsis.

Methods
Animal Studies
To study the impact of treatment with HC or CRH during sepsis, 24-week-old (mature adult) male C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) were randomly
allocated to a healthy control group or 1 of 3 intervention sepsis groups (Fig. 1A). Mice randomized to 1 of the sepsis groups were anesthetized with xylazine/ketamine and were implanted with a subcutaneous osmotic pump (ALZET Osmotic Pumps, Cupertino, CA, USA), delivering HC (1.2 mg per day, equivalent of human doses between 200 and 400 mg [28]; “HC-treated sepsis”), CRH (1 µg per day, sufficient to restore pituitary pulsatile ACTH secretion in CRH knockout C57BL/6 mice [27]; “CRH-treated sepsis”), or PlasmaLyte (“placebo-treated sepsis”). Next, the left internal jugular vein was cannulated, followed by a median laparotomy and cecal ligation and puncture to induce polymicrobial abdominal sepsis [described in detail in Muglia et al [27]]. In the first 24 h after surgery, septic mice received continuous intravenous fluid resuscitation (4:1 mixture of crystalloids/colloids) at 0.3 mL/h. Thereafter, parenteral nutrition (Olcinomel N7E, Baxter, Braine-l’Alleud, Belgium) was administered at 0.2 mL/h to mimic the illness-induced lack of feeding in human sepsis patients (up to 45% of the normal daily caloric intake) until the end of the study period. All septic mice were treated with broad-spectrum antibiotics (Imipenem/Cilastin, Aurobindo Pharma, Hyderabad, India) and opioid-analgetics [buprenorphine (Vetergesic), Patheon UK Ltd, Covingham, UK] via subcutaneous injection twice daily. Healthy control mice did not receive any intervention and received ad libitum feeding and free access to water. All animal cages were kept in an animal cabinet under controlled temperature (27°C) and 12-h light and dark cycles. After a 7-day study period, all mice were sacrificed via cardiac puncture and whole blood and tissue samples were collected.

To study the adrenocortical steroidogenic capacity of full-length POMC, healthy, 24-week-old (mature adult) male C57BL/6j mice (Janvier Labs, Le Genest-Saint-Isle, France) were anesthetized with xylazine/ketamine and whole adrenal glands were dissected out. The protocol was based on previously published studies [30,31]. Briefly, after dissection, each adrenal gland was quickly rinsed in 10 mL of ice-cold sterile phosphate buffered saline and subsequently individually suspended in 200 µL of culture medium (Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12, 0.1% bovine serum albumin) in a 96-well culture plate for 4 h at 37°C. Next, culture medium was replaced by 200 µL of incubation medium (Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12, 0.1% bovine serum albumin) containing purified POMC (100 nM; MyBioSource, San Diego, CA, USA), ACTH (100 nM; MyBioSource, San Diego, CA, USA), or no additive (basal incubation medium). For each mouse, 1 adrenal gland was treated with either POMC or ACTH, whereas the contralateral adrenal gland was treated with placebo. After overnight incubation at 37°C, the incubation medium was collected and stored at –80°C until further analysis. CORT concentration in the medium was measured with enzyme-linked immunosorbent assay (Cat. no. EIA-5186 from DRG International Inc, Springfield, NJ, USA; sensitivity 6.1 ng/mL; intra- and interassay variation, <8.9% and <8.2%, respectively; RRID: AB_2893407; https://antibodyregistry.org/search?q=AB_2893407).

All animals were treated according to the Principles of Laboratory Animal Care (US National Society of Medical Research) and to the European Union Directive 2010/63/EU concerning the welfare of laboratory animals. All animal procedures were approved by the Institutional Ethical Committee for Animal Experimentation (P181-2018) and complied with the ARRIVE guidelines [32].

Sample Size Calculation, Survival, and Behavior Analysis

For the animal study investigating the impact of treatment with HC or CRH during sepsis-induced critical illness, based on previously published data of our research group [6,8], we estimated that 14 surviving animals per group would suffice to detect a doubling in plasma ACTH and a normalization of adrenal melanocortin 2 receptor (MC2R) gene expression in the CRH-treated sepsis group with an α-error ≤ 0.05 and β-error ≤ 0.20. The study was stopped early due to the outbreak of the COVID-19 pandemic and the policy of the KU Leuven University to cease inclusion of all ongoing animal studies. A total of 74 animals were randomized to a healthy control group (n = 11), HC-treated sepsis (n = 20), CRH-treated sepsis (n = 21), or placebo-treated sepsis (n = 18). Four animals were excluded from any analysis due to death prior to the start of the study period (perioperative death). From the 70 animals included in the survival analysis, 50 animals survived until the end of the study period (Fig. 1B). Two surviving animals were excluded from tissue analysis due to absolute or functional loss of the central venous catheter after completion of fluid resuscitation and inability to start parenteral nutrition. Mice who lost their central venous catheter after completion of fluid resuscitation were kept in the analysis. Pain/discomfort was assessed twice daily based on the Mouse Grimace Score and analyzed as a cumulative score across the 7-day study period (min 0, max 30) [33]. Biting behavior of the animal, which results in absolute or functional loss of the central venous catheter, is referred to as “excessive biting.”

For the animal study investigating the adrenocortical steroidogenic capacity of full-length POMC, we calculated that 8 adrenal glands per stimulation group (POMC, ACTH) would suffice to detect a similar response in
adrenocortical steroidogenesis as previously reported (31), with an \( \alpha \)-error \( \leq 0.05 \) and \( \beta \)-error \( \leq 0.05 \).

**Plasma Hormones**

Plasma concentrations of ACTH were quantified with radioimmunoassay (Cat. no. 65.1 from Brahms Diagnostics, Hennigsdorf, Germany; analytical sensitivity 1.2 pg/mL; intra- and interassay variation of <7.9% and <16.2%, respectively; RRID: AB_2893408; https://antibodyregistry.org/search?q=AB_2893408). Plasma concentrations of POMC and CORT were quantified with enzyme-linked immunosorbent assay (for POMC: Cat. no. CEB311Mu from Cloud-Clone Corp., Katy, TX, USA; sensitivity, 48.4 pg/mL;
Intra- and interassay variation, <10% and <12%, respectively; RRID: AB_2893406; https://antibodyregistry.org/search?q=AB_2893406 and for CORT: Cat. no. EIA-5186 from DRG International Inc., Springfield, NJ, USA; sensitivity 6.1 ng/mL; intra- and interassay variation, <8.9% and <8.2%, respectively RRID: AB_2893407; https://antibodyregistry.org/search?q=AB_2893407).

In Situ Hybridization

We performed chromogenic RNAscope in situ hybridization (Advanced Cell Diagnostics, Newark, CA, USA) to quantify gene expression of CRH and AVP in the hypothalamic paraventricular nucleus (PVN), as previously described in detail (6). In short, formalin-fixed (10% neutral formalin buffer), paraffin-embedded whole brain was cut in 5 µm thick coronal sections. On microscopically selected slides containing a coronal section though the PVN, RNAscope 2.5 HD Duplex Assay with specific anti-CRH (blue channel, Cat. no. 316091) and anti-AVP (red channel, Cat. no. 401391) probes was performed, as per the manufacturer’s instructions (34). Images were captured with a Leica DM3000 bright-field microscope (Leica Camera, Wetzlar, Germany) and digital camera with LAS V4.10 software (Heerbrugg, Switzerland). A machine learning classifier was developed to identify anti-CRH and anti-AVP stained pixels within the cell nuclei in the predefined region of interest. Data were analyzed as anti-CRH and anti-AVP stained pixels per nucleus.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction Analysis

In the pituitary, we quantified gene expression of (1) the receptors through which the main effects of the hypophysiotropic hormones CRH and AVP and glucocorticoids are signaled, the CRHR1, AVPR1B, and GR (and its main isoforms, the GRα and GRβ), respectively; (2) the ACTH precursor POMC; (3) the main processing enzyme cleaving POMC into smaller fragments, proprotein convertase 1 (PC1/3); and (4) pro-inflammatory cytokines [tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β)]. In the adrenal gland, we quantified gene expression of (1) key regulators of adrenal steroidogenesis [melanocortin receptor 2 (MC2R) and MC2R accessory protein]; (2) PC1/3; (3) receptors and enzymes involved in intracellular cholesterol availability (high-density lipoprotein receptor, low-density lipoprotein receptor, 3-hydroxy-3-methylglutaryl coenzyme A reductase); (4) enzymes involved in CORT synthesis [steroidogenic acute regulatory protein, cholesterol side-chain cleavage (P450scc) and steroid 11β-hydroxylase]; and (5) markers of inflammation (TNF-α and IL-1β). In brief, RNA was isolated from pituitary and adrenal tissue with Macherey-Nagel Nucleospin RNA kit (Macherrey-Nagel, Düren, Germany) and reverse-transcribed into complementary DNA with random hexamers (Invitrogen, Waltham, MA, USA). Next, complementary DNA was quantified in real time with the use of commercial TaqMan assays (Applied Biosystems Waltham, MA, USA) for all gene expressions except for the GR isoforms. The GRα and GRβ isoforms were quantified with SYBR Green and customized forward/reverse primers (common forward primer: AAAGAGCTAGGAAAAAGCCATTGTC; GRα reverse primer: TCGCTAATCATCTGGGAATTCA; GRβ reverse primer: CTGTCTTTGGGCTTTTGAGAG). Data of gene expression are normalized to a stable housekeeping gene (HPRT for the pituitary, SDHA for the adrenal) and are presented as fold difference of the median of the healthy controls.

Protein Isolation and Immunoblotting

In the pituitary, we quantified the protein content of POMC, ACTH, and PC1/3 with Western blot. In brief, proteins, isolated with Macherey-Nagel Nucleospin RNA/protein kit from whole pituitary were separated on a 16.5% tris-tricine polyacrylamide gel (POMC and ACTH) or 4% to 20% tris-glycine polyacrylamide gel (PC1/3; all from Bio-Rad, Hercules, CA, USA), subsequently electroblotted on a nitrocellulose membrane and incubated overnight at 4°C with primary antibodies (anti-ACTH and anti-PC1/3 from Abcam, Cambridge, UK; RRID: AB_1280736; https://antibodyregistry.org/search.php?q=AB_1280736 and RRID: AB_303882; https://antibodyregistry.org/search.php?q=AB_303882). Afterwards, the immunoblots were incubated with HPR-linked secondary antibodies (Dako, Glostrup, Denmark; RRID: AB_2617138; https://antibodyregistry.org/search.php?q=AB_2617138) at room temperature for 1 h. Finally, the immunoblots were visualized using chemiluminescence plus technology (PerkinElmer, Zaventem, Belgium) and imaged using G:BOX XRQ (SynGene, Cambridge, UK). Bands were quantified with Syngene software (SynGene, Cambridge, UK). Data are presented as fold difference of the median of the healthy controls.

Structural Integrity, Cholesterol Ester Content, and Presence of Macrophages in the Adrenal Cortex

To evaluate cholesterol ester content and presence of macrophages (CD68+ cells) in the adrenal cortex, snap-frozen adrenal glands were cut at 8 µm thickness with a microtome-cryostat and serially collected on Superfrost
Results

The Impact of Treatment With HC or CRH on Behavior, Body Composition, and Survival During Sepsis-induced Critical Illness

During the study period of 7 days, excessive biting of the animals was observed more frequently in the HC-treated sepsis group (30%, n = 6) than in the other sepsis groups [n = 1 (5.6%) and n = 1 (5.6%) in the placebo- and CRH-treated sepsis groups, respectively; P = 0.05 for the comparison between HC-treated sepsis and placebo-treated sepsis]. Cumulative pain/discomfort scores were not different among the 3 sepsis groups (all Ps > 0.05) (Fig. 1C).

After 7 days of sepsis-induced critical illness, a substantial and comparable reduction in body weight was observed in all septic groups [proportionate loss of body mass, medians (IQR): -22.1% (-24.8% to -19.1%), -25.5% (-28.2% to -17.6%), and -12.0% (-19.6% to -9.3%) in the placebo-, HC- and CRH-treated sepsis groups, respectively; all Ps < 0.01 vs healthy controls] (Fig. 1C). The presence of macrophages was assessed with immunohistochemistry. In short, cross-sections of adrenal glands were incubated with an anti-CD68 primary antibody (Abcam, Cambridge, UK; RRID: AB_10975465; https://antibodyregistry.org/search.php?q=AB_10975465) overnight at 4°C and subsequently with a secondary HRP-linked antibody (Dako, Glostrup, Denmark) and visualized with diaminobenzidine. Images were captured with a Leica DM3000 bright-field microscope (Leica Camera, Wetzlar, Germany) and digital camera with LAS V4.10 software (Heerbrugg, Switzerland). The percentage of the positively stained adrenal cortex was quantified with ImageJ 1.52a.

Statistical Analysis

Data are presented as box plots with median, interquartile range (IQR; 25th-75th percentiles), and the furthest points within 1.5 times the IQR. Differences between each sepsis group and the healthy controls group were pairwise analyzed with use of Mann-Whitney U. Differences between the placebo-treated sepsis group and both the HC- and the CRH-treated sepsis group were also analyzed with use of Mann-Whitney U. No corrections for multiple comparisons were performed. A 2-sided P-value equal or less than 0.05 was considered statistically significant. All statistical analyses were done with JMP pro 14 (SAS Institute Inc., Cary, NC, USA).

HPA Axis Plasma Hormones Were Substantially Affected by HC and CRH Treatment During Sepsis-induced Critical Illness

Plasma ACTH concentrations of septic mice receiving a 7-day continuous infusion of HC were substantially lower than those of placebo-treated septic mice (P < 0.001), whereas plasma ACTH concentrations of CRH-treated septic mice were higher than those of septic mice receiving placebo (P = 0.01) and within the range of unstressed healthy control mice (P = 0.24) (Fig. 2A). Plasma concentrations of the ACTH precursor, POMC, were increased in all sepsis groups (all Ps ≤ 0.05 vs healthy controls), regardless of treatment (all Ps > 0.05 in comparison with placebo-treated sepsis) (Fig. 2B). Plasma CORT, the endogenous main glucocorticoid in rodents, was substantially and similarly increased in both placebo-treated and CRH-treated septic mice (P < 0.0001 vs healthy controls) but not in HC-treated septic mice (P = 0.31 vs healthy controls) (Fig. 2C).

HC and CRH Infusion During Sepsis-induced Critical Illness Affected the Hypothalamic-Pituitary Regulation of Synthesis and Secretion of ACTH

The sepsis-induced rise in hypothalamic paraventricular messenger RNA (mRNA) expression of CRH (P ≤ 0.05 placebo-treated sepsis vs healthy controls) was not present in HC- or CRH-treated septic mice (both Ps > 0.05 vs healthy controls), while paraventricular mRNA expression of AVP was suppressed in only HC-treated septic mice (P ≤ 0.05 vs healthy controls) (Fig. 3A-C). At the level of the pituitary, gene expression of the receptors through which CRH and AVP exert their signaling effects, the CRHR1 and AVPR1B, respectively, was unaffected by CRH treatment (both Ps > 0.05 vs placebo-treated sepsis) (Fig. 3D), whereas it was substantially suppressed in HC-treated septic mice (both Ps ≤ 0.01 vs placebo-treated sepsis) (Fig. 3D). Pituitary mRNA levels of the GR and its main isoforms (α and β) were similar across all groups (all Ps > 0.05) (Fig. 3E).

While gene expression of the precursor hormone POMC was increased, mRNA levels of the main processing enzyme cleaving POMC into ACTH and other fragments, PC1/3, were suppressed in all sepsis groups (all P ≤ 0.01 vs healthy controls) (Fig. 4A). Protein expression of PC1/3 was suppressed in both placebo- and HC-treated septic mice (both

Statistical Analysis

Data are presented as box plots with median, interquartile range (IQR; 25th-75th percentiles), and the furthest points within 1.5 times the IQR. Differences between each sepsis group and the healthy controls group were pairwise analyzed with use of Mann-Whitney U. Differences between the placebo-treated sepsis group and both the HC- and the CRH-treated sepsis group were also analyzed with use of Mann-Whitney U. No corrections for multiple comparisons were performed. A 2-sided P-value equal or less than 0.05 was considered statistically significant. All statistical analyses were done with JMP pro 14 (SAS Institute Inc., Cary, NC, USA).
Ps ≤ 0.05 vs healthy controls), but not in the CRH-treated sepsis group (P > 0.05) (Fig. 4A). HC and CRH but not placebo infusion in septic mice resulted in elevated pituitary mRNA levels of the inflammatory marker IL-1β (both Ps ≤ 0.05 vs healthy controls), whereas TNF-α gene expression was increased only in the CRH-treated sepsis group (P ≤ 0.05 vs healthy controls) (Fig. 4B).

Pituitary POMC protein content was similar across all groups (all Ps > 0.05), whereas pituitary ACTH protein content was decreased only in the HC-treated sepsis group (P ≤ 0.001 vs healthy controls and placebo-treated sepsis) (Fig. 4C-D).

HC, but Not CRH, Infusion Substantially Affected the Sepsis-induced Adrenocortical Phenotype

In comparison with placebo-treated septic mice, mice receiving HC treatment had lower adrenal mRNA levels of signaling receptors and associated proteins (MC2R and MC2R accessory protein) (Fig. 5A), but higher mRNA levels of the POMC processing enzyme, PC1/3 (Fig. 5A) (all Ps ≤ 0.01). Gene expression of receptors and enzymes involved in intracellular cholesterol availability (SCARB1 and 3-hydroxy-3-methylglutaryl coenzyme A reductase) (Fig. 5B) and of enzymes involved in corticosterone synthesis (steroidogenic acute regulatory protein, CYP11A1, and CYP11B1) (Fig. 5C) were also lower in HC-treated septic mice as compared with the placebo-treated group (all Ps ≤ 0.01). However, despite the substantially suppressed plasma ACTH concentrations, gene expression of these markers of adrenocortical steroidogenesis did not differ from those in healthy control mice (all Ps > 0.05). In contrast, septic mice treated with CRH had a similar adrenocortical gene expression profile as placebo-treated septic mice (all Ps > 0.05).

Remarkably, HC-treated septic mice, but not placebo- or CRH-treated septic mice had elevated adrenal mRNA levels of the inflammatory markers TNF-α and IL-1β (both Ps ≤ 0.05 vs healthy controls) (Fig. 6A) and an abnormally abundant presence of CD68+ cells in the adrenal cortex and more specifically in the CORT-producing zona fasciculata (Fig. 6B). Adrenocortical cholesterol ester content was substantially lower in placebo- and CRH-treated septic mice (both P < 0.01 vs healthy controls), but not in septic mice receiving HC-treatment (P = 0.91 vs healthy controls) (Fig. 6C).

Adrenocortical Steroidogenic Capacity of the ACTH-precursor POMC

We next investigated whether the precursor of ACTH, POMC, of which the plasma concentrations were uniformly increased during sepsis-induced critical illness, potentially has a role in maintained adrenocortical steroidogenesis (Fig. 7A). We found that CORT secretion from adrenal explants of healthy mice was indeed significantly increased in response to both ACTH (100 nM) and POMC (100 nM) (both Ps ≤ 0.05 vs nonstimulated contralateral adrenal glands), and this similarly by equimolar doses of ACTH and POMC (P > 0.05) (Fig. 7B).
Discussion

In the present study, we have provided insights into how treatment with either stress doses of HC or a rescue dose of CRH alters behavior but not mortality and impacts the functioning and integrity of the HPA-axis during sepsis-induced critical illness. Whereas 7 days continuous...
infusion with HC aggravated the known sepsis-induced decrease in plasma ACTH, predominantly through reducing hypothalamic-pituitary positive forward signaling, infusion with CRH was able to normalize circulating ACTH, following restored pituitary PC1/3-mediated processing of POMC into ACTH. At the level of the adrenal cortex, HC infusion abrogated the sepsis-induced rise in markers of adrenocortical steroidogenesis, while markers of inflammation were increased. Infusion of CRH and subsequent normalization of plasma ACTH appeared to have no effect on adrenocortical structure and function. In addition, we have shown that POMC can activate adrenocortical steroidogenesis, offering a potential explanation for keeping up the plasma endogenous CORT concentrations within the normal range despite substantially suppressed circulating ACTH in HC-treated septic mice.

Figure 4. Pituitary expression of pro-opiomelanocortin (POMC) and adrenocorticotropic hormone (ACTH). (A) Pituitary gene expression of the ACTH-precursor POMC and gene and protein expression of the main POMC-processing-enzyme PC1/3. (B) Pituitary gene expression of inflammatory markers tumor necrosis factor alpha and interleukin 1 beta. (C) Pituitary protein content of POMC and ACTH. (D) Representative image of Western blot analysis of pituitary POMC (31 kDa) and ACTH (4.5 kDa) protein content (1 of 2 gels). Box-and-whiskers represent median, interquartile range (IQR), and the furthest points within 1.5 times the IQR. *, **, *** indicates significance between the respective sepsis group and healthy controls (P ≤ 0.05, P ≤ 0.01, P ≤ 0.001, respectively). †, ††, ††† indicates significance between the respective sepsis group and the placebo-treated sepsis group (P ≤ 0.05, P ≤ 0.01, P ≤ 0.001, respectively). Number of samples per group: healthy controls n = 11; placebo-treated sepsis n = 14; hydrocortisone-treated sepsis n = 12; corticotropin-releasing hormone-treated sepsis n = 11. Statistical tests used: Mann-Whitney U for all pairwise comparisons. Abbreviations: AU, arbitrary units; CRH, corticotropin-releasing hormone-treated sepsis; H, healthy controls; HC, hydrocortisone-treated sepsis; M, marker; P, placebo-treated sepsis.
as an adjunctive vasopressor therapy (septic shock). Nevertheless, the mortality benefit across some of the largest RCTs in these fields is inconsistent (11-16). Here, treatment with HC did not result in improved survival of sepsis-induced critically ill mice. In addition, HC-treated septic mice exhibited excessive biting behavior during the 7-day study period. Although neuropsychiatric outcomes, such as new-onset delirium, are known to be associated with worse short- and long-term outcome of critically ill patients (35-37) and glucocorticoid treatment is a known independent risk factor for the development of delirium in the critically ill (38), most RCTs investigating the potential benefit of HC treatment during sepsis and septic shock did not systematically study or report such potentially harmful behavioral outcomes (11-14). At least, the findings of increased biting behavior in septic mice receiving infusion with HC suggest that future research investigating the use of glucocorticoids in patients with sepsis should include changes in behavior and new-onset delirium as safety outcome parameters.

![Diagram](https://academic.oup.com/endo/article/163/1/bqab222/6410739)
Outside the context of critical care medicine, oral and parenteral glucocorticoid therapies are well-known to suppress the HPA axis, even when given in low-doses or as a short course (39,40). Patients with an intact, normal functioning HPA axis who are treated with glucocorticoids, such as patients with hematological malignancies, transplant recipients, or patients with chronic inflammatory conditions, may exhibit multilevel suppression of the entire HPA axis. This is characterized by suppression of hypothalamic expression of CRH (41), suppression of pituitary POMC gene expression (42), PC1/3-mediated processing of POMC into ACTH (43), and secretion of mature ACTH into the bloodstream (44). As a result, circulating levels of ACTH start to fall, in turn causing a loss of essential trophic

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**Figure 6.** Inflammation and cholesterol ester storage in the adrenal gland. (A) Gene expression of inflammatory markers tumor necrosis factor alpha and interleukin 1 beta. Box-and-whiskers represent median, interquartile range (IQR), and the furthest points within 1.5 times the IQR. Gray area represents IQR of healthy controls (B) Presence of macrophages within the adrenal cortex. Left panel show box-and-whiskers representing the median, IQR, and the furthest points within 1.5 times the IQR of CD68+ stained area of each sepsis group, relative to the total adrenal cortex. Middle and right panels show representatives images of a healthy controls and hydrocortisone (HC)-treated septic mice, respectively. Arrows point out clustering of CD68+ staining within the adrenal cortex, more specifically within the zona fasciculata (C) Cholesterol ester storage. Box-and-whiskers show the median, IQR, and the furthest points within 1.5 times the IQR of Oil-Red-O–stained area of each sepsis group, relative to the total adrenal gland. Middle and right panels show representatives images of each group (healthy controls, placebo-, HC-, and corticotropin-releasing hormone-treated sepsis). *, ** indicates significance between the respective sepsis group and healthy controls (P ≤ 0.05, P ≤ 0.01, respectively). †, †† indicates significance between the respective sepsis group and the placebo-treated sepsis group (P ≤ 0.05, P ≤ 0.01, respectively). Number of samples per group: healthy controls n = 10; placebo-treated sepsis n = 14; HC-treated sepsis n = 11; corticotropin-releasing hormone-treated sepsis n = 11. Statistical tests used: Mann-Whitney U for all pairwise comparisons. Abbreviations: AU, arbitrary units; ORO, Oil-Red-O.
and especially between glucocorticoid target tissues, such as the GR is differentially regulated between various tissues at the protein level (46-48). A possible explanation could be that experimental and human sepsis both at the mRNA and protein content and further suppressed plasma concentrations of POMC within the pituitary (6). The latter leaches unprocessed into the systemic circulation and causes a rise in plasma POMC concentrations during sepsis-induced critical illness (6). In the current study, as hypothesized, we found that continuous infusion with HC, equivalent to the recommended and commonly used dosage in human septic shock (18,19), substantially suppressed pituitary ACTH protein content and further suppressed plasma concentrations of ACTH. HC infusion reduced the expression of AVP in the hypothalamus and the expression of the CRH- and AVP-receptors in the pituitary but did not affect the expression of the GR (and its main isoforms GRα and GRβ). It appears that the earlier observed sepsis-induced preservation of positive CRH- and AVP-signaling is mitigated by further increasing the systemic glucocorticoid availability through HC infusion, while unaltered pituitary GR expression allows increased negative feedback signaling. Ongoing gene expression of the active GR isoform within the pituitary stands in contrast with several studies documenting altered GR expression profiles within various tissues during experimental and human sepsis both at the mRNA and protein level (46-48). A possible explanation could be that the GR is differentially regulated between various tissues and especially between glucocorticoid target tissues, such as the heart, lungs, liver, and immune cells and tissues that are part of the HPA axis itself, such as the pituitary.

Despite mitigated positive CRH and AVP signaling and increased systemic glucocorticoid availability with preserved negative feedback signaling, pituitary gene and protein expression, and plasma concentrations of POMC were still similar between HC-treated and placebo-treated septic mice. This suggests that other, yet to be identified, CRH-, AVP-, and GR-independent factors are involved in the maintenance of pituitary POMC production during sepsis-induced critical illness.

In addition, the HC-induced mitigation in CRH- and AVP-driven positive feed-forward signaling did not result in a further reduction in pituitary expression of PC1/3. Indeed, while pituitary and plasma ACTH levels were substantially suppressed in HC-treated septic mice, both pituitary gene and protein expression levels of the main processing enzyme of POMC yielding mature ACTH, PC1/3, were not different than those in placebo-treated septic mice. PC1/3 is a Ca2+-dependent serine endopeptidase, produced as a zymogen in the endoplasmic reticulum. Only after proper packaging into dense core secretory granules (DCSGs), requiring a minimal availability of cholesterol (49), and subsequent maturation of the DCSG with tight regulation of intragranular environment [low pH and high (Ca2+)], PC1/3 can exert its enzymatic activity (50). Although glucocorticoids are known to suppress gene and protein expression of PC1/3 (43), their impact on the formation and function of the environment within DCSGs, thus indirectly affecting PC1/3 activity at the posttranslational level, is not known and requires further investigation.

In contrast, in septic mice receiving a 7-day continuous infusion of CRH (1 µg/day), which has been shown before to rescue normal pulsatile pituitary ACTH secretion in CRH-knockout mice (27), pituitary protein expression...
of PC1/3 was restored as well as plasma concentrations of ACTH. Unexpectedly, a normalization of plasma ACTH concentrations did not coincide with any changes in adrenocortical phenotype. Indeed, gene expression of the most important markers of adrenocortical steroidogenesis as well as cholesterol-ester storage and plasma CORT concentrations were all similar between CRH- and placebo-treated septic mice. Conversely, the sepsis-induced rise in markers of adrenocortical steroidogenesis and rise in plasma CORT concentrations were abrogated in HC-treated septic mice. This is not an unsurprising finding, considering that ACTH, the primary adrenal steroidogenic and trophic factor (45), was dramatically suppressed by the HC treatment. In addition, proinflammatory cytokines were more expressed and phagocytic cells from the monocye lineage were more abundantly present in septic mice who received HC treatment. Although glucocorticoids are often prescribed as a monotherapy or part of a broader therapeutic strategy to counteract systemic and/or tissue inflammation, a proinflammatory effect of glucocorticoid treatment within the adrenal gland, as we document here, has been observed previously (26).

We have previously documented that plasma concentrations of the precursor of ACTH, POMC, are several-fold elevated in septic mice and in human patients with sepsis who did not receive any form of glucocorticoid treatment and have already speculated on a potential biological role for POMC (6). In the present study, HC treatment of septic mice resulted in further suppression of plasma ACTH, while plasma POMC remained elevated. And, although the rise in markers of adrenocortical steroidogenesis was abrogated, mRNA levels of the key regulators of CORT synthesis and plasma CORT concentrations were still similar in HC-treated septic mice and in healthy, unstressed controls. As adrenocortical steroidogenesis appears to be preserved, at least to some extent in these mice, a role for POMC in adrenocortical steroidogenesis was again suggested. In a second animal study, we therefore investigated the adrenocortical steroidogenic capacity of POMC by quantifying the total CORT content in the incubation medium in which explanted adrenal glands were suspended and subsequently exposed to either POMC, ACTH, or no additive. In line with earlier published studies, ex vivo overnight stimulation of adrenal glands with 100 nM of ACTH resulted in a substantial increase in de novo CORT production and secretion (30,31). As hypothesized, stimulation with full-length POMC also resulted in a substantial increase CORT production. Moreover, the adrenocortical response to equimolar concentrations of ACTH and POMC was similar, both approximately 2-fold of basal stimulation. Whether POMC is indeed an important steroidogenic factor in vivo, preserving at least some adrenocortical production of CORT during sepsis-induced critical illness, remains uncertain. In addition, it remains to be scrutinized how exactly POMC exerts the observed steroidogenic effects. It has been proposed by some authors that POMC, in sufficiently high concentrations, may bind directly to the ACTH receptor, MC2R, as POMC contains the sequence necessary for receptor binding (7,51). Alternatively, POMC could be cleaved into ACTH locally in the adrenocortical target cells (7). Indeed, our results indicate that PC1/3 is expressed at the mRNA level within the adrenal gland. In addition, gene expression of PC1/3 was upregulated in the HC-treated sepsis group (ie, the group with the lowest plasma ACTH levels) but still maintained plasma corticosterone concentrations in face of elevated plasma POMC levels.

This study has some limitations. First, although CLP-induced mouse models of sepsis are often used in preclinical and translational sepsis research, translation to the human setting have to be done carefully, and validation of the current results in human sepsis patients is needed. Second, all septic mice received intravenous fluid resuscitation, parenteral nutrition, broad-spectrum antibiotic therapy, and opioid-analgetics whereas healthy control mice did not undergo any intervention to serve as a true reference group for the “healthy state.” This study design does not allow us to point out which specific aspect of our treated mouse model of sepsis is causing the alterations within the HPA axis of the placebo-treated septic mice. In addition, the different study drugs (HC and CRH) were given only to the septic mice and not to the healthy controls. As such, the comparison of HC or CRH treatment with untreated healthy controls has to be interpreted within the context of sepsis.

In conclusion, we have shown that infusion of stress doses of HC during sepsis in mice further impaired integrity and function of the HPA axis at the level of the pituitary and the adrenal cortex while CRH infusion restored pituitary processing of POMC into ACTH though without affecting the adrenal cortex. High-circulating POMC may be responsible for some ongoing adrenocortical steroidogenesis, despite low ACTH in sepsis.

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Additional Information

Correspondence: Greet Van den Berghe, MD, PhD, Herestraat 49, B-3000 Leuven, Belgium. Email: greet.vandenbergh@kuleuven.be.

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Data Availability: Some or all data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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