Hypothalamic-pituitary-adrenal axis in lethal canine *Staphylococcus aureus* pneumonia

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Cortés-Puch I, Hicks CW, Sun J, Solomon SB, Eichacker PQ, Sweeney DA, Nieman LK, Whitley EM, Behrend EN, Natanson C, Danner RL. Hypothalamic-pituitary-adrenal axis in lethal canine *Staphylococcus aureus* pneumonia. Am J Physiol Endocrinol Metab 307: E994–E1008, 2014. First published September 30, 2014; doi:10.1152/ajpendo.00345.2014.—The clinical significance and even existence of critical illness-related corticosteroid insufficiency is controversial. Here, hypothalamic-pituitary-adrenal (HPA) function was characterized in severe canine *Staphylococcus aureus* pneumonia. Animals received antibiotics and titrated life-supportive measures. Treatment with dexamethasone, a glucocorticoid, but not deoxycorticosterone, a mineralocorticoid, improves outcome in this model. Total and free cortisol, adrenocorticotrophic hormone (ACTH), and aldosterone levels, as well as responses to exogenous ACTH were measured serially. At 10 h after the onset of infection, the acute HPA axis stress response, as measured by cortisol levels, exceeded that seen with high-dose ACTH stimulation but was not predictive of outcome. In contrast to cortisol, aldosterone was largely autonomous from HPA axis control, elevated longer, and more closely associated with survival in early septic shock. Importantly, dexamethasone suppressed cortisol and ACTH levels and restored ACTH responsiveness in survivors. Differing strikingly, nonsurvivors, sepsis-induced hypocortisolemia, and high ACTH levels as well as ACTH hyperresponsiveness were not influenced by dexamethasone. During septic shock, only serial measurements and provocative testing over a well-defined timeline were able to demonstrate a strong relationship between HPA axis function and prognosis. HPA axis unresponsiveness and high aldosterone levels identify a septic shock subpopulation with poor outcomes that may have the greatest potential to benefit from new therapies.

septic shock; sepsis; corticosteroids; adrenal insufficiency; cortisol; adrenocorticotropic hormone; aldosterone

“RELATIVE ADRENA DEFICIENCY” in critically ill patients with otherwise normal hypothalamic-pituitary-adrenal (HPA) function was recently renamed “critical illness-related corticosteroid insufficiency” (CIRCI) (33). However, there are no widely accepted criteria for the diagnosis and treatment of this hypothetical condition (40, 41, 57, 58). No test of HPA function reliably predicts outcome, and the relative importance of endogenous glucocorticoid (cortisol) vs. mineralocorticoid (aldosterone) activity to survival is not known. Importantly, patient selection for corticosteroid therapy in sepsis has varied in clinical practice and across randomized trials, in which benefit has not been reproducible (4, 40, 41, 57).

To date, HPA testing in patients with septic shock has focused primarily on criteria for inadequate cortisol production using both random cortisol levels and adrenocorticotropic hormone (ACTH)-stimulated adrenal responsiveness. However, both low and high cortisol levels have been associated with worst outcomes (5, 23, 39, 52). Total cortisol increases of less than 9 μg/dl in response to high-dose ACTH (250 μg) (5) or a random total cortisol below an arbitrary cutoff (e.g., <10 to <25 μg/dl) (6, 20, 23, 33–35) have been proposed to define CIRCI (33). However, neither high- nor low-dose (1 μg) ACTH stimulation testing has been shown to identify septic patients that reproducibly benefit from exogenous corticosteroids, and the use of stimulation testing to guide therapy is not currently recommended (2, 33, 56). Free instead of total cortisol has been proposed as a better measure of adrenal function, because cortisol-binding globulin is depleted during critical illness (27). However, this test is cumbersome, and its clinical utility during sepsis has not been established (9, 20, 39). Furthermore, no consensus exists for what random cortisol value defines relative adrenal insufficiency in septic shock or indicates the need for exogenous therapy (3, 6, 17, 20, 23, 25, 33–35). This lack of reliable diagnostic criteria has led to skepticism regarding the existence of CIRCI and hampered trials evaluating corticosteroid replacement therapy.

Although cortisol levels and ACTH stimulation testing of adrenal function have been extensively studied during septic shock, other HPA axis attributes such as dexamethasone suppression and aldosterone responses have been relatively unexplored. In a canine model of severe septic shock due to *Staphylococcus aureus* pneumonia, we previously demonstrated that deoxycorticosterone pivalate, a selective mineralocorticoid agonist, started 72 h before sepsis (25), or therapeutic dexamethasone, a selective glucocorticoid agonist, given at the onset of sepsis either alone or with deoxycorticosterone (26), reversed shock and improved survival. How-

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http://ajpendo.org
ever, dexamethasone started 48 h before sepsis, and desoxy-
corticosterone started at the onset of infection, were not ben-
eficial in this *S. aureus* pneumonia model (25). Thus, the effect of
selective corticosteroids on outcomes in this model of septic
shock has been previously fully described (25, 26). In these
studies, total, bound, and free cortisol, ACTH, and aldosterone
were serially measured, and low- and high-dose ACTH stim-
ulation tests were performed daily. Presented here is an anal-
ysis of the combined hormonal data from 101 animals that
provides clear evidence for a distinct type of HPA axis dys-
function during severe sepsis in otherwise normal canines.
Some tests distinguished survivors from nonsurvivors and
potentially could be used to identify a subpopulation of septic
patients with poor outcomes for whom new therapeutic ap-
proaches might provide the greatest benefit.

**METHODS**

All experiments were conducted under a protocol approved by
the Animal Care and Use Committee of the Clinical Center at
the National Institutes of Health. A detailed description of our canine
*S. aureus* pneumonia-induced sepsis model has been published (25, 26, 42, 59). One hundred one purpose-bred beagles (12–28 mo old, 9–12.5 kg) were studied prospectively for 96 h as part of four sets of
experiments designed to compare the effects of desoxycorticosterone
(DOC) and dexamethasone (DEX) given 48–72 h before or at the
onset of sepsis, individually or combined, as shown in Table 1. Each
study week, two animals were randomly allocated to treatment and
one or two to a control group. All animals were continuously moni-
tored and received standard intensive-care ancillary supportive ther-
apy (see below). Care was provided throughout the 96-h study by staff
blinded to group assignment. Eighty-three of the 101 animals included
in this analysis were reported on previously (25, 26).

**Study design.** Experimental procedures have been described in
greater detail previously (42). Briefly, on day 0, anesthetized animals
were instrumented (external jugular vein catheter, femoral artery
catheter, and urinary catheter), followed by placement of a balloon-
tipped pulmonary arterial thermodilution catheter and a tracheostomy.
Animals were then weaned off inhalation anesthesia, followed by the
initiation of continuous sedation (fentanyl, midazolam, and medeto-
midine) and mechanical ventilation via the tracheostomy tube. At
*time 0 h*, *S. aureus* isolates were prepared and administered via a broncho-
scope into the right caudal lung lobe (42). Corticosteroid therapy
produced an overall survival benefit (25, 26) at the *S. aureus* doses
[1.5 (*n* = 89), 1.75 (*n* = 3), 2 (*n* = 3), 3.5 (*n* = 3) or 7.5 (*n* = 3) ×
10⁹ CFU/kg suspended in 1 ml of PBS] included in this study. At 4 h,
when symptoms of sepsis were fully developed, antibiotic treatment
with intravenous (iv) oxacillin (30 mg/kg) was initiated and admin-
istered every 8 h until 96 h or death. All animals received conven-
tional intensive care unit support including fluids, vasopressor therapy
with norepinephrine infusion, mechanical ventilation, prophylaxis to
prevent gastrointestinal ulcers (famotidine 1 mg/kg iv every 12 h), and
deep venous thrombosis (heparin 3,000 IU SQ every 8 h), and routine
care to prevent pressure ulcers (regular position change). Mechanical
ventilation, fluid boluses, and vasopressor therapy were titrated to
physiological and laboratory endpoints using previously described
algorithms (25, 26, 42). Animals alive at 96 h were considered
survivors and euthanized while still sedated (Beuthanol, 75 mg/kg iv).

**Corticosteroid dosing.** The dexamethasone treatment regimen used
(0.014 mg·kg⁻¹·h⁻¹ iv started after bacterial intrabronchial challenge)
has ~10 times more glucocorticoid activity than the daily unstressed
production of cortisol and is equivalent to stress-dose therapy with
hydrocortisone (300 mg daily) in humans (30). Furthermore, animals
received an amount of dexamethasone during each successive 8-h
period that is comparable to the bolus dose of the standard 8-h
suppression test used to diagnose hyperadrenocorticism (Cushing’s
syndrome) in canines (7). Animals assigned to starting dexamethasone
48 h before the onset of infection received 0.17 mg/kg subcutaneously
every 12 h followed by the same dexamethasone dosing regimen
described above for treatment. This group simulated septic patients
already on corticosteroids with adrenal gland suppression at the onset
of septic shock.

Prophylactic desoxycorticosterone was given as a single subcuta-
neous injection of desoxycorticosterone pivalate (2.2 mg/kg) 72 h
before bacterial inoculation. This dose is used clinically to treat
mineralocorticoid deficiency in canines (30). A single injection pro-
vides physiologically adequate levels of mineralocorticoid for up to
28 days (61). Animals assigned to desoxycorticosterone treatment
starting immediately after bacterial challenge received a loading dose
of desoxycorticosterone acetate (0.2 mg/kg dissolved in DMSO 0.45
ml/kg and diluted in 250 ml of 0.9% saline given iv over 45 min),
followed by daily subcutaneous injections (0.17 mg/kg dissolved
in sesame oil).

Animals assigned to the control groups received equivalent vol-
umes and administration routes of the normal saline, DMSO, or
sesame oil vehicles without dexamethasone or desoxycorticosterone
at the same time as concurrently studied animals in the treatment
arms.

**HPA axis function.** Serial determinations of serum total, and free
cortisol, plasma ACTH and serum aldosterone levels were performed
throughout the 96-h study period at 0 h (baseline), 10, 24, 48, 72, and
96 h after bacterial challenge. Bound cortisol levels were calculated
by subtracting free from total cortisol levels. At the same time points,
sequential ACTH stimulation tests were performed with low (1.0 µg
iv) and high (5.0 µg/kg iv) doses of synthetic ACTH (cosyntropin; Cortrosyn,
Amphastar Pharmaceuticals, Rancho Cucamonga, CA). High-dose tests immediately followed the completion of each low-
dose test. Additional blood samples for measurement of cortisol and
aldosterone were drawn before the tests were performed, 1 h after
administration of low-dose ACTH (immediately before the high-dose
test) and 1 h after administration of high-dose ACTH. Daily urinary
total cortisol levels were also measured. All hormones were assayed
using previously described methods (59).

Table 1. Treatment groups, corticosteroid treatments, number of animals, identification of animals previously published, and
doze of *S. aureus*

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Subgroups</th>
<th>No. of Animals (n)</th>
<th><em>S. aureus</em> Dose, ×10⁹ CFU/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DEX</td>
<td>Controls (no corticosteroids)</td>
<td>29*††</td>
<td>1.5 (<em>n</em> = 26), 2 (<em>n</em> = 1), 3.5 (<em>n</em> = 1)</td>
</tr>
<tr>
<td></td>
<td>Therapeutic DEX</td>
<td>6*</td>
<td>1.5 (<em>n</em> = 6)</td>
</tr>
<tr>
<td></td>
<td>Propylactic DEX</td>
<td>20*</td>
<td>1.5 (<em>n</em> = 19), 1.75 (<em>n</em> = 1)</td>
</tr>
<tr>
<td>Therapeutic DEX</td>
<td>Therapeutic DEX</td>
<td>12*</td>
<td>1.5 (<em>n</em> = 6), 2 (<em>n</em> = 2), 3.5 (<em>n</em> = 2), 7.5 (<em>n</em> = 2)</td>
</tr>
<tr>
<td></td>
<td>Therapeutic DEX and DOC</td>
<td>9†</td>
<td>1.5 (<em>n</em> = 9)</td>
</tr>
<tr>
<td></td>
<td>Therapeutic DEX and propylactic DEX</td>
<td>12</td>
<td>1.5 (<em>n</em> = 10), 1.75 (<em>n</em> = 2)</td>
</tr>
<tr>
<td>Prophylactic and therapeutic DEX</td>
<td>Propylactic and Therapeutic DEX</td>
<td>13*</td>
<td>1.5 (<em>n</em> = 13)</td>
</tr>
</tbody>
</table>

DEX, dexamethasone; DOC, desoxycorticosterone. *Results concerning some or all of these animals were published in Ref. 25. †Results concerning some or all of these animals were published in Ref. 26.
Histology. Immediately post mortem, both adrenal glands were obtained from randomly selected animals not receiving dexamethasone. Adrenal tissue was fixed in 10% neutral buffered formalin, processed for paraffin embedding, sectioned, stained with hematoxylin and eosin using standard methods, and evaluated by an experienced pathologist (E. M. W). Different areas of the gland were scored using the criteria in Table 2. The total cortical score (sum of both adrenal glands) was used to differentiate mild, moderate, and severe injury.

Table 2. Pathological criteria for adrenocortical injury

<table>
<thead>
<tr>
<th>Zonae</th>
<th>Score</th>
<th>Histologic Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zona glomerulosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion</td>
<td>1</td>
<td>Space around erythrocytes within capillary, can discriminate individual erythrocytes</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Erythrocytes are 2–3 across in capillary, rounded profiles of vessel wall</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Vessels compress adjacent cells</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>1</td>
<td>No hemorrhage or a few scattered extravascular RBCs</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild hemorrhage between cortical cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Foci of hemorrhage that replace cortical cells</td>
</tr>
<tr>
<td>Edema/shrinkage</td>
<td>1</td>
<td>Cells attached to fibrovascular stroma</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Small space between cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Cells separated from fibrovascular stroma by large vacuoles or thin remnant strands</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>1</td>
<td>Distinct cell borders, fine intracytoplasmic vacuoles</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Distinct cell borders, moderate number of fine intracytoplasmic vacuoles</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Distinct cell borders, multiple foci of cytoplasmic granularity or large intracytoplasmic vacuoles</td>
</tr>
<tr>
<td>Nuclei</td>
<td>1</td>
<td>Oval to elongated nuclei, dispersed to finely stippled chromatin</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Oval to elongated nuclei, some mildly to moderately pyknotic</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Some to many pyknotic, karyorrhectic or karyolytic nuclei</td>
</tr>
<tr>
<td>Zona fasciculata-reticularis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>1</td>
<td>No hemorrhage</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild hemorrhage between cortical cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Scattered foci of hemorrhage that replaces cortical cells</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Marked hemorrhage</td>
</tr>
<tr>
<td>Deep cortical venous plexus</td>
<td>1</td>
<td>Inapparent</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mildly congested</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Dilated to ~15–24 μm (2–3 erythrocytes widths)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Endothelium</td>
<td>1</td>
<td>Normal, flat, adherant endothelium</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Endothelial cells ovoid</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Detached endothelial cells</td>
</tr>
<tr>
<td>Cord organization in zona fasciculata and zona reticularis</td>
<td>1</td>
<td>Cortical cells arranged in well-organized, anastomosing cords</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Cord arrangement completely lost in &gt;70% of region</td>
</tr>
<tr>
<td>Cell membrane</td>
<td>1</td>
<td>Very crisp cell margins</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderately crisp cell margins</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Indistinct cell margins</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Necrotic or lysed cells</td>
</tr>
<tr>
<td>Cytoplasmic vacuoles</td>
<td>1</td>
<td>Very distinct vacuoles</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderately distinct vacuoles or moderately swollen vacuoles</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Indistinct vacuoles or markedly swollen vacuoles</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Vacuoles not observed</td>
</tr>
<tr>
<td>Nuclear: cytoplasmic ratio (zona fasciculata)</td>
<td>1</td>
<td>1 to 3–4 (normal)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1 to 2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1 to 1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Nuclear structure</td>
<td>1</td>
<td>~80–100% dispersed chromatin; no apoptosis</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>~50–80% dispersed chromatin, some condensed nuclei</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50–80% condensed nuclei (apoptosis or pyknosis)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>80–100% apoptotic or pyknotic nuclei</td>
</tr>
</tbody>
</table>

Severity Groups  | Cortical Total Score (sum of both glands) |
-----------------|------------------------------------------|
Severe           | >50                                      |
Moderate         | 41–50                                    |
Mild             | <41                                      |

Pathological criteria and scoring system used to evaluate the severity of injury of adrenal cortex are shown. According to the total score sum, animals were divided into 3 severity groups, as denoted in the key below the table.
of 1.0 is perfect separation of survivors from nonsurvivors, and a value of 0.5 represents no discrimination (i.e., chance).

ACTH levels were not measured after administration of exogenous ACTH. The “theoretical” ACTH peak levels after low- and high-stimulation tests were estimated using the ACTH dose and calculated blood volumes based on animal body weight. While actual ACTH measurements would be desirable, differences from the calculated levels should be nonconsequential, since the same dose of ACTH was given serially to each animal, and the logarithm of ACTH concentrations was used in the regression analysis. A linear regression was used (instead of the more typical nonlinear models like the Emax model) because we had only three data points for each animal (baseline and 1 h after each ACTH dose).

Survival times were analyzed using a stratified Cox proportional hazard model to account for potential cycle effect. All P values are two-tailed and considered significant if P ≤ 0.05. SAS version 9.3 (Cary, NC) was used for all analyses.

RESULTS

HPA axis responses to sepsis: serial mean basal cortisol and endogenous ACTH levels during sepsis. The natural response of the HPA axis during fluid and vasopressor resuscitation in antibiotic-treated septic shock in the absence of exogenous corticosteroids is shown in Fig. 1. At 10 and 24 h after S. aureus intrapulmonary challenge, mean basal (pre-ACTH stimulation) total, bound, and free serum cortisol and ACTH levels were markedly elevated compared with 0-h baseline values (all P < 0.0001; Fig. 1, A and B). However, these values then decreased and were similar to baseline levels by 48 h for cortisol and by 72 h for ACTH (P = NS for all).

Exogenous ACTH stimulation as a test of adrenal reserve. Among control animals, administration of low or high doses of ACTH at 0 h produced a significant increase in cortisol levels (Fig. 1, C and D). In contrast, ACTH administration did not significantly increase cortisol at 10 or 24 h after the bacterial challenge, suggesting that the adrenal gland was already maximally stimulated at these time points (P = NS for all; Fig. 1, C and D). At 10 h after the septic challenge, basal total cortisol levels (before ACTH administration) were significantly higher than stimulated levels after supraphysiological exogenous ACTH administration at 0 h (P < 0.0001). Cortisol responses to exogenous ACTH administered at 48, 72, and 96 h, after basal cortisol had returned to baseline levels, were similar to baseline increases at 0 h (all, P = NS; Fig. 1, C and D).

Dexamethasone suppression of the HPA axis during sepsis. Next, HPA axis responses were examined in the presence and absence of dexamethasone initiated at the onset of sepsis. Cortisol and ACTH responses to dexamethasone were similar whether or not desoxycorticosterone was given (all P = NS; data not shown); so data from animals that received and did not receive desoxycorticosterone were combined for analysis. Overall, when survivors and nonsurvivors were combined, dexamethasone did not significantly suppress mean cortisol levels (total, bound, or free), ACTH levels, or cortisol responses to low or high doses of ACTH stimulation at 10, 24,

Fig. 1. Serial basal cortisol (total, bound, and free), ACTH levels, and total cortisol level responses to low- and high-dose ACTH stimulation tests. Mean ± SE values of cortisol (total, bound, and free; A) and ACTH (B) levels are plotted over time. C and D: change (delta) in cortisol levels with ACTH stimulation is shaded gray, between cortisol levels pre- (○) and post- (●) stimulation test. Actual P values for comparisons are denoted by different symbols explained in the key to the right of the figures. Data are presented as actual values to show the potential variability among animals.
and 48 h following *S. aureus* challenge (for all, *P* = NS vs. no dexamethasone treatment; Fig. 2, A–F). However, at later time points, the HPA axis stress response was suppressed by dexamethasone. At both 72 and 96 h, dexamethasone significantly suppressed free cortisol levels (Fig. 2B) as well as the total cortisol response to low-dose ACTH stimulation (Fig. 2E).

**HPA axis and survival.** Next, the relationship between HPA axis function and survival was examined. At 10 h after the onset of sepsis, total cortisol levels, free cortisol levels, endogenous ACTH levels, and change (Δ) in cortisol after exogenous ACTH administration correlated weakly or not at all with survival time in nonsurvivors regardless of whether animals received dexamethasone or not (Fig. 3, A–H). Although discrimination (c-statistic) between nonsurvivors and survivors for total and free cortisol improved modestly in animals treated with dexamethasone (Fig. 3, E and F), generally HPA axis measures were poorly predictive of outcome in septic shock at 10 h.

Later, at 24 h, in animals not receiving therapeutic dexamethasone, these same four measures of HPA axis function moderately but significantly correlated with survival time in nonsurvivors (*r* = −0.56, *P* = 0.02; *r* = −0.56, *P* = 0.02; *r* = −0.57, *P* = 0.01; and *r* = 0.58, *P* = 0.02 for total cortisol, free cortisol, ACTH, and Δcortisol, respectively; Fig. 3I–L). Each parameter also moderately discriminated between survivors and nonsurvivors (c-statistic 0.70, 0.71, 0.76, and 0.62, respectively). Notably, within nonsurvivors receiving therapeutic dexamethasone there was loss at 24 h of this moderate correlation between the four parameters and survival time (*r* = −0.13; *P* = 0.62; *r* = −0.21, *P* = 0.46; *r* = −0.39, *P* = 0.12; *p* < 0.05 Dexamethasone Therapeutic vs. No Dexamethasone at this timepoint).

### Table 1: cortisol and ACTH levels in septic shock

<table>
<thead>
<tr>
<th>Time (h) after intrabronchial <em>S. aureus</em> challenge</th>
<th>No Dexamethasone (n=55)</th>
<th>Dexamethasone Therapeutic (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>24</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>48</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>72</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>96</td>
<td>600</td>
<td>600</td>
</tr>
</tbody>
</table>

### Fig. 2. Effect of dexamethasone therapy after bacterial challenge on cortisol and ACTH levels and cortisol responses to exogenous ACTH stimulation. Total (A), free (B), and bound cortisol (C), endogenous ACTH (D) and total cortisol levels response to low- (E) and high-dose (F) ACTH stimulation tests are plotted at serial time points after bacterial challenge for animals with (●) and without therapeutic dexamethasone (○). Serial mean (±SE) changes from baseline are plotted from a common origin, which represents the mean value of all animals at baseline (T = 0). Significant differences are denoted by symbols in each panel, and the corresponding *P* values are provided at the bottom of the figure.

# *p* < 0.05 Dexamethasone Therapeutic vs. No Dexamethasone at this timepoint
and $r = 0.001$, $P = 0.99$, for total cortisol, free cortisol, ACTH, and $\Delta$cortisol, respectively; Fig. 3, M–P). However, at 24 h with dexamethasone treatment, all four HPA axis measures discriminated consistently and almost perfectly between nonsurvivors and survivors ($c$-statistic 0.97, 0.98, 0.93, and 0.98, respectively; Fig. 3, M–P). Dexamethasone appeared to reduce cortisol levels in survivors alone, thereby increasing differences between survivors and nonsurvivors. This result suggests that dexamethasone suppressed cortisol production during sepsis only in animals likely to survive, and this suppression markedly increased the ability of total and free cortisol, ACTH, and $\Delta$cortisol after ACTH stimulation to discriminate between survivors and nonsurvivors.

**HPA axis recovers faster in survivors vs. nonsurvivors.** The mechanism responsible for the almost perfect $c$-statistic for all four HPA axis measures at 24 h after dexamethasone suppression was explored further by analyzing the relevant subgroups. Survivors and nonsurvivors with and without dexamethasone exposure were compared over time for differences in total and free cortisol and endogenous ACTH levels (Fig. 4). In the absence of dexamethasone, total cortisol, free cortisol, and endogenous ACTH levels were similarly elevated at 10 h in survivors and nonsurvivors (Fig. 4, A–C). However, among animals treated with dexamethasone, survivors had lower total and free cortisol levels than nonsurvivors at 10 h ($P = 0.03$ and $P = 0.06$, respectively; Fig. 4, D and E), even though endogenous ACTH levels were similar ($P = NS$; Fig. 4F). Thus, as early as 10 h after the onset of sepsis, the HPA axis was more responsive to dexamethasone suppression in survivors than in nonsurvivors.

Among animals not receiving dexamethasone, at 24 h cortisol levels began to decrease and were lower in survivors than in nonsurvivors ($P = 0.07$ and 0.01 for total and free cortisol levels, respectively; Fig. 4, A and B). Likewise, a similar pattern was seen for endogenous ACTH in the absence of dexamethasone ($P = 0.06$; Fig. 4C). However, among animals treated with dexamethasone, cortisol levels were markedly lower in survivors compared with nonsurvivors at 24 h (for total cortisol $P = 0.0002$, for free cortisol $P = 0.0005$; Fig. 4, D and E). Likewise, ACTH strongly distinguished between survivors and nonsurvivors at 24 h in the presence of dexamethasone ($P = 0.0002$; Fig. 4F). Importantly, differences...
between survivors and nonsurvivors at 24 h were greater with vs. without dexamethasone for total cortisol ($P = 0.05$ for an interaction; Fig. 4, A and D) and trended toward being greater for endogenous ACTH ($P = 0.06$ for an interaction; Fig. 4, C and F). Within 24 h after the onset of sepsis, the HPA axis of survivors compared with nonsurvivors was more suppressible by dexamethasone. Dexamethasone suppression of the HPA axis in survivors, but not nonsurvivors, increased the ability of these measures to predict outcome.

We next examined whether the responsiveness of cortisol to exogenous ACTH stimulation also returned toward presepsis levels faster in survivors compared with nonsurvivors. To compare changes over time, at each time point, dose-response plots were created comparing ACTH levels (endogenous ACTH and estimated peaks from exogenous ACTH challenges) vs. their associated cortisol responses (Fig. 5). We did not analyze the absolute changes in Fig. 5 but rather how the slope and intercept of each animal changed over time in response to the same exogenous ACTH stimulus. The slope of these lines in Fig. 5 represents changes in cortisol levels in response to changes in ACTH levels. The $y$-axis intercept of the lines in Fig. 5 represents the level of cortisol at very low ACTH levels (50 pg/ml). The $y$-axis intercept was estimated at 50 pg/ml instead of 0 pg/ml to avoid extrapolation to ACTH levels for which data were not available. For each panel in Fig. 6, we show only the slope plotted vs. intercept for each animal from Fig. 5. A 95% confidence region, represented by a gray ellipse, denotes the expected slope vs. intercept for all animals based on aggregated presepsis results (baseline at time 0 h).

Before the onset of sepsis (0 h), ACTH-cortisol dose-response slopes were on average very steep with relatively low intercepts, indicating that baseline cortisol levels were relatively low but increased markedly with exogenous ACTH stimulation (Fig. 6, A–D). By 24 h in the absence of dexamethasone therapy, survivors (Fig. 6E) and nonsurvivors (Fig. 6F) alike had decreased slopes and increased intercepts that were mostly outside of the presepsis ranges, particularly in nonsurvivors. In contrast, dexamethasone therapy at 24 h had largely restored ACTH responsiveness of cortisol to the presepsis state in survivors (Fig. 6G) but not in nonsurvivors (Fig. 6H). By 48–96 h, there was almost full recovery to high slopes and low intercepts similar to presepsis levels in most survivors, whereas nonsurvivors showed at best only partial recovery (Fig. 6, I–L).

In summary, in survivors, lowering cortisol and ACTH levels at 24 h as well as return of greater cortisol responsiveness to ACTH denoted a faster recovery of the HPA axis. Dexametha-
asone suppressed cortisol and ACTH levels and improved ACTH responsiveness, but only in survivors, resulting in even greater discrimination between survivors and non-survivors. Starting dexamethasone prior to sepsis also improved differentiation of survivors from nonsurvivors.

Compared with no dexamethasone therapy, dexamethasone started 48 h before *S. aureus* challenge and continued during sepsis significantly blunted sepsis-induced elevations in basal total cortisol levels, endogenous ACTH levels, and the total cortisol response to low and high doses of ACTH for up to 72 h following bacterial challenge (Fig. 7, A–D). Despite this blunting and similarly to animals receiving dexamethasone only after the onset of sepsis, HPA axis function as measured by total and free cortisol, ACTH, and Δcortisol still modestly to strongly discriminated survivors from nonsurvivors at 10 h (c-statistic 0.65, 0.60, 1.00, and 0.90, respectively) and at 24 h (0.75, 0.92, 0.92, and 0.92, respectively; Fig. 7, E–L).

Serial 24-h urinary cortisol excretion. Prophylactic dexamethasone significantly reduced urinary cortisol excretion on the first day of sepsis (*P* = 0.0004; data not shown). In animals receiving dexamethasone after the onset of sepsis, the reduction in urinary cortisol was modest and not seen until the last day of study, at 96 h (*P* = 0.03; data not shown). These findings are consistent with the early decreases in serum...
cortisol noted at 24 h, when dexamethasone was started before sepsis, and with the later decreases at 72–96 h noted in animals that received dexamethasone after the onset of sepsis.

Survival. Animals receiving dexamethasone treatment after the onset of sepsis in this pooled analysis, consistent with our previous studies, had significantly better survival rates compared with time-matched controls (Hazard ratio 0.34 (95% CI), 0.13 to 0.85, P = 0.02; Fig. 8A). In animals starting dexamethasone 2 days before the onset of sepsis, there was no significant beneficial effect on survival compared with controls (0.81 (95% CI), 0.29 to 2.28, P = 0.68; Fig. 8B).

Mineralocorticoid response. Similar to cortisol and ACTH (Fig. 1, A and B, respectively), by 10 h after the onset of sepsis there were marked increases in aldosterone levels among control animals (vs. baseline, P < 0.0001; Fig. 9A). However, different from the effect seen on basal cortisol and endogenous ACTH levels (which overall returned to baseline levels by 48 and 72 h, respectively), basal aldosterone levels remained significantly elevated at 72 h (P < 0.0001) compared with baseline. We modeled our data (using multiple linear regression; see Statistical analysis) to compare the relative strength of the correlation between aldosterone or cortisol and ACTH.

Fig. 6. Analysis of relationship between total cortisol and ACTH stimulation testing using intercepts and slopes from the dose-response plots. Slopes (y-axis) vs. intercepts (x-axis) of lines created for individual animals in Fig. 5 are plotted at 0 h (A–D), acutely at 24 h (E–H) after infection, and at recovery from 48 to 96 h (I–L) in survivors and nonsurvivors of septic shock without and with dexamethasone therapy. Specifically in these panels, for individual animals (○), the intercept of the line shown in Fig. 5 (cortisol level at ACTH level of 50 pg/mL; x-axis) is plotted vs. the slope of the line (change in cortisol in response to change in ACTH level; y-axis). Gray ellipses in each panel represent the 95% confidence region for baseline values of intercepts vs. slopes (0 h). Shift to right or left in intercepts out of the gray ellipses indicates a rise or fall in pre-ACTH cortisol levels, respectively; shift up or down out of the gray ellipses of slopes indicates a rise or fall in the cortisol response to ACTH, respectively.

No Dexamethasone Therapy

| Change in Total Cortisol level (nmol/L) with Increasing ACTH levels (Slope) |
|------------------|---|---|---|---|
|                | Survivors (n=15) | Non Survivors (n=40) |
|                | A | B | C | D |
|                | E | F | G | H |
|                | I | J | K | L |

Total Cortisol level at ACTH level of 50 pg/mL (Intercept)
Dexamethasone started 48 h before sepsis

levels. After accounting for both aldosterone and cortisol levels in the model, the association between aldosterone and ACTH was not significant ($P = 0.34$), whereas that between cortisol and ACTH was highly significant ($P < 0.0001$). Finally, unlike cortisol and other HPA measures, sepsis-associated increases in aldosterone were not suppressed by dexamethasone when administered for 2 days before bacterial challenge (Fig. 9B).

In contrast to our findings with cortisol, early increases in aldosterone had a moderate to strong correlation with survival time in nonsurvivors not treated with dexamethasone ($10 \mathrm{~h} r = -0.50, P = 0.001$, and $24 \mathrm{~h} r = -0.87, P < 0.0001$; Fig. 10, A and B). Based on these correlations, data were modeled at both 10 and 24 h using multiple linear regression to investigate whether aldosterone levels had stronger associations with survival than any of the four HPA axis measures previously evaluated (serum total cortisol, free cortisol, endogenous ACTH, and $\Delta$ corticosterone in response to ACTH stimulation; Fig. 2). Aldosterone levels were significantly correlated with survival at both 10 and 24 h after bacterial challenge (both $P < 0.0001$). After adjusting for aldosterone, the four HPA axis measures no longer demonstrated any significant correlation with survival time. Thus, only aldosterone levels were independently associated with survival time.

Similar to the pattern observed with total cortisol, free cortisol, endogenous ACTH, and $\Delta$ corticosterone, administering dexamethasone (either 2 days before or at the onset of sepsis) weakened the correlation between aldosterone levels and survival time at both 10 and 24 h in nonsurvivors (Fig. 10, C–F). However, dexamethasone administration strengthened the ability of aldosterone levels to discriminate survivors from nonsurvivors (c-statistic 0.76 and 0.91 at 10 and 24 h, respectively, for dexamethasone started after the onset of sepsis and 0.95 and 1.00 at 10 and 24 h, respectively, for dexamethasone started 2 days before the onset of sepsis; Fig. 10, C–F). Dexamethasone therapy increased overall survival and appeared to eliminate animals with low aldosterone levels from
the nonsurvivor group, presumably by converting them into survivors. The survival of almost all animals with low aldosterone levels at 10 and 24 h increased the aldosterone level differences between survivors and nonsurvivors, improving the c-statistic. This finding suggests that animals unlikely to survive despite low aldosterone levels could be specifically salvaged with dexamethasone therapy.

**Histology.** Adrenal tissue was obtained immediately post mortem from 12 randomly selected animals not treated with dexamethasone. There was a significant negative association between survival time and the degree of injury (mild, moderate, or severe, calculated according to the criteria in Table 2) to the adrenal cortex (slope, \(-0.23 \pm 0.07, P = 0.01\) for the relationship between survival time and degree of injury) and the whole adrenal gland (\(-0.34 \pm 0.12, P = 0.02\)).

**DISCUSSION**

Lethal *S. aureus* challenge resulted in a HPA stress response characterized by large, acute increases in total and free cortisol, ACTH, and aldosterone. Notably, basal cortisol levels during the early stages of septic shock were higher than those produced in response to supraphysiological doses of exogenous ACTH. Previous studies have shown that inflammatory mediators released during sepsis and in this model (25) can increase glucocorticoid production or levels independently of ACTH in rodents (1, 10, 36) and humans (14, 49, 63, 65). Cytokine release and metabolic derangements in critical illness can also impair cortisol inactivation (19, 39) and clearance from the circulation (35). Consistent with other reports (15, 54), early peak cortisol and ACTH levels did not correlate with survival in the present study, suggesting that the acute phase of the HPA axis response to septic shock is not very sensitive to disease severity or eventual outcome. However, time to recovery of the HPA axis toward an unstressed state did reflect severity. Cortisol and ACTH levels returned toward baseline after 24 h in survivors but not in nonsurvivors. Importantly, recovery of ACTH responsiveness, dexamethasone suppression of cortisol, aldosterone levels, and the severity of adrenal gland pathology were all associated with survival.

Outside the critical care setting, the presence of persistent hypercortisolism in the absence of primary adrenal pathology, pituitary tumors or ectopic ACTH production has been referred to as a pseudo-Cushing state or as functional hypercortisolism (60). Functional hypercortisolism can occur in active alcoholism, metabolic syndrome, major depression, and severe obesity and has been ascribed to an increase in corticotropin-releasing hormone (CRH) with an otherwise normal HPA axis. Dexamethasone suppression of cortisol production can therefore
distinguish functional hypercortisolism from Cushing’s syndrome because in the former, negative feedback mechanisms are intact (67). Also, unlike Cushing’s syndrome (45) and perhaps due to intact negative feedback, the ACTH response to CRH stimulation testing is blunted in functional hypercortisolism. Lethal septic shock, a state of extreme stress, seemingly differs from functional hypercortisolism because, like Cushing’s syndrome, dexamethasone administration was unable to suppress the very high cortisol levels. However, this finding might be explained, at least in part, by the prolongation of cortisol half-life in sepsis (13, 38) rather than as a true inability to suppress production. Nonetheless, lethal sepsis appears to cause a distinct form of HPA axis dysfunction that has not been fully described.

Cortisol production during lethal sepsis is driven both by sustained elevations in ACTH levels and by ACTH-independent mechanisms (63). Early in all animals, and persisting longer among nonsurvivors, we found that the adrenal gland did not respond to exogenous ACTH. Whether this finding reflects adrenal exhaustion (9, 63), a maximum threshold for cortisol production, or ACTH insensitivity is not clear. In support of the adrenal exhaustion hypothesis, Beishuizen et al. (8) found that nonsurvivors of septic shock or trauma had the highest cortisol levels combined with lowest levels of dehydroepiandrosterone sulfate (DHEAS), suggesting that adrenal corticosteroid synthesis may be shifted away from androgens and toward excessive cortisol production. DHEA and DHEAS are, in health, the most abundant steroids secreted by the adrenal cortex, and the decrease in DHEAS levels during lethal septic shock may be a sign of exhausted adrenal reserves (8, 63). Clinical sepsis studies also have reported that a poor response to ACTH stimulation correlated with non-survival (5, 53). However, this criterion has not been predictive of benefit from exogenous corticosteroid administration in clinical trials (4, 12, 16, 37, 47). In the current study, acutely at 10 h, survivors and nonsurvivors alike had high basal cortisol levels, and both similarly had no increase in cortisol in response to exogenous ACTH. Therefore, neither basal nor Δcortisol in response to ACTH distinguished survivors from nonsurvivors at relatively early time points during septic shock. A lack of
association between survival and acute cortisol/ACTH responses has been previously documented in coronary syndromes (18, 48), major surgery (44), trauma (66), burns (28, 46), and hemorrhage (29).

Aldosterone regulation appeared to be largely independent of the HPA axis stress response. The time course of aldosterone release during septic shock was distinct from that of ACTH and cortisol. Furthermore, unlike total cortisol, free cortisol, ACTH, and the Δcortisol response to exogenous ACTH, aldosterone was not suppressed by dexamethasone when started 48 h before sepsis. Although partially regulated by ACTH under normal conditions, aldosterone is primarily regulated by the renin-angiotensin system (24, 43). Our data also indicate that ACTH is not the principal regulator of aldosterone during lethal septic shock. In contrast to cortisol and ACTH, aldosterone levels in nonsurvivors were independently correlated with survival time early in septic shock. The role of aldosterone in salt and water metabolism and in the maintenance of intravascular volume (21, 22, 64) may explain, in part, the association with survival. Intravascular volume depletion, decreased peripheral resistance and metabolic acidosis are potent stimuli for aldosterone production during septic shock (21, 25). Aldosterone counteracts shock by expanding plasma volume, retaining sodium, and increasing peripheral resistance (21, 25, 64). The association of aldosterone levels with survival in our study suggests that its production is modulated by shock severity.

Our results demonstrate that single determinations of cortisol and isolated ACTH stimulation tests can be misleading. Serial evaluations were critical to understanding the evolution of HPA axis dysfunction over the course of severe sepsis. In patients with septic shock, Bouachour et al. (15) similarly found that cortisol levels were elevated acutely in survivors and nonsurvivors alike and that nonsurvivors maintained persistently elevated levels over time. In canines with parvoviral diarrhea, Schoeman et al. (54) found, similar to our model of experimental pneumonia, that on admission, survivors and nonsurvivors had similarly elevated cortisol levels, but by 24 h, cortisol was significantly higher among animals that eventually died from the infection. Also consistent with our results, Flierl et al. (22) found in a rat model of cecal ligation and puncture that aldosterone levels remained significantly elevated for prolonged periods of time after sepsis, whereas cortisol levels started decreasing shortly after 24 h. However, heterogeneous patient populations with septic shock, unlike controlled animal models, have poorly defined time lines and multiple sources of stress. The variable response over time may explain, in part, why ACTH stimulation tests have not reproducibly identified patients in clinical studies that might benefit from corticosteroid therapy (32, 39, 41).

Unlike ACTH stimulation testing alone, dexamethasone suppression of the HPA axis, performed in the first 24 h after the onset of septic shock, may help discriminate between survivors and nonsurvivors. The inability of dexamethasone to suppress the high levels of cortisol and ACTH in severe sepsis, followed by a lack of response to ACTH stimulation, identified animals with severe HPA axis dysfunction and a high likelihood of death. Previous clinical studies have also demonstrated that HPA axis negative feedback mechanisms are profoundly altered during critical illness and/or sepsis (6, 50, 51). Reincke et al. (51) showed that dexamethasone did not suppress hypercortisolism in ICU patients compared with healthy controls. Similarly, Perrot et al. (50) found that cortisol levels in patients with shock, both septic and nonseptic, were not suppressible with dexamethasone within the first 24 h after the onset of shock. Even in non-shock states, such as the surgical removal of brain tumors, cortisol and ACTH levels are resistant to dexamethasone suppression for at least 48 h during severe stress (6). Decreased cortisol clearance might result in a false negative dexamethasone suppression test. Consistent with this notion, cortisol half-life may be particularly prolonged in nonsurvivors compared with survivors (13, 38); however, this cannot explain why ACTH levels were not suppressed by dexamethasone in nonsurvivors. The mechanistic relationship, if any, between dexamethasone suppression of cortisol levels and dexamethasone-induced increases in survival time is unclear. Nonetheless, the pharmacological administration of corticosteroids rescued some animals with a high risk of death.

The interpretability of our findings has a number of important limitations. Prior studies in nonseptic canines suggest that a portion of cortisol responses in the present study may be attributed to sedation, intubation, mechanical ventilation, and repeated ACTH stimulation testing (59). However, after accounting for the potential stimulatory effects of critical care support and repeated ACTH stimulation tests, most of the increase in cortisol levels was due to the sepsis-induced stress response. Importantly, our experimental model does not recapitulate all of the complexities found in patients with septic shock. For example, it is unknown whether the current findings would apply to infections caused by bacteria other than S. aureus or to other sites of infection. Furthermore, use of continuous infusion dexamethasone, rather than bolus treatments, may have affected the results. Although some animals received desoxycorticosterone with dexamethasone, exogenous mineralocorticoid administration did not significantly alter cortisol, ACTH, or aldosterone levels during the course of septic shock. Moreover, our findings do not necessarily rule out a relative glucocorticoid deficiency during septic shock. Alterations in glucocorticoid receptor (GR) signaling may also have a role in the HPA dysfunction during sepsis and critical illness (11). Even in the presence of high levels of cortisol, a relative insufficiency can occur due to reduced levels or impaired function of GR. This may be a consequence of GR downregulation by cytokines (31, 55), decreased ligand affinity (62), or an altered expression profile of the GR isoforms (31). Finally, in retrospect, measurements of DHEA, DHEAS, CRH, and the renin-angiotensin system may have added valuable information.

In conclusion, sepsis produces an initial cortisol and ACTH response that is unrelated to survival time. However, compared with nonsurvivors the high cortisol and ACTH levels, as well as the HPA axis responses to dexamethasone and ACTH, normalized faster in survivors. Nonsurvivors exhibited a distinct HPA axis dysfunction characterized by persistently high cortisol and ACTH levels with blunted responses to exogenous ACTH stimulation and dexamethasone suppression. Aldosterone during septic shock was independent of HPA axis control, remained elevated longer than cortisol, and was independently associated with survival. These findings suggest that the early HPA axis response to stress is not closely calibrated to illness severity, whereas aldosterone regulation may be more directly linked to the degree of shock and potential for death. Finally,
during sepsis-induced hypercortisolemia, dexamethasone suppression followed by ACTH stimulation testing may further help to distinguish likely survivors from nonsurvivors during sepsis. Collectively, these tests may identify a patient population with a poor prognosis that might be more likely to benefit from new therapeutic strategies.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


