Characterization of an Interleukin-6- and Adrenocorticotropin-Dependent, Immune-to-Adrenal Pathway during Viral Infection

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There has been longstanding interest in the capacity of the immune system to access immunomodulatory glucocorticoid responses without invoking upstream neuroendocrine secretagogues, including CRH and ACTH. Here, we investigate the role of CRH and ACTH in adrenal glucocorticoid responses to murine cytomegalovirus (MCMV). Mice infected with MCMV exhibit IL-6-dependent glucocorticoid responses that peak at 36 h post infection and protect against cytokine (TNFα)-mediated lethality. Acute administration of a CRH-antibody (Ab) completely eliminated ACTH responses to both low- and high-dose MCMV. However, corticosterone responses in CRH-Ab-treated animals remained apparent in mice infected with low-dose MCMV and were robust in mice infected with high-dose MCMV. CRH-knockout (KO) mice exhibited robust corticosterone responses to both MCMV doses, despite reduced baseline and MCMV-induced ACTH. Interestingly, robust corticosterone responses in CRH-Ab-treated and CRH-KO mice were associated with exaggerated IL-6 levels, and IL-6 and corticosterone concentrations in infected CRH-Ab-treated animals were significantly correlated. Neutralization of IL-6 responses in infected CRH-KO mice reduced corticosterone responses by approximately 70%. Finally, MCMV-infected mice deprived of ACTH by hypophysectomy failed to elicit glucocorticoid responses, despite elevated plasma IL-6 concentrations. Taken together, these results suggest that a greater than normal induction of IL-6 compensates for the absence of a normal CRH-dependent ACTH surge during viral infection. This enhanced IL-6 response, in turn, may mediate a direct immune-adrenal pathway that can become a predominant driving force for glucocorticoid induction in the absence of CRH. However, the presence of ACTH appears to serve as a necessary permissive factor, enabling direct cytokine actions on the adrenal gland. (Endocrinology 145: 3580–3589, 2004)

Although hypothalamic CRH is considered a primary mechanism by which cytokines stimulate adrenal glucocorticoid responses (cortisol in humans and non-human primates; corticosterone in rodents), there has been longstanding interest in the potential contribution of alternative, CRH-independent pathways that include a direct action of cytokines at the level of the pituitary and/or adrenal glands (for reviews, see Refs. 1–8). Indeed, in the early 1980s, Blalock and colleagues (9) proposed the concept of a lymphoid-adrenal axis, where ACTH produced by virus-stimulated lymphocytes was able to directly stimulate corticosterone release in the absence of pituitary-derived ACTH. However, subsequent studies (10, 11) failed to replicate Blalock’s findings and reported that extrapituitary sources of ACTH were not sufficient to stimulate adrenal steroidogenesis. Nevertheless, more recent data from in vitro studies indicate that cytokines may have the capacity to stimulate the adrenal gland directly, and therefore may activate glucocorticoid release independent of central nervous system neuroendocrine pathways (see below).

Considerable data exist describing the pathways by which purified cytokines or endotoxin [lipopolysaccharide (LPS)] induce glucocorticoid release (for reviews, see Refs. 1–8); however, few studies have examined these pathways during viral infection. To further investigate the neuroendocrine pathways by which virus-induced immune responses access a glucocorticoid response, our laboratories have been studying mice infected with murine cytomegalovirus (MCMV). MCMV is a cytopathic herpes virus that induces an early natural killer (NK) cell-mediated, antiviral defense. The antiviral immune response is characterized by high levels of IL-12 and NK cell-produced interferon-γ (12), and NK cell-mediated liver inflammation (13). In addition to the induction of antiviral cytokines, the proinflammatory cytokines, TNFα, IL-1, and IL-6, are induced during the innate immune response to MCMV infection. Peak serum cytokine concentrations occur 36–44 h after infection and are paralleled by peak neuroendocrine (ACTH and corticosterone) responses, which occur at 36 h after MCMV infection (14). Previous studies have shown that the MCMV-induced corticosterone response is dependent on IL-6 (14) and that this glucocorticoid response is essential for protection against TNFα-mediated lethality (15).

Relevant to neuroendocrine pathways by which IL-6 may induce a glucocorticoid response, IL-6 receptors (IL-6Rs) have been detected in the brain (16, 17), pituitary (18, 19)
[mouse corticotropes, in particular (18)], and adrenal glands (18, 20–23) of several species. In support of IL-6 activation of the hypothalamic-pituitary-adrenal (HPA) axis at the hypothalamic level, it has been demonstrated that lesions of the paraventricular nucleus of the hypothalamus (PVN) or the administration of an anti-CRH-Ab has profound effects on reducing IL-6-induced ACTH and corticosterone responses (24–27). In vitro, IL-6 has been shown to stimulate the rapid release of CRH from rat hypothalamic explants (28), correlating well with the in vivo findings that IL-6 induces a rapid rise in ACTH after iv administration. In addition, IL-6 is capable of acting directly on CRH nerve terminals in the median eminence (29, 30) and may thereby increase CRH release without necessarily increasing CRH synthesis in the PVN.

In vitro studies also have provided increasing evidence in support of IL-6 activation of extrahypothalamic mechanisms of glucocorticoid release. For example, IL-6 has been shown to stimulate proopiomelanocortin expression/activity or ACTH release from cultured pituitary cells (29), hemipituitaries (31), and AtT-20 (mouse corticotrophic tumor cell line) cells (32–34). Likewise, IL-6 has been shown to stimulate the release of corticosterone from cultured adrenal cells, alone (20, 22, 23, 35–37) and in synergy with low levels of ACTH (20, 36).

In the present study, we endeavored to characterize the relative contribution of various levels of the HPA axis to the endogenous glucocorticoid response to MCMV infection. To determine whether CRH mediates the MCMV-induced ACTH and corticosterone responses, neuroendocrine responses were measured in mice treated with a CRH-antibody (Ab) or in CRH-knockout (KO) mice. Although CRH-KO mice have been shown to exhibit markedly attenuated corticosterone responses to nonimmunological stressors, such as restraint, ether, and fasting (38), they are able to mount a significant corticosterone response to a variety of immunological stressors, such as LPS, carageenan, and turpentine (18, 39, 40). However, none of these studies examined the ACTH and corticosterone responses to an immune challenge in the face of an acute elimination of CRH. In addition, we assessed the role of IL-6 and arginine vasopressin (AVP) in mediating the pituitary-adrenal response to MCMV in the absence of CRH by administering a respective immunoneutralizing Ab to infected CRH-KO mice. Finally, we investigated the importance of ACTH in immune-induced glucocorticoid responses. The requirement of ACTH in MCMV-induced corticosterone release was addressed in infected hypophysectomized mice.

Materials and Methods

Subjects

Male mice (ages 7–14 wk) were housed in the Emory University Animal Care Facility for at least 1 wk before use and were kept on a 12-h light, 12-h dark cycle with lights on at 0700 h. Breeder pairs of homologous CRH-KO mice (C57BL/6 × 129/SvJ background) were kindly provided by Dr. Katia Karalis (Children's Hospital, Harvard Medical School, Boston, MA) and bred at Emory University. CRH-KO breeder pairs were kept on corticosterone-treated water (10 mg/ml, Sigma, St. Louis, MO) to ensure survival of offspring (personal communication with Dr. Maria Venilhaki, Children's Hospital, Harvard Medical School, Boston, MA). Once weaned, the CRH-KO offspring were maintained on untreated water. Wild-type (WT) controls (B6;129SF2) were obtained from The Jackson Laboratory (Bar Harbor, ME). Hypophysectomized, sham-operated, and normal C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival, hypophysectomized mice were placed on corticosterone-treated water (10 mg/ml, Sigma, St. Louis, MO) and given a 5% sucrose solution to drink throughout the duration of the experiment. A hypophysectomy was considered complete if less than 15 mg/ml plasma ACTH (and no corticosterone response) was detected in response to a 90-s forced swim. Blood was collected from the retro-orbital cavity, under methoxyflurane (Metofane) anesthesia, 15 min post swim. Subsequent challenges (e.g., cortrosyn, MCMV) were administered 1–2 d post retro-orbital bleed. (Mice with ACTH >15 pg/ml and a significant swim-induced corticosterone response were not included in data analysis.) Except for designated experimental manipulations, mice were minimally disturbed. Protocols were in accordance with institutional guidelines for animal care and use.

Virus

Stocks of salivary gland-extracted MCMV, Smith strain, were generated by Dr. Christine Biron (Brown University, Providence, RI) as previously described (41). Mice were infected ip with MCMV [5 × 104 plaque-forming units (PFU)/mouse (low-dose) or 1 × 105 PFU/mouse (high dose)] in 100 µl of vehicle or control injected with 100 µl of vehicle alone [1× media 199 (Invitrogen Life Technologies, Grand Island, NY) and 3% FBS (HyClone, Logan, UT)] between 2000 and 2100 h. Plasma IL-6 was measured in all experiments to determine evidence of a successful infection. (MCMV-infected mice with IL-6 <100 pg/ml were not included in data analysis.)

In vivo treatments

Polyclonal antibodies (Ab) to CRH (sheep antirat/human CRH serum) and AVP (rabbit) were generously provided by Dr. Wylie Vale (The Salk Institute, La Jolla, CA). Mice were injected ip with the CRH- or AVP-Ab 8 h before the peak MCMV-induced corticosterone response (200 µl/mouse, 28 h post infection). This dose has been determined by the ability of the Ab to block a restraint-induced ACTH response and has been shown to be effectively neutralizing for 8 h (42). Control mice for the CRH-Ab and AVP-Ab experiments received injections of normal sheep serum (NHS) or normal rabbit serum (NRS), respectively (200 µl/mouse, Colorado Serum Co., Denver, CO). The IL-6-Ab (monoclonal rat antinmune) was prepared and purified as described below. Mice were injected ip with the IL-6-Ab 16 h before the peak MCMV-induced corticosterone response (1 mg/mouse, 20 h post infection). This dose was determined by the ability of the Ab to block a recombinant mouse IL-6 (1 µg/mouse)-induced corticosterone response. Control mice for the IL-6-Ab experiments received injections of purified rat IgG (1 mg/mouse, Sigma, St. Louis, MO). To examine the adrenal integrity of hypophysectomized mice and test their ability to release corticosterone in response to an ACTH challenge, hypophysectomized mice were injected ip with Cortrosyn (ACTH24–25, 25 µg/mouse, Organon, West Orange, NJ). Trunk blood was collected 30 min post injection for measurement of plasma corticosterone.

Preparation and purification of monoclonal antibodies against mouse IL-6

A hybridoma cell line (rat-mouse hybridoma) secreting antinmune IL-6-Ab (6B4, rat IgG1) was kindly provided by Dr. Jacque Van Snick (Ludwig Institute for Cancer Research, Brussels, Belgium). The cells initially were grown in hybridoma serum-free media (HSFM 1×, Invitrogen Life Technologies) supplemented with 10% FBS (HyClone) and 2 ng/ml recombinant human IL-6 (R&D Systems, Minneapolis, MN) and adapted to grow in HSFM with no FBS and 0.5 ng/ml recombinant human IL-6. Cells were incubated at 37°C and 5% CO2, Cells were passed in mid log-phase growth (~1×106 cells/ml). Supernatant was collected and dialyzed for buffer exchange with the binding buffer (0.01 M sodium phosphate, 0.15 M NaCl, and 0.01 M EDTA (pH 7.0)) to be used for purification by protein G affinity chromatography [HiTrap Protein G High Performance (HP) column; Amersham Biosciences, Piscataway, NJ]. After the dialyzed supernatant was applied to the column was washed with binding buffer until the absorbance at 280
nm returned to baseline. The Abs were then eluted with 0.1 M glycine-HCl (pH 3.0), and the protein fractions were collected in tubes already containing 1 M Tris-HCl (pH 9.0) to immediately neutralize the eluate. The concentration of Abs was quantified by reading the absorbance at 280 nm, and the fractions with the highest protein concentration were pooled and buffer exchanged with 0.9% saline. The Ab concentration of the final preparation was then quantified by bicinchoninic acid (BCA) Protein Assay (Pierce, Rockford, IL). This Ab has been well characterized in Dr. Van Snick’s laboratory (43, 44) and has been shown, in vitro, to inhibit the proliferation of an IL-6-dependent cell line (7TD1) (45).

Forced swim

Mice were placed in a 4-liter beaker of 20 C water for 90 sec and then returned to their home cage until the time they were killed, 15 min post stress.

Plasma collection

At 36 h post infection (between 0800 h and 0900 h), trunk blood was collected under low stress conditions (within 3 min of exposure to isoflurane anesthesia). Blood was collected into EDTA tubes, kept on ice, and centrifuged at 4 C. Plasma was aliquoted and stored at −80 C until assayed for hormone/cytokine levels.

Hormone and cytokine assays

Plasma ACTH and corticosterone were measured by RIA. [ACTH: Allegro HS-ACTH, Nichols Institute Diagnostics (San Juan Capistrano, CA); limit of detection = 5 pg/ml; intraassay coefficient of variation (CV) = 3.1%; interassay CV = 7.3%; CORT: ImmunoChem, ICN Biomedicals (Costa Mesa, CA); limit of detection = 5 ng/ml; intraassay CV = 7.5%; interassay CV = 6.9%] Plasma IL-6 was measured by sandwich ELISA (Quantikine murine kit, R&D Systems; limit of detection = 3 pg/ml; intraassay CV = 4.5%; interassay CV = 7.1%).

Statistical analysis

Values are presented as means ± SEM. A one-way ANOVA was used to assess the effect of treatment (infection/Ab) on dependent variables. A two-way ANOVA was used to evaluate genotype or surgical manipulation, as well as treatment (infection/Ab) effects. The Student-Newman-Keuls method was used for post hoc tests of significant differences between group means. Where indicated, P values were obtained by comparing groups using a Student’s t test. The level of significance was set at P < 0.05, and all tests of significance were two tailed.

Results

ACTH and corticosterone responses to MCMV in CRH-Ab-treated mice

To determine the effectiveness of the CRH-Ab, we examined its ability to block the corticosterone response to a stressor that is known to be CRH dependent. Administration of the CRH-Ab 8 h before a forced-swim stress completely eliminated the stress-induced ACTH and corticosterone responses (Fig. 1, A and B).

As found in mice exposed to swim stress, mice infected with either a low (5 ⋅ 10⁴ PFU/mouse) or high (1 ⋅ 10⁵ PFU/mouse) dose of MCMV who were administered the CRH-Ab failed to exhibit an ACTH response (Fig. 2A). In fact, plasma ACTH concentrations in CRH Ab-treated animals infected with MCMV were lower than in noninfected control animals. In contrast to ACTH, corticosterone responses to low-dose MCMV in CRH-Ab-treated mice were apparent, albeit significantly reduced, and corticosterone responses to high-dose MCMV remained robust, reaching concentrations similar to those seen in infected, non-CRH-Ab-treated controls (Fig. 2B). Interestingly, plasma IL-6 levels in CRH-Ab-treated mice infected with high-dose MCMV were significantly greater than non-CRH-Ab-treated, MCMV-infected animals, whereas there was no significant difference in IL-6 induction between CRH-Ab-treated and non-CRH-Ab-treated mice infected with low-dose MCMV (Fig. 2C). Plasma IL-6 and corticosterone concentrations (across both doses of virus) were highly correlated (r = 0.86) in MCMV-infected, CRH-Ab-treated animals (Fig. 3). A similar correlation was found in infected, non-CRH-Ab-treated mice (r = 0.78).

ACTH and corticosterone responses to MCMV in CRH-KO mice

As in the case of CRH-Ab-treated, MCMV-infected mice, CRH-KO mice infected with MCMV (1 ⋅ 10⁵ PFU/mouse) exhibited a robust corticosterone response despite reduced baseline and MCMV-induced ACTH relative to WT animals (Fig. 4, A and B). Of note, in contrast to MCMV-infected CRH-Ab-treated mice, CRH-KO mice did exhibit a small but significant increase in ACTH in response to MCMV. Infected CRH-KO mice also exhibited a 3-fold greater elevation in plasma IL-6 concentrations in comparison with infected WT animals (Fig. 4C). Similar results were found in mice infected with a lower dose of virus (5 ⋅ 10⁴ PFU/mouse) (data not shown).
Role of IL-6 in the MCMV-induced ACTH and corticosterone responses in CRH-KO mice

Previous data from our labs have shown IL-6 to be the pivotal mediator of the glucocorticoid response to MCMV (14). Accordingly, an IL-6-Ab was administered to determine whether IL-6 played a similar role in the corticosterone response of MCMV-infected mice rendered CRH deficient. Neutralization of IL-6 in the CRH-KO mice infected with MCMV (5 × 10^4 PFU/mouse) resulted in an approximately 70% decrease in both the ACTH and corticosterone responses, compared with that of untreated, infected CRH-KO mice (Fig. 5, A and B). Similar to our previously reported results in infected IL-6-KO mice (14), infected WT mice ad-
ministered the IL-6-Ab exhibited reduced ACTH and corticosterone responses to MCMV compared with that of untreated, infected WT mice (Fig. 5, A and B). Although infected CRH-KO mice administered the IL-6-Ab exhibited a dramatic decrease in their corticosterone response, there was no increase in sickness behavior or lethality observed at 36 h post infection.

Role of AVP in the MCMV-induced ACTH and corticosterone responses in CRH-KO mice

To assess the role of other hypothalamic ACTH secretagogues in regulating pituitary-adrenal axis activity in the absence of CRH, the role of AVP, known to play a synergistic role with CRH in stimulating ACTH release from the anterior pituitary, was examined in MCMV-infected CRH-KO mice treated with an AVP-Ab. Administration of the AVP-Ab did not have a significant effect on the ACTH response to MCMV (1 × 10^5 PFU/mouse) in WT or CRH-KO mice. Nevertheless, infected CRH-KO mice administered the AVP-Ab exhibited a significant attenuation (~30%) in their corticosterone response (MCMV/NRS, 219 ± 23 ng/ml; MCMV/AVP-Ab, 155 ± 21 ng/ml). Moreover, AVP-Ab-treated, infected CRH-KO mice demonstrated a significantly greater IL-6 induction in comparison to untreated, infected CRH-KO mice (MCMV/NRS, 2655 ± 169 pg/ml; MCMV/AVP-Ab, 3423 ± 309 pg/ml).

The corticosterone response to MCMV in hypophysectomized mice

Although MCMV-induced ACTH increases were eliminated in CRH-Ab-treated mice and reduced in CRH-KO mice, low levels of ACTH were still present. Therefore, the absolute role of ACTH in the MCMV-induced corticosterone response was investigated in infected hypophysectomized mice. In the absence of pituitary-derived ACTH, MCMV-infected mice failed to elicit a corticosterone response to either a low (data not shown) or high dose of MCMV, despite a significantly greater induction of IL-6 relative to infected sham controls (Fig. 6, B and C). The lack of a corticosterone response to MCMV in these animals was not due to adrenal insensitivity to ACTH because hypophysectomized animals were able to produce a corticosterone response 30 min after a Cortrosyn (ACTH1–24, 25 μg/mouse, ip) challenge (hypox/saline, 0.38 ± 0.38 ng/ml; hypox/Cortrosyn, 176 ± 50 ng/ml).

FIG. 5. Effect of the administration (ip) of an IL-6-Ab on plasma ACTH (A) and corticosterone (B) levels in WT and CRH-KO mice after injection with MCMV (5 × 10^5 PFU/mouse) or vehicle. The IL-6-Ab (1 mg/mouse) was administered 16 h before the peak MCMV-induced corticosterone response (20 h post infection). Trunk blood was collected from mice at 36 h post infection (n = 4–8 animals per group). *, P < 0.05; ***, P < 0.001 (t test for WT/MCMV/IL-6-Ab vs. WT/media/IgG); compared with media/IgG group within genotype. #, P < 0.05; ###, P < 0.001; compared with MCMV/IgG group within genotype. –, P > 0.05; +, P < 0.01 (t test); ++, P < 0.001; compared with respective WT group. Two-way ANOVA was used (unless otherwise indicated). Because of limited amounts of Ab, a media/IL-6-Ab group was not included in this experiment.

FIG. 6. Plasma ACTH (A), corticosterone (B), and IL-6 (C) levels after injection with MCMV (1 × 10^5 PFU/mouse) or vehicle in sham-operated or hypophysectomized (hypox) C57BL/6 mice. Trunk blood was collected from mice at 36 h post infection (n = 3–11 animals per group). ***, P < 0.001; compared with media group within surgical manipulation. ++, P < 0.01; ++++, P < 0.001; compared with respective sham group. Two-way ANOVA indicated a significant interaction of surgery and infection on corticosterone and IL-6 responses to MCMV.
Discussion

The present study demonstrates that in the case of both acute (CRH-Ab administration) and chronic (CRH-KO) CRH deficiency, alternative (CRH independent) pathways can contribute to glucocorticoid induction during viral infection. The presence of corticosterone responses in infected CRH-Ab-treated mice, despite the failure to elicit an ACTH response, indicates that nonpituitary-ACTH factors, such as cytokines (IL-6), can act directly on the adrenal gland to drive glucocorticoid release. Previous studies from our laboratories have shown IL-6 to be a pivotal mediator of the MCMV-induced corticosterone response in CRH-intact mice (14). Interestingly, CRH-deficient mice (CRH-Ab-treated and CRH-KO mice) that exhibited significant corticosterone responses to MCMV, demonstrated dramatically greater IL-6 responses compared with infected CRH-intact mice. This IL-6 response proved to be critical for glucocorticoid induction in the absence of CRH because neutralization of IL-6 in CRH-KO mice greatly attenuated the MCMV-induced corticosterone response. Interestingly, MCMV-infected mice deprived of pituitary-ACTH by hypophysectomy failed to elicit glucocorticoid responses, despite elevated IL-6. Taken together, these data suggest that IL-6 can directly activate adrenal glucocorticoid responses. However, ACTH appears to serve as a necessary permissive factor, enabling this direct immune-to-adrenal pathway in viral infection.

Based on our data, it appears that the direct action of cytokines on subhypothalamic levels of the HPA axis is not completely independent of upstream neuroendocrine secretagogues. Our findings in hypophysectomized mice indicate that ACTH is required for immune-induced glucocorticoid release during viral infection. These data are supported by other in vivo findings demonstrating that hypophysectomized animals fail to exhibit a glucocorticoid response to various immune challenges, including IL-1 and Newcastle disease virus (10, 11, 46), unless pretreated with a low dose of ACTH (47). Thus, the low levels of ACTH present in our MCMV-infected mice treated with the CRH-Ab (and in infected CRH-KO mice) may reveal a direct action of IL-6 (elevated levels) on the adrenal gland. Indeed, Barney et al. (20) have shown that ACTH and IL-6 act synergistically to induce corticosterone release from adrenal cell cultures at time points when IL-6 alone is ineffective. The mechanism by which ACTH supports the direct action of cytokines, such as IL-1 and IL-6, on the adrenal gland has not been elucidated. One possibility includes an ACTH-induced up-regulation of adrenal gland cytokine receptor expression. Such up-regulation of cytokine receptors by upstream neuroendocrine factors has been found in the pituitary gland after administration of CRH (see below).

On the other hand, the high levels of IL-6 may increase adrenal sensitivity to submaximal doses of ACTH. IL-6R mRNA has been detected in both the adrenal cortex and medulla of multiple species, including mice (18, 20–23). In addition to adrenal immune cells, such as macrophages, expressing the IL-6R, steroid-secreting cells of the adrenal cortex have been shown to express IL-6R. Moreover, IL-6 mRNA has been detected in macrophages and steroid-producing cells of the adrenal cortex of numerous species (48) and its release is stimulated by LPS, IL-1, and ACTH (49–51). Adrenal sensitivity to ACTH is regulated by adrenal innervation (for example, splanchnic nerve stimulation) with the subsequent release of catecholamines and vasoactive neuropeptides that affect adrenal blood flow and hence, increase the presentation of ACTH to the steroidogenic cells of the adrenal cortex and result in an increase of glucocorticoid release (52–62). However, the role of IL-6, whether circulating or intradrenal, in this interplay requires further investigation.

The requirement of upstream secretagogues to enable neuroendocrine activation at subhypothalamic levels of the HPA axis also is apparent at the level of the pituitary gland. Our data are consistent with previous reports showing that acute elimination of CRH (by the administration of a CRH-Ab) blocks the ACTH response to various purified cytokines (24, 26, 63, 64). The lack of an observed rise of ACTH in the context of a rise in corticosterone in this study cannot be effectively explained as the result of glucocorticoid-negative feedback on the pituitary because the CRH-Ab-treated mice infected with the low dose of MCMV or subjected to the swim stress exhibited a mild or absent corticosterone response, respectively, and still failed to exhibit any ACTH response. Besides actively driving ACTH release, CRH has been shown to increase pituitary sensitivity to circulating cytokines. For example, Payne et al. (65) demonstrated that low levels of CRH sensitize the pituitary to the direct effects of IL-1 on ACTH release. Interestingly, once sensitized by CRH, IL-1 induction of ACTH release is no longer inhibited by a CRH antagonist. Previous data have demonstrated that CRH up-regulates pituitary IL-1 receptor expression (66, 67), thus providing a potential mechanism for these effects.

In contrast to cytokine-challenged animals with an acute elimination of CRH (via a CRH-Ab), our data and others indicate that CRH-KO or CRHR1-KO mice are capable of producing an ACTH response to an immune challenge (18, 39, 42). These data suggest that (genetically induced) chronic CRH deficiency may induce compensatory mechanisms acting at the level of the pituitary gland, including cytokines and other upstream ACTH secretagogues. In fact, the MCMV-infected CRH-KO mice administered an IL-6-Ab exhibited an approximately 70% attenuation in their ACTH response, suggesting that IL-6 plays an important CRH-independent role in stimulating ACTH release in these animals. In addition to cytokines acting directly at the pituitary gland, another possible mechanism involves hypothalamic arginine vasopressin (AVP) (3). AVP is known to play a synergistic role with CRH in stimulating ACTH release from the anterior pituitary. However, administration of an AVP-Ab did not have a significant effect on the ACTH response to MCMV in either infected WT or CRH-KO mice. Turnbull et al. (42) also have shown that the turpentine-induced ACTH response in CRHR1-KO mice is AVP independent. Of note, elimination of AVP did significantly attenuate the corticosterone response in MCMV-infected CRH-KO mice. These results suggest that AVP may exert a direct action on the adrenal gland to stimulate glucocorticoid release in CRH-KO mice. In this case, the source of AVP would be the magnocellular, rather than the parvocellular, cells of the PVN, releasing AVP from the posterior pituitary into the general circulation. AVP receptors, of the V1 subtype, are present on the adrenal gland.
stimulatory effects on adrenal glucocorticoid production in several species, in vivo (71–73) and in vitro (74, 75). Moreover, the direct adrenal effect of AVP has been shown to be dependent on the presence of low level ACTH (73, 74). Of note, increased plasma AVP concentrations are found in response to long-term systemic IL-6 injections in man (76).

The marked increase in IL-6 that was observed in infected CRH-deficient mice seems to compensate for a hypoactive CRH-dependent ACTH surge, allowing for a direct action of IL-6 on subhypothalamic levels of the HPA axis. This greater than normal induction of IL-6 observed in response to a viral challenge in the absence of CRH drive is consistent with other studies, in which CRH-KO or CRHR1-KO mice treated with various immunological stimuli, such as carrageenan (39), turpentine (40), and LPS (18, 39, 42), are able to produce near-normal corticosterone responses in the presence of dramatically elevated IL-6 levels relative to infected WT mice. However, CRH-KO studies alone provide limited insight into how the neuroendocrine profile, in response to various immune challenges, is affected by acute elimination of CRH. Indeed, studies using immunoneutralization techniques (acute elimination) to examine the CRH dependency of ACTH and corticosterone responses during MCMV infection, avoid compensatory mechanisms that may develop in the CRH-KO mice, hence giving a more accurate portrayal of a physiologically normal mouse. In addition, rather than working with ceiling IL-6 levels in infected CRH-KO mice, the use of a CRH-Ab produces intermediate levels of MCMV-induced IL-6, which then enables the assessment of the effects of differential IL-6 levels on adrenal corticosterone release and determination of correlations between the two factors.

Although the exaggerated IL-6 levels exhibited by CRH-Ab-treated mice infected with the high dose of MCMV were sufficient to stimulate a robust corticosterone response, when IL-6 levels were not sufficiently elevated to compensate for a lack of CRH function (as in the case of CRH-Ab-treated mice infected with the low dose of MCMV) corticosterone responses remained greatly attenuated relative to infected CRH-intact mice (although greater than baseline values). Therefore, even normal levels of IL-6 induction may exert a weak effect directly on the adrenal to increase glucocorticoid release in the absence of CRH. According to such a dose-response relationship, incremental increases in IL-6 would result in progressively greater activity in stimulating glucocorticoid release at the level of the adrenal, in which an exaggerated IL-6 response may allow IL-6 to assume the predominant role for driving glucocorticoid secretion in the absence of CRH/ACTH drive. The importance of IL-6 in glucocorticoid induction is further substantiated by the strong positive correlation between IL-6 and corticosterone in our infected, CRH-Ab-treated mice.

Although the Bethin (18) and Veniha (40) CRH-KO studies have evaluated the contribution of IL-6 in compensating for CRH deficiency by analysis of CRH/IL-6 double KOs, we have chosen to immunoneutralize IL-6 in the CRH-KO mice. Their findings that CRH/IL-6 double KO mice injected with various immune challenges exhibit reduced corticosterone responses relative to immune-stimulated CRH-KO mice are in agreement with our findings showing a significant attenuation of MCMV-induced corticosterone in CRH-KO mice administered an IL-6-Ab in comparison with that of non-Ab-treated CRH-KO mice. However, acutely blocking the MCMV-induced IL-6 response with an anti-IL-6-Ab, rather than chronically via genetic manipulation, allows a more natural progression of the initial immune response to the virus, and thus avoids the development of additional compensatory mechanisms that may ensue due to the chronic elimination of IL-6.

In accordance with our data suggesting that direct adrenal effects of IL-6 may be important for steroidogenesis in the relative absence of the upstream neuroendocrine factors, there are observations from clinical and animal studies in which glucocorticoid levels can remain elevated during immune activation, despite declining CRH and/or ACTH concentrations (77). Indeed, elevated levels of corticosterone and ACTH (or proopiomelanocortin mRNA) in the face of reduced hypothalamic CRH mRNA have been observed in animal models of inflammatory disorders, such as arthritis (78), multiple sclerosis (79), and lupus (80). Clinical conditions also have exhibited a dissociation between ACTH and cortisol levels, including sepsis (81–83), major surgery (84, 85), and burn trauma (86). In these latter situations, it is possible that once ACTH levels have normalized, a sustained cortisol response may be driven by factors other than pituitary-derived ACTH, such as cytokines. In support of this notion, IL-6 production is increased in many clinical situations characterized by tissue injury and chronic inflammation (87), and long-term IL-6 administration in humans stimulates elevated levels of cortisol in the presence of low levels of ACTH (76, 88, 89). In addition, IL-6 has been shown to play an important role in the maintenance phase of the corticosterone response to turpentine-induced inflammation in rats (90) and in a murine model of colitis (91). These observations lend support to the idea that IL-6 may contribute to a sustained glucocorticoid response by directly stimulating its release from the adrenal gland once CRH/ACTH drive has retreated.

In conclusion, the present study demonstrates the existence of alternative, CRH-independent pathways of glucocorticoid induction during viral infection. Such alternative pathways include a direct IL-6 action at the level of the adrenal gland, requiring the presence of ACTH. Together with our data and others, we hypothesize that in an intact animal, CRH is necessary for the initiation of HPA axis activation (to drive an ACTH surge) to immune stimuli. However, when a sustained glucocorticoid response is needed to keep inflammation in check, proinflammatory cytokines, such as IL-6, assume the role of prime mediators in glucocorticoid release. This condition is reflected by a dissociation between ACTH and glucocorticoid levels and may occur in situations of chronic inflammation or sepsis. With at least low levels of ACTH (and CRH) present during the transition from neuroendocrine to immune drive of glucocorticoid release, the adrenal (and pituitary) gland may be primed (possibly via increased cytokine receptor expression) to receive direct cytokine signals. Therefore, ACTH may be important in this process, by both directly stimulating adrenal glucocorticoid release and facilitating direct IL-6 effects.
on the adrenal gland. However, in the absence of either CRH (acutely) or ACTH, which would leave the pituitary and adrenal glands (respectively) in a state insensitive to cytokine signals, a longer exposure period to circulating cytokines or exaggerated cytokine responses (as we observed) may be necessary to reveal their direct subhypothalamic effects. This hypothesis also may explain why the majority of in vitro studies examining the direct effects of cytokines on adrenal glucocorticoid release in the absence of ACTH, require prolonged incubation times (>12 h) to observe responses. In vivo experiments, as performed here, are important because systemic cytokine actions at the adrenal gland and their effect on glucocorticoid synthesis/release may be dependent upon intricate intraadrenal interactions between cortical and medullary cells, the vascular supply, neural input, and locally synthesized cytokines and neuropeptides (e.g. vasoactive intestinal peptide, an intraadrenal CRH-ACTH system) (77, 92–95). However, the mechanisms by which cytokines interact with these other factors to influence steroidogenesis remain to be clarified. Given the importance of glucocorticoids in maintaining a balance between the beneficial, anti-viral and the detrimental, toxic effects of proinflammatory cytokines, redundant and complementary pathways of glucocorticoid induction appear to exist to prevent the onset of septic shock-induced lethality and thereby ensure the survival of the host during viral infection.

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