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Corticotropin-Releasing Hormone Contributes to the Peripheral Inflammatory Response in Experimental Autoimmune Encephalomyelitis

Christina Benou, Yue Wang, Jaime Imitola, Lilian VanVlerken, Christina Chandras, Katia P. Karalis, and Samia J. Khoury

Peripheral corticotropin-releasing hormone (CRH) is thought to have proinflammatory effects. We used the model of experimental autoimmune encephalomyelitis (EAE) to study the role of CRH in an immune-mediated disease. We showed that CRH-deficient mice are resistant to EAE, with a decrease in clinical score as well as decreased cellular infiltration in the CNS. Furthermore, Ag-specific responses of primed T cells as well as anti-CD3/anti-CD28 TCR costimulation were decreased in crh<sup>−/−</sup> mice with decreased production of Th1 cytokines and increased production of Th2 cytokines. Wild-type mice treated in vivo with a CRH antagonist showed a decrease in IFN-γ production by primed T cells in vitro. This effect of CRH is independent of its ability to increase corticosterone production, because adrenalectomized wild-type mice had similar disease course and severity as control mice. We found that 1α,25D<sub>3</sub> phosphorylation induced by TCR cross-linking was decreased in crh<sup>−/−</sup> T cells. We conclude that peripheral CRH exerts a proinflammatory effect in EAE with a selective increase in Th1-type responses. These findings have implications for the treatment of Th1-mediated diseases such as multiple sclerosis. The Journal of Immunology, 2005, 174: S407–S413.

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4 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; ACTH, adrenocorticotropic; CRH, corticotropin-releasing hormone; HPA, hypothalamic-pituitary-adrenal; MOG, myelin oligodendrocyte glycoprotein.
(29, 30), CRH-deficient mice were shown to have reduced NF-κB activity in thymus and spleen during inflammatory challenge by LPS, which was mediated by protein kinases A and C.

There are few data on the peripheral effects of CRH in EAE. In one study, Lewis rats that received synthetic human CRH systematically had increased glucocorticoid levels and significantly milder EAE compared with untreated controls. Adrenalectomized animals receiving dexamethasone supplementation also had milder disease when treated with CRH (31). The data obtained by this group are difficult to interpret due to the intrinsic defect in central CRH-producing neurons in Lewis rats, that is the basis for their lower glucocorticoid levels. In addition, it has been shown that immune activation of this strain causes overexpression of peripheral CRH, which acts as a proinflammatory factor (32).

We investigated the role of peripheral CRH in EAE using crh−/− mice. We found that these mice have a milder form of EAE and decreased IFN-γ production. We also present evidence that CRH promotes Th1 cell priming independently of its glucocorticoid-inducing effects. The role of CRH in enhancing Th1 differentiation appears to be correlated to NF-κB activity.

Materials and Methods

Animals and reagents

C57BL/6 mice were obtained from The Jackson Laboratory. The crh−/− mice were on a C57BL/6 × 129 genetic background and were generated in our laboratory (33) (backcrossed for 10 generations). Wild-type littermates of these mice were used as controls. All mice were housed according to National Institutes of Health guidelines, and the animal care committee of Harvard University approved all experiments. Mice were used at 6–8 wk of age. Astressin was provided by D. Grigoriadis (Neurorine, San Diego, CA). Astressin was administered i.p. at a dose of 50 ng twice a day on days 11–16 after immunization with myelin oligodendrocyte glycoprotein (MOG).

EAE induction with myelin oligodendrocyte protein

MOG peptide 35–55 (MOG35–55; M-E-V-G-W-Y-R-S-F-R-O-V-H-L-Y-R-N-G-K), corresponding to mouse sequence, was synthesized by QCB (a division of BioSource International) and purified by HPLC. Pletic purity was >99% after HPLC. Mice were immunized s.c. in the flanks with an emulsion of 150 μg of MOG peptide in 100 μl of PBS and 100 μl of CFA containing 400 μg of Mycobacterium tuberculosis (H37Ra; Difco) and were i.p. injected with 200 ng of pertussis toxin (List Biological Laboratories) on the day of immunization and 48 h later. EAE was scored as previously described (34, 35): 0, no disease; 1, limp tail or isolated weakness of gait without limp tail; 2, partial hind limb paralysis; 3, total hind limb paralysis; 4, quadreparesis; and 5, moribund or dead animal.

Generation of primed T cells and cell culture

Mice were immunized in one hind footpad and in the flank with an emulsion of 100 μl of PBS and 100 μl of CFA containing 200 μg of MOG35–55 and i.p. injected with 200 ng of pertussis toxin on the day of immunization and 48 h later. Mice were killed 10–12 days later, and a single-cell suspension was prepared from the spleens and the popliteal lymph nodes. CD4+ T lymphocytes were purified by MACS (Miltenyi Biotec) to 95% purity. To expand the MOG-specific CD4+ T cells in vitro, the purified cells were cultured with gamma-irradiated MOG-loaded APCs supplemented with 5 ng/ml IL-2 and IL-7 (BD Pharmingen) for 3 days, followed by rest 7 days with 5 ng/ml IL-2 and IL-7. The APCs were purified from naive B6 splenocytes, which were incubated with MOG35–55 (100 μg/ml) for 4 h, and irradiated with a gamma irradiator at 3200 rad. The medium used for in vitro cell expansion was DMEM (BioWhittaker) with 10% heat-inactivated FBS, 75 mM/M glutamine, 100 U/ml penicillin and streptomycin, 1% 100× concentrated nonessential amino acid solution, 0.1 mM/ml HEPES, 1 mM/ml sodium pyruvate (all from BioWhittaker), and 0.05 mM/ml 2-ME (Sigma-Aldrich). Cells were incubated at 37°C in humidified air containing 5% CO2.

Proliferation assay

For MOG-specific proliferation assay, CD4+ T cells were cultured in 96-well plates (Corning-Costar) at 2 × 105 cells/ml in 200 μl serum-free medium with irradiated Ag-loaded APCs at different stimulator to responder ratios (1:1, 5:1, or 10:1). For anti-CD3/anti-CD28 costimulation, 96-well plates were precoated with anti-CD3 and anti-CD28 Ab (BD Pharmingen) at various concentrations. CD4+ T cells were cultured at 2 × 106 cells/ml in 200 μl of serum-free medium. After 48 h of culture, 1 μCi of [3H]thymidine (NEN Life Science Products) was added in 20 μl of medium to each well for another 16 h. Cells were harvested and dried on filter mats, and the radioactivity incorporated was measured by a Microbeta scintillation counter (PerkinElmer).

Th1 differentiation experiment

CD3+ splenocytes (2 × 106/ml) were obtained from the spleens of naive crh−/− and crh+/− mice as described previously and were cultured for 5 days in 96-well plates in medium with plate-bound anti-CD3 and anti-CD28 Abs (5 μg/ml) (BD Pharmingen). On day 5, cells were washed, and new medium was added containing IL-2 (10 ng/ml; R&D Systems) as well as IL-12 (10 μg/ml; R&D Systems) or IL-4 (4 ng/ml; BD Pharmingen). Supernatants were collected on days 5–8. IFN-γ and IL-5 levels were measured in the supernatants by ELISA.

ELISAs

Supernatants from the proliferation assay were collected after 48 h of culture. Quantitative ELISAs for IFN-γ and IL-5, and IFN-γ were performed using paired Abs and recombinant cytokines from BD Pharmingen according to the manufacturer’s recommendations.

Pathology

Mice were killed on days 15 and 24 after immunization and were perfused with PBS containing heparin. Two or three animals were used for each experimental group. Spinal cords, brains, and lymph nodes were embedded in OCT (Sakura Finetek USA), flash-frozen in liquid nitrogen, and kept at −80°C until use. Cryostat sections of 10 μm were obtained and fixed with acetone. For immunohistochemical staining, the sections were processed using the avidin-biotin technique (VectorStain Elite kit; Vector Laboratories), visualized with diaminobenzidine (Vector Laboratories), and counterstained with hematoxylin. Omission of the primary Ab served as a negative control. Each specimen was evaluated at three different levels of sectioning.

Adrenalectomy

Adrenalectomy and sham adrenalectomy were performed via the retroperitoneal route under Avertin (tribromoethanol; Sigma-Aldrich) anesthesia. Corticosterone/cholesterol pellets (10/90 g) weighing 200 mg, prepared in our laboratory, were implanted s.c. in the adrenalectomized mice at the time of adrenalectomy to provide a constant amount of circulating corticosterone equivalent to approximately twice the low (morning) basal normal levels (3). Adrenalectomized animals were provided with 0.9% normal saline as drinking water and were left for 6 days to recover before the induction of EAE.

Corticosterone measurements

Mice were housed individually for 16 h preceding blood drawings. Blood samples were obtained at the same time each day. Plasma was extracted by centrifugation of the samples for 8 min at 3000 × g at 4°C. Samples were stored at −20°C until used to measure corticosterone levels by a commercially available IRA kit (ICN Biomedicals) (3).

Fluorescence-mediated cell sorting

A single cell suspension in 1% BSA/PBS was obtained from primed splenocytes harvested as described previously. Cells were stained with Abs to CD4, CD8, CD19, CD80, CD86, CD25, CD28, CD69, IAa, and CTLA-4 (all from BD Pharmingen) after washing twice with 1% BSA/PBS. Appropriate isotype controls were used. Cells were incubated on ice for 20 min, then washed twice with 1% BSA in PBS. Cells were resuspended at a concentration of 107/ml in PBS and analyzed on a FACScan (BD Biosciences).

Protein extraction and Western blot analysis

Splenocytes from naive crh−/− and age-matched wild-type control mice were stimulated with 5 μg/ml plate-bound anti-CD3 and anti-CD28 (BD Pharmingen) in DMEM-based serum-free medium. Cells were harvested at different time points and washed with ice-cold PBS. Whole cell lysates were prepared by extraction in lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 3 mM sodium pyrophosphate, 10% glycerol, 2 mM sodium orthovanadate, and 20 μM Protease Arrest (Genotech). Protein concentration was determined by the Bradford protein.
assay (Bio-Rad). Forty micrograms of protein was analyzed by SDS-PAGE using 10% Tris-glycine gels (Invitrogen Life Technologies), followed by transfer to Hybond-P membranes (Amersham Biosciences). Membranes were blocked in 5% BLOT-QuickBlocker (Genotech) in TBS containing 0.05% Tween 20 for 1 h and incubated with primary Abs at 4°C overnight. The detection step was performed with HRP-coupled anti-rabbit IgG Abs (Genotech). Primary Abs were rabbit anti-phospho-IκBα (1/100; Santa Cruz Biotechnology) and rabbit anti-IκBα (1/100; Upstate Biotechnology). Blots were developed with the ECL Plus chemiluminescence detection system (Amersham Biosciences).

Results

crh−/− mice develop a mild form of EAE

We tested the susceptibility of crh−/− mice to EAE. As shown in Fig. 1A, crh−/− animals had significantly milder disease, with a mean maximal score of 1.71 ± 0.8 compared with 0.81 ± 0.9 (p < 0.05, by Mann-Whitney U test). Day of onset was similar in the two groups (day of onset, 12.66 ± 1.9 for crh−/− vs 12.4 ± 1.9 for controls), whereas incidence was dramatically decreased in the crh−/− animals (6/13 vs 14/14; p < 0.05, by Fisher’s exact test). Immunohistochemical staining showed reduced inflammatory infiltration of CD4 T cells and macrophages in the CNS of crh−/− mice compared with controls (Fig. 1B).

Specific blockade of CRH in wild-type mice decreases disease severity

Astrin, a specific CRH antagonist administered to C57BL/6 mice in vivo during the peak of the disease, significantly suppressed clinical disease compared with saline-treated controls (mean maximal grade, 2.4 ± 0.42 vs 3.83 ± 1.26; p < 0.05, by Mann-Whitney U test). Day of onset was similar in the two groups (Fig. 2A). Furthermore, splenocytes harvested from Astrin-treated crh+/+ mice secreted significantly less IFN-γ compared with control saline-treated mice (p < 0.001; Fig. 2B).

CRH effect on EAE is independent of its glucocorticoid-inducing effect

One of the main functions of hypothalamic CRH is to induce glucocorticoid release by the adrenal glands. Because glucocorticoids have known anti-inflammatory effects, it was important to distinguish whether the proinflammatory effects of CRH are independent of its effects on the HPA axis. We measured corticosterone levels in the serum of crh−/− and crh+/+ animals at various time points during EAE. A correlation between disease severity and stress-induced corticosterone production was apparent in the crh+/+ mice. Serum corticosterone levels increased from 5.55 ± 0.7 ng/ml before the onset of EAE (day 5 after immunization) to 46 ± 5.6 ng/ml at the early onset (day 14; mean score, 1.18 ± 1.53; Fig. 3). Crh−/− animals (not shown) had had minimal or no clinical disease and no change in corticosterone levels compared with control mice.

We also examined the contribution of glucocorticoid production by the adrenal glands by performing experiments in adrenalectomized, steroid-supplemented mice. Thus, we adrenalectomized wild-type animals and induced EAE 1 wk later. Some adrenalectomized animals were supplemented with corticosterone pellets at the time of surgery. Adrenalectomized nonsupplemented mice died soon after immunization because of adrenal insufficiency. The disease course was similar among the adrenalectomized, corticosterone-supplemented animals and the sham-adrenalectomized control group (not shown).

No major differences were detected in the distribution of cell subpopulations in crh−/− immune organs

We compared the absolute number of splenocytes between crh−/− and crh+/+ mice and found significantly increased cellularity in naive crh−/− mice (p < 0.05) compared with naive crh+/+ animals. In immunized mice, there was no significant difference in splenocyte numbers. Splenocytes from both groups were analyzed for CD4, CD8, CD19, IAb, CD80, CD86, CD25, CD28, and F4/80.
Cdh−/− T cells have no intrinsic defect in their ability to differentiate into Th1 effector cells in vitro

T cells can produce CRH in response of inflammation stimuli (13) and express CRH receptors (11). We examined whether there was an intrinsic defect in the differentiation of cdh−/− T cells to Th1 effector cells using standard protocols of nonspecific stimulation of T cells in the presence of Th1-promoting cytokines. When cultured with anti-CD3/anti-CD28 for 5 days, followed by treatment with additional IL-2 and IL-12 or anti-IL-4 for 3 days, both cdh+/+ and cdh−/− cells effectively differentiated toward Th1 cells, as illustrated by the large production of IFN-γ detected by ELISA. Thus, there was no intrinsic defect in Th1 differentiation of cdh−/− T cells (Fig. 4).

Impaired responses to TCR triggering by cdh−/− T cells

Purified CD4+ T cells from naive cdh+/+ and cdh−/− mice were stimulated with various doses of plate-bound anti-CD3 or anti-CD3/anti-CD28 for 48 h. We observed a dose-dependent response of both cdh+/+ and cdh−/− CD4+ T cells to TCR stimuli, but cdh−/− T cell proliferation was significantly decreased compared with that in wild-type controls (Fig. 5, A and B). IL-2 production in the 48-h culture supernatant was consistent with the proliferation result, with a significantly decreased level of IL-2 in cdh−/− CD4+ T cells (p < 0.0001, by two-way ANOVA; Fig. 5C).

We assessed proliferation and cytokine production of MOG-specific CD4+ T lymphocytes isolated from immunized cdh+/+ and cdh−/− mice. As shown in Fig. 6A, both primed cdh+/+ and cdh−/− CD4+ T cells responded to MOG-loaded APC, but not to the irrelevant Ag OVA. Furthermore, both cdh+/+ and cdh−/− CD4+ T showed a dose response to increase the APC to responder T cell ratio (Fig. 6B), but the cdh−/− T cells had significantly less proliferation than cdh+/+ T cells (Fig. 6, A and B). There was also a significant reduction in Th1 cytokines IL-2 (Fig. 6C; p < 0.0001) and IFN-γ (Fig. 6D; p = 0.0001, by two-way ANOVA test) in the supernatants. These results suggest that the CRH-deficient T cells have a defect in TCR-stimulated activation, but this defect is overcome in the presence of Th1-promoting cytokines in vitro.

TCR stimulation leads to Th2 cytokine shift of cdh−/− T cells in vitro

The imbalance of the Th1/Th2 cytokine paradigm is thought to contribute to the pathology of immunological diseases. To address the question of whether Th2 cytokine production is altered in cdh−/− T cells, we next examined IL-5 production of the cdh−/− CD4+ T cells with both Ag-nonspecific and Ag-specific stimulations. We found that cdh−/− T cells produce significantly higher amounts of IL-5 than cdh+/+ T cells with either a strong TCR stimulus of anti-CD3/anti-CD28 (Fig. 7A) or a weak Ag (MOG peptide; Fig. 7B). Interestingly, increased IL-5 production by cdh−/− T cells lost significance with increased stimulation strength as the dose of anti-CD28 Ab increased. In contrast, when stimulated with weak Ag MOG, IL-5 production of cdh−/− T cells was higher than that of cdh+/+ T cells at all doses. These data also suggest that CRH deficiency is more important in T cell responses to weak Ag stimulation.

Cdh−/− APCs have impaired Ag presenting function to MOG-specific T cells

CRH binding sites are present on a variety of immune cells, including monocyte-macrophages (11) and microglia (36). Veniahki
et al. (30) have shown that CRH-deficient mice have impaired splenocyte response to LPS. We compared the Ag-presenting function of APCs from crh−/− and crh+/+ of CD4+ T cell-depleted splenocytes. The APCs were incubated in vitro with MOG<sub>35-55</sub> peptide and added to crh+/+ MOG-specific CD4+ T cells after irradiation. As shown in Fig. 8, T cell proliferation to Ag-loaded crh−/− APCs was significantly lower than that to crh+/+ APCs (p < 0.0001). These data suggest that CRH-deficient APCs are poor Ag presenters.

Delayed and compromised anti-CD3- and anti-CD28-induced IkBα phosphorylation in crh−/− T cells

The NF-κB family of molecules plays a major role in the activation of several genes encoding costimulatory molecules, cytokines, chemokines, adhesion molecules, and inflammatory enzymes in EAE. NF-κB1- and c-Rel-deficient mice are partially resistant to EAE and fail to generate Th1 responses, which is manifested by their decreased ability to secrete IFN-γ and IL-2 (37, 38). Anti-CD3/anti-CD28 costimulation induces IkB degradation and NF-κB nuclear translocation (39). Karalis et al. (29, 30) have shown that CRH is also important for NF-κB activation, and that crh−/− splenocytes and thymocytes have impaired NF-κB binding capacity in response to LPS.

We assessed IkBα phosphorylation on crh−/− splenocytes with plate-bound anti-CD3/anti-CD28 stimulation. Wild-type splenocytes show IkBα phosphorylation within 20 min of anti-CD3/anti-CD28 stimulation. In contrast, the IkBα phosphorylation of...
**Discussion**

As the relationship between the neuroendocrine and immune systems unfolds, we are trying to understand the regulation of the HPA axis to immune activation and, alternatively, HPA axis-mediated regulation of immune function. Immune activation is one of the strongest stressors for the HPA axis. We and others (33, 40) have shown that acute inflammation is the only stressor able to induce some pituitary-adrenal activation in the crh−/− mice. During EAE, the animals are subject to a strong stress response manifested by significant weight loss and elevation of corticosterone levels.

Despite the increasing evidence for a proinflammatory role of peripheral CRH in acute or subacute inflammation, its contribution in chronic inflammatory conditions has not yet been elucidated. In one study of mycobacterium-induced arthritis in rats (41), it was shown that CRH was increased in the early phase of the disease. We show that crh−/− mice are resistant to EAE, with <50% of the mice having mild clinical symptoms and no significant inflammatory infiltrates in the CNS. Crh−/− mice do not have an obvious phenotype in postnatal life (33), but have low serum corticosterone levels. However, disease resistance in crh−/− mice appears to be independent of serum glucocorticoid production, as evidenced by the inhibition of EAE by astressin, a CRH antagonist that has independent of serum glucocorticoid production, as evidenced by densitometric analysis.

Rather, the inhibition of disease appears to be related to impaired priming and differentiation of MOG-specific cells in the crh−/− animals. Our data do not support a generalized immunosuppressed phenotype, because the crh−/− mice have similar allocation of immune cell subpopulations in the spleen as the wild-type mice. Furthermore, crh−/− T cells are able to fully differentiate into Th1 cells in vitro when we used strong Th1-promoting cytokine stimulation protocol. However, with a lower strength stimulus, the crh−/− T cells have a defective activation response. T cells can produce CRH in response to inflammatory stimuli (13), and CRH binding sites are present on a variety of cells and tissues in the periphery, including monocyte-macrophage lineage cells and CD4+ T cells (11, 36). Therefore, CRH could function as an autocrine or paracrine molecule for optimal T cell activation, especially with weak Ag stimulation. It could be argued that the clonal frequency of MOG-specific T cells is lower in crh−/− compared with crh+/+ in the in vitro experiments, leading to a decrease in proliferation and cytokine production. However, the decreased response of crh−/− cells to anti-CD3 stimulation would argue against that being an important factor. Nonetheless, we are planning to cross crh−/− mice with MOG TCR transgenic mice to obtain equal number of MOG-specific T cells for these experiments.

We also found a functional defect of APCs in crh−/− mice. Interestingly, this defect was not corrected by coculture with crh+/+ T cells that are capable of producing CRH. The CRH receptor expression by crh−/− APCs needs to be further investigated.

The cytokine profile of primed crh−/− T cells shows a Th1 to Th2 bias, which is consistent with protection from EAE. However, it is unclear whether the increase in Th2 cytokines is consequent to the impairment of Th1 cytokine production or whether it is an independent phenomenon.

The NF-κB family of molecules plays a major role in the activation of several genes encoding costimulatory molecules, cytokines, chemokines, adhesion molecules, and inflammatory enzymes in EAE. NF-κB regulates proinflammatory cytokine production, such as IL-2, IFN-γ, TNF-α, IL-2R, IL-12 p40, ICAM-1, etc. (42). NF-κB- and c-Rel-deficient mice are partially resistant to EAE and fail to generate Th1 responses (37, 38). In our study we found delayed and compromised IκB phosphorylation in anti-CD3/anti-C28-stimulated crh−/− T cells, which is consistent with the previous findings that CRH can induce NF-κB activation, and crh−/− thymocytes and splenocytes exhibit a defective LPS-induced response (29). These data also suggest that the impaired Th1 response of crh−/− T cells may, at least in part, be the consequence of insufficient NF-κB activation.

The CRH receptor signals through G protein coupling, leading to stimulation of adenylate cyclase and activation of protein kinase A and other cAMP pathway events in a variety of brain-derived and peripheral cell lines (43). The cross-talk between CRH receptor-initiated signal transduction and activation of NF-κB in T cell is not clear. However, there is evidence to suggest that CRH receptor can regulate phosphorylation of CREB and MAPK (44, 45), which can regulate NF-κB activation. Zhong et al. (46) have shown activation of NF-κB by IκB and protein kinase A catalytic unit-associated regulation, suggesting the possibility of direct regulation of NF-κB activation by CRH receptor-associated signal pathways. The pathways of CRH-induced NF-κB activation need to be further identified.

Our results reveal a novel contribution of the HPA axis to the regulation of Th1-mediated inflammation. CRH appears to participate in inflammation in EAE, and peripheral pharmacologic inhibition of CRH protects from disease. These data have implications for the development of novel treatment strategies for autoimmune diseases.

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**FIGURE 9.** Anti-CD3/anti-CD28 costimulation induces IκBα phosphorylation in crh+/+ and crh−/− T cells. Splenocytes isolated from naïve crh+/+ and crh−/− mice were stimulated with plate-bound anti-CD3/anti-CD28 (5 μg/ml) for the indicated time periods. A, A total of 40 μg of whole cell lysate was examined by Western blot analysis. Phosphorylated and nonphosphorylated IκBα were detected by specific Abs. B, The ratio of phosphorylated to nonphosphorylated IκBα was determined by densitometric analysis.
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Disclosures
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