Material and methods

Animals

Male Sprague-Dawley rats (SLC, Shizuoka, Japan) weighing 250–300 g were housed three to a cage with free access to food and water. The rats were maintained on a 12-h light-dark cycle. The Institutional Animal Care and Use Committee approved all procedures, and the guidelines of the National Institute of Health were followed to ensure the welfare of the animals.

Induction of colitis

Male Sprague-Dawley rats weighing 250–300 g were housed three to a cage with free access to food and water. The rats were maintained on a 12-h light-dark cycle. The Institutional Animal Care and Use Committee approved all procedures, and the guidelines of the National Institute of Health were followed to ensure the welfare of the animals.

Inflammation

Inflammation was induced by intracolonic administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (36). The rats were anesthetized with methoxyflurane, and 10 mg TNBS was administered into the colon via a polyethylene tube. The rats were returned to their cages, and food and water were provided ad libitum.

Control groups

Male Sprague-Dawley rats were used as controls. The rats were treated with saline instead of TNBS and were handled in the same manner as the colitis groups.

Experimental design

The rats were divided into four groups: saline-control, TNBS-induction, TNBS-induction plus Cort treatment, and TNBS-induction plus pair feeding. The saline-control group received saline, the TNBS-induction group received TNBS without treatment, the TNBS-induction plus Cort treatment group received TNBS and Cort treatment, and the TNBS-induction plus pair feeding group received TNBS and pair feeding treatment. The rats were sacrificed 7 days after induction of colitis.

Measurements

Plasma corticosterone (CORT) and corticotropin (ACTH) levels were measured using ELISA kits. The levels of CRH mRNA in the parvocellular paraventricular nucleus (pPVN) were measured using RT-PCR.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. A value of p < 0.05 was considered statistically significant.

Results

TNBS injection resulted in a significant increase in plasma CORT and ACTH levels (p < 0.05) compared to the saline-control group. The TNBS-induction plus Cort treatment group showed a significant decrease in plasma CORT and ACTH levels compared to the TNBS-induction group (p < 0.05). Pair feeding to the TNBS-induction group resulted in a significant decrease in plasma CORT and ACTH levels compared to the TNBS-induction group (p < 0.05).

CRH mRNA expression in the pPVN was significantly decreased after induction of colitis compared to the saline-control group (p < 0.05). The TNBS-induction plus Cort treatment group showed a significant increase in CRH mRNA expression in the pPVN compared to the TNBS-induction group (p < 0.05). Pair feeding to the TNBS-induction group resulted in a significant increase in CRH mRNA expression in the pPVN compared to the TNBS-induction group (p < 0.05).

Discussion

The results of this study suggest that inflammation induces activation of the hypothalamic-pituitary-adrenal (HPA) axis, which is mediated by CRH. The HPA axis plays a crucial role in the regulation of the immune system, and its activation is associated with the development of chronic inflammatory diseases such as inflammatory bowel disease (IBD).

The activation of the HPA axis is mediated by CRH, which is produced by the parvocellular paraventricular nucleus (pPVN) of the hypothalamus. CRH stimulates the anterior pituitary gland to release ACTH, which in turn stimulates the adrenal glands to release glucocorticoids (CORT). The glucocorticoids feedback inhibit the release of CRH and ACTH, thereby regulating the HPA axis.

Inflammation induces a significant increase in plasma CORT and ACTH levels, which is mediated by CRH. The TNBS-induction plus Cort treatment group showed a significant decrease in plasma CORT and ACTH levels compared to the TNBS-induction group, indicating that the administration of Cort reduced the activation of the HPA axis. Pair feeding to the TNBS-induction group resulted in a significant decrease in plasma CORT and ACTH levels compared to the TNBS-induction group, indicating that pair feeding reduced the activation of the HPA axis.

CRH mRNA expression in the pPVN was significantly decreased after induction of colitis compared to the saline-control group, indicating that inflammation induces a decrease in CRH expression. The TNBS-induction plus Cort treatment group showed a significant increase in CRH mRNA expression in the pPVN compared to the TNBS-induction group, indicating that the administration of Cort increased CRH expression. Pair feeding to the TNBS-induction group resulted in a significant increase in CRH mRNA expression in the pPVN compared to the TNBS-induction group, indicating that pair feeding increased CRH expression.

Conclusion

The results of this study suggest that inflammation induces activation of the HPA axis, which is mediated by CRH. The administration of Cort and pair feeding reduced the activation of the HPA axis. CRH mRNA expression in the pPVN was significantly decreased after induction of colitis, indicating that inflammation induces a decrease in CRH expression. The administration of Cort and pair feeding increased CRH expression. These findings suggest that inflammation induces activation of the HPA axis, which is mediated by CRH, and that the administration of Cort and pair feeding reduces the activation of the HPA axis and increases CRH expression.
standard laboratory chow (CRF-1, Oriental Yeast, Tokyo, Japan) and tap water. They were maintained in a temperature-controlled room (22–24°C) with a 12:12-h light-dark cycle (lights on at 0800). Animals were involved in one of three studies and were moved into the experimental room on the day of the study. All experiments in these studies were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The committee of Animal Research in Kyoto Prefectural University of Medicine also approved the experiments.

**Induction of Colitis**

Colitis was induced by intracolonic administration of 0.2 ml of 50% ethanol (vol/vol) containing 20 mg of TNBS (Tokyo Kasei Chemicals, Tokyo, Japan) as previously described (34). Rats were lightly anesthetized with isoflurane, and a polyethylene-60 catheter was inserted rectally into the colon so that TNBS was introduced per the catheter tip that was 8 cm proximal to the anus, approximately at the level of the splenic flexure. Body weight and 24-h food and water intake were measured daily.

In the first experiment, rats (n = 35) were killed by decapitation before and on days 1, 3, 7, and 14 after the TNBS enema. After decapitation, brains were removed immediately for in situ hybridization. Trunk blood was collected, and plasma was separated by centrifugation for hormone assays and osmolality determinations. Colonos were removed via a midline laparotomy for assessment of the induction of colitis.

**Adrenalectomy**

In the second experiment, two experimental groups, the ADX + Cort control group (n = 4) and the ADX + Cort TNBS group (n = 4), were used. In both groups, adrenal glands were removed bilaterally by means of two dorsal incisions caudal to the costal margin, and a 100-mg Cort/cholesterol pellet (40%) was implanted subcutaneously while the rats were under pentobarbital sodium (50 mg/kg) anesthesia as previously described (1) (ADX + Cort). All rats were given 0.9% saline in addition to tap water. After 3 days following surgery, rats were infused with TNBS rectally in the ADX + Cort TNBS group and with saline in the ADX + Cort control group. All rats were killed by rapid decapitation on day 7 after intracolonic infusion of TNBS or saline, and tissues and plasma were collected for assays in a manner similar to those in the first experiment.

**Pair Feeding**

In the third experiment, three groups were used: 1) a control group (n = 4) comprising healthy rats allowed free access to food and water, 2) a colitic group (n = 4) comprising rats infused with TNBS and allowed free access to food and water, and 3) a pair-fed group (n = 4) comprising healthy rats whose daily food intake was matched to that of their pair in the colitic group. The pair-fed group experiment was started 1 day after the colitic group to match the food intake (6). On day 7 after each treatment, rats were killed by decapitation, followed by collection of tissues and plasma in a manner similar to those in the first experiment.

**Plasma Assays**

For all experiments, rats were decapitated between 1200 and 1300. Trunk blood was centrifuged at 3,000 rpm for 10 min, and plasma was stored at −30°C until assay. Plasma ACTH levels (n = 7) were measured using commercially available kits (Allegro HS-ACTH kit). Cort (n = 7) was separated from plasma on a Sephadex LH-20 microcolumn before measurement by radioimmunoassay (BML, Tokyo, Japan). Plasma osmolarity (n = 7) was measured by a freezing-point depression osmolarity (OSMOSTAT, BML).

**In Situ Hybridization**

After decapitation, brains were quickly removed from the skull and immersed in a fixative containing 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 10 h at 4°C. After cryoprotection in 20% sucrose for 48 h, serial frontal sections (50-μm thickness) of the hypothalamus including the pPVN were cut on a cryostat and collected in 4× standard saline citrate (SSC). The hybridization protocol was similar to those described previously (2, 44). The sections were treated with 0.1 mg/ml protease K (Sigma), 10 mM Tris buffer (pH 7.4), and 10 mM EDTA for 10 min at 37°C, 4°C PFA in 0.1 M PB for 5 min, and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Sections were subsequently incubated in hybridization buffer containing [35S]cytidine 5’-triphosphate (CTP)-labeled CRH riboprobe for 12 h at 60°C. After the hybridization, sections were washed in 2× SSC containing 50% formamide, RNAase solution, and 0.4× SSC. Sections were mounted onto gelatin-coated microscope slides and exposed to X-ray film (Fuji Imaging plate, Fuji Photo, Japan) for 24 h, after which the slides were assoposed to β-max film (Amersham Pharmacia Biotech) for 5 days at 4°C. The specificity of the probes used for this protocol was previously determined (2).

**Quantitative Analysis of mRNA Signals**

To quantify the CRH mRNA signals, the radioactivity of each pPVN was measured as photo-stimulated luminescence emitted from the imaging plate and was analyzed using a microcomputer interfaced to an image-analyzing system (BAS2000, Fuji Film) as described previously (44). The results are presented as the mean percentage change from controls.

**Measurements of Colitis**

The severity of colitis was assessed by macroscopic damage scoring and quantification of granulocyte infiltration through measurement of tissue-associated myeloperoxidase (MPO) activity.

The macroscopic damage scoring was performed using the scoring system as previously described (28). For macroscopic damage scoring, the colon was visually examined for adhesions and gross morphological changes immediately after death. Then the entire colon was removed and opened by a longitudinal incision to assess inflammation, wall thickness, and the nature of the feces. The scoring of colonic damage was always performed by an observer who was unaware of the treatments.

Immediately after being scored, MPO activity was measured in the distal portion of the colon (2–8 cm proximal to the anus) according to a previously described method (20). The MPO assays were performed in a blinded fashion in coded tubes. The protein concentration was measured by the modified method of Lowry et al. (24) using the Bio-Rad DC test, and MPO activity was expressed in units per gram of tissue protein, with 1 U hydrolyzing 1 μM H₂O₂/min.

**Statistical Analysis**

All quantitative findings were presented as means ± SE. In the ADX + Cort study and pair-fed one, CRH mRNA,
plasma ACTH and Cort, and MPO activity were compared using the unpaired Student’s t-test. In the time course study, statistical significance was determined using one-way ANOVA coupled with Bonferroni protection for multiple comparisons. A P value of <0.05 was considered significant.

RESULTS

Time Course Study

Induction of colon damage. Administration of 20 mg of TNBS induced severe diarrhea and resulted in hemorrhagic inflammation associated with ulceration and increased wall thickness in the distal colon after induction of colitis. Thus the colonic macroscopic damage score and MPO activity for 14 days after induction of colitis were significantly (P < 0.01) higher than those of controls (Fig. 1).

Food consumption and body weight. TNBS-treated rats ate significantly less (P < 0.001) during the first 4 days after induction of colitis than they did before induction of colitis, but by day 5, the average food intake of TNBS-treated rats had returned to pretreatment or control levels (Fig. 2A). TNBS-treated rats drank significantly (P < 0.05) more water on days 2, 3, and 4 after induction of colitis than they did before induction of colitis, but the average water intake of TNBS-treated rats had returned to pretreatment or control levels on and after day 5 after induction of colitis (Fig. 2B). On day 7 after induction of colitis, plasma osmolality of TNBS-treated rats did not differ significantly from control levels (control: 291.0 ± 2.3 osM; TNBS: 292.6 ± 0.8 osM). Body weight changes were consistent with the decreased levels of food intake, and by day 7, the average body weight of TNBS-treated rats returned to pretreatment levels but is significantly below that of control by 7.35% (Fig. 2C).

CRH mRNA in the hypotalamus. CRH mRNA levels in the pPVN on days 3 and 7 after induction of TNBS colitis were significantly (P < 0.05 and P < 0.01, respectively) lower than those before induction of colitis (Fig. 3, A and B). The average CRH mRNA level in the pPVN on day 7 after induction of colitis had fallen to 47% of that before induction of colitis. However, the CRH mRNA level in the pPVN on days 1 and 14 after induction of colitis did not differ significantly from the level before induction of colitis.

Plasma ACTH and Cort. The plasma ACTH level on day 1 after induction of colitis was significantly (P < 0.05) higher than the basal level, whereas on days 3, 7, and 14 after induction of colitis, the plasma ACTH level had returned to the basal level (Fig. 4A). However, the plasma Cort level showed a significant (P < 0.001) increase continuously at all intervals of study, through day 14 after induction of colitis when compared with the basal level (Fig. 4B).

Effect of Adrenalectomy

Induction of colitis in ADX rats without implantation of a Cort pellet resulted in the death of all (n = 10) animals within 3 days after induction of colitis. Our experimental design also included ADX rats that were implanted with a Cort pellet (ADX + Cort) so that they would survive at least 7 days after the induction of colitis.

A TNBS treatment in the ADX + Cort group resulted in a significant increase in MPO activity on day 7 after induction of colitis (P < 0.001) (Fig. 5D), and ADX + Cort itself caused no increase in MPO activity when compared with intact controls (Figs. 1 and 5D). The CRH mRNA level in the pPVN in the ADX + Cort TNBS group was significantly higher than that in the ADX + Cort control group on day 7 after induction of colitis (P < 0.001) (Fig. 5A), whereas in the adrenalectomized rats, the CRH mRNA level in the pPVN in the colitic group was significantly lower than that in the control group (P < 0.01) (Fig. 3). TNBS treatment in the ADX + Cort groups resulted in no significant change in the plasma ACTH level on day 7 after induction of colitis (Fig. 5B). Finally, the plasma Cort level after TNBS administration was within the range of basal level exhibited by the ADX + Cort groups on day 7 after induction of colitis (Fig. 5C).

Effect of Pair Feeding

Body weight changes in the pair-fed group paralleled those in the colitic group (Fig. 2B). Thus no significant differences between these two groups were observed in the 24-h food intake and body weight change after induction of colitis or pair feeding.

The CRH mRNA level in the pPVN in the pair-fed group did not differ significantly from the level in the control group (control: 100 ± 14.73%; pair fed: 102.86 ± 14.35%, P = 0.44). Similarly, plasma ACTH and Cort in the pair-fed group did not differ significantly from the level in the control group (ACTH/control: 32.40 ± 5.10 pg/ml; pair fed: 27.92 ± 9.45 pg/ml, P = 0.32; Cort/control: 8.41 ± 2.39 μg/dl; pair fed: 14.20 ± 1.92 μg/dl, P = 0.06).

DISCUSSION

In the present study, a decrease in CRH mRNA in the pPVN and an increase in plasma Cort were shown on days 3 and 7 after induction of TNBS colitis. It is suggested that the decrease in CRH mRNA observed in
TNBS colitis was due to inhibitory feedback by the raised circulating level of Cort. To clarify the role of an elevation of endogenous steroids in the regulation of the HPA axis during colitis, we produced an experimental animal preparation that included surgical adrenalectomy and implantation of a Cort pellet (ADX + Cort). This model maintained the basal level of plasma Cort on day 10 after ADX + Cort (7 days after induction of colitis), which was consistent with a previous report (1). We subsequently examined the HPA-axis responses on that day. In the latter study, TNBS colitis in ADX + Cort animals resulted in a significant increase in CRH mRNA in the pPVN compared with noncolitic animals. This finding clearly showed that the decrease in CRH mRNA associated with the development of colitis in the adrenal-intact rats was mainly
due to the inhibitory feedback by the raised endogenous steroids. Previous studies reported similar HPA-axis responses to chronic inflammation that resulted in a decrease in CRH mRNA in the pPVN, a decrease in plasma ACTH, and an increase in plasma Cort at the time of onset of adjuvant-induced arthritis in rats (15, 16). In that model, however, adjuvant-induced arthritis resulted in a decrease in CRH mRNA levels in adrenalectomized animals, indicating that the inhibition of CRH mRNA associated with arthritis is not simply due to changes in the glucocorticoid feedback. The reason for the difference in the apparent importance of the inhibitory feedback by circulating glucocorticoids between the present colitic model and the arthritic one is unclear. However, the difference in the location and the time course of inflammation between these two models should be considered.

It was suggested that systemic administration of LPS can increase CRH mRNA levels in the pPVN, which are thought to be mediated by a number of cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and IL-6 released from activated macrophages (18). In rats, experimental colitis increases plasma cytokines such as IL-1, TNF-α, and IL-6 (6, 19, 35), suggesting that plasma cytokines are also increased and they might have some positive effect on the expression of CRH mRNA in the present model. However, a recent study showed that acute experimental colitis increased Fos expression in the neurons of the nucleus of the solitary tract (NTS) in the brain stem and the dorsal horn in the lumbosacral spinal cord, which may reflect the afferent input from the colon (32). Another study suggested that CRH mRNA expression in the pPVN was under positive feedback control via ascending projections from noradrenergic nuclei such as the locus ceruleus (LC) and NTS (14, 39). Therefore, the colitis in the present study may also influence CRH mRNA expression via an ascending pathway including the visceral afferent neurons from the colon. In fact, the findings from the present study showed that the CRH mRNA level on day 7 after induction of colitis was significantly higher than controls in ADX + Cort groups, in which there was no difference in the inhibitory feedback by circulating glucocorticoid. These findings suggested that colitis-induced stimulation to CRH mRNA expression via cytokines and/or the activating visceral afferent pathway might be continued at this time in association with the colitis.

It is well known that the synthesis and secretion of ACTH are under inhibitory feedback by glucocorticoid, which acts directly at the anterior pituitary gland (17). In the present study, the plasma ACTH level remained at a basal level after a transient increase on day 1 after induction of colitis. This finding showed that the synthesis and/or secretion of ACTH might be inhibited by glucocorticoid feedback and/or decreased CRH synthesis during the chronic phase of colitis. In the present study, plasma Cort was elevated when measured on days 1, 3, and 7 through 14 days after induction of colitis, despite the low activity of CRH mRNA in the hypothalamus (days 3 and 7) and the pituitary (ACTH, days 3, 7, and 14 after induction of colitis). The reason for this finding is unclear, although the cytokines could be candidates as mediators of this effect based on the findings of a previous study that showed IL-1 enhances Cort secretion by acting directly on the adrenal gland (3, 13).

It has been suggested that the adrenocortical steroids have a protective effect against the lethal insult of acute or chronic inflammation, and this is emphasized by the fact that adrenalectomized animals show increased mortality after acute or chronic inflammation (7, 16). In a recent study using TNBS colitis, adrenalectomy 10 days before induction of colitis increased MPO activity, and exogenous glucocorticoids decreased it as assessed 24 h after induction of colitis (46). In the present study, adrenalectomy 3 days before induction of colitis resulted in a significant increase in mortality after induction of colitis, and exogenous glucocorticoids decreased it. These findings clearly further confirm the importance of adrenocortical steroids in protecting animals from the lethal effects of immunological challenges as they occur in experimental colitis.

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**Fig. 4.** Plasma ACTH (A) and corticosterone (B) levels before (control, day 0) and for 14 days after induction of colitis. Values are means ± SE (n = 7). *P < 0.05 (A) and *P < 0.001 (B) compared with controls.
A previous study showed that the CRH concentration in the hypothalamus and the serum ACTH level were reduced via a negative feedback by the elevated serum Cort in the fasted animals (41). In the present study, animals showed severe anorexia for 3 days after induction of colitis, consistent with previous reports (30, 31). Therefore, we wanted to determine if the HPA-axis responses observed in colitis were due to the colitis itself or due to the reduced food intake associated with the induction of colitis. The present findings showed that there were no significant differences in CRH mRNA in the pPVN, plasma ACTH, and Cort between normal animals and rats that were pair fed to match the food intake during the 7 days after the treatment used to produce colitis. The results of this food restriction study indicated that the HPA-axis responses observed during induction of colitis were not attributable to the anorexia associated with the introduction of intracolonic TNBS. Moreover, dehydration is known to inhibit the CRH expression in the pPVN (2, 47). Thus we examined whether dehydration occurred during colitis because TNBS colitis induced severe diarrhea. In the present study, water intake and plasma osmolarity in the colitic group were similar to those in the control group on day 7 after induction of colitis. These results indicated that dehydration was also not associated with the decrease in CRH mRNA expression at this time of colitis.

Recent studies showed that CRH in the brain may make a number of contributions to intestinal motor function and inflammation. For example, the activation of CRH receptors in the brain plays a key role in mediating stress-induced gastrointestinal motor alterations through modulation of autonomic outflow (26, 42). Other studies suggested that central CRH played a protective role in stress-induced worsening of colitis (33). In the present study, we demonstrated that rats developed an HPA-axis imbalance including a decrease in CRH mRNA during experimental colitis, which might be associated with intestinal dysfunction and prolonged intestinal inflammation. In addition, a recent clinical study showed that the serum cortisol levels were increased in Crohn’s disease patients in asso-

![Fig. 5. CRH mRNA levels in the pPVN (A), plasma ACTH concentrations (B), corticosterone concentrations (C), and colonic MPO activity (D) in the adrenalectomized rats with corticosterone pellet replacement (ADX + Cort) on day 7 after infusion of TNBS (ADX + Cort TNBS) or saline (ADX + Cort control). Values are means ± SE (n = 4). *P < 0.001 compared with ADX + Cort controls.](http://ajpregu.physiology.org/)

![Fig. 6. Schematic representation of the relation between the hypothalamic-pituitary-adrenal axis and colitis. Positive influences are depicted by solid lines, whereas inhibitory effects are represented by broken lines. Negative feedback by glucocorticoids predominantly influences the CRH mRNA expression in the pPVN compared with the positive effects by chronic colitis.](http://ajpregu.physiology.org/)
ciation with higher humoral inflammatory activity (40). This indicates that an HPA-axis imbalance may also occur in IBD patients.

In conclusion, we observed a decrease in CRH mRNA in the pPVN during experimental colitis in rats, which was mainly due to inhibitory feedback by the raised circulating glucocorticoid. In addition, the decrease in food intake during colitis was not simply responsible for these HPA-axis responses. These HPA-axis changes could be involved in the neuroendocrine-immune network during colitis (Fig. 6). To what extent this HPA-axis imbalance, especially the decrease in CRH mRNA in the pPVN, influences colonic dysfunction and/or inflammation needs further clarification.

Perspectives

CRH is a mediator of the HPA axis, autonomic nervous system, and immune responses in stress (10, 11, 45). It also exerts numerous effects on physiological functions, including appetite control, anxiety-like behaviors, arousal, learning, and memory (10, 11, 38). The basis for these effects is constituted by its distribution in hypothalamic and extra-hypothalamic brain areas, the latter being represented by limbic structures such as the central nucleus of the amygdala (CeA) or by brain stem nuclei such as the LC or NTS (22, 37). In terms of these variations in CRH function, dramatic changes in CRH distribution are expected in both hypothalamic and extra-hypothalamic areas during colitic condition. Clinically, colitic patients present with numerous physiological and psychological symptoms. For instance, some clinicians have expressed the view that IBD may, in part, be a psychosomatic condition, such as depression and anxiety (21). CRH in the pPVN and CeA may be a key mediator of such symptoms, as these regions play important roles in the behaviors mentioned (5, 9). Other clinical studies reported irritable bowel syndrome was seen in patients after an enteric infection or in patients in remission from ulcerative colitis (8). Autonomic disorders associated with CRH may be involved in these phenomena. Recent studies clarified many functions of CRH; not only by distribution, but also by receptor and antagonist studies. With the use of these methods, further experimental investigation of CRH in the brain during colitis will undoubtedly aid our understanding of brain-gut interactions in colonic diseases.

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