EXPERIMENTAL / LABORATORY STUDIES

Acute Stress Ameliorates Colitis via Central Corticotropin-Releasing Factor and Serotonin (5-HT)-3 Receptors*

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Abstract: The central nervous system may modulate many aspects of inflammation through alterations of the autonomic nervous system and/or the hypothalamo-pituitary-adrenal (HPA) axis. The aim of present study was to examine the impact of controllable acute stress on the course of experimental colitis and to elucidate the roles of serotonin (5-HT) and corticotropin-releasing factor (CRF) in the central modulation of the stress. In the stress group, acute electric shock (AES; 0.3-0.6 mA) was applied, while in the central agonist group, rats were injected with central 5-HT agonist (400 µg/kg). In the antagonist groups, 10 min before the application of AES or central agonist, rats were injected with astressin (10 µg/kg; icv) or ramosetron (40 µg/kg; icv). Hexamethonium (15 mg/kg; ip) or RU-486 (10 mg/kg; ip) were given before and after stress or central agonist application. The severity of colonic damage was evaluated by the assessment of macroscopic score, histological analysis and tissue myeloperoxidase activity. The data are expressed as means ± SE and analyzed using Student's t-test or the Mann-Whitney U test. Both AES and central 5-HT decreased colonic damage scores seem to be mediated by central CRF and 5-HT3 receptors and by adrenal corticosteroids and sympathetic ganglia. These results show that the anti-inflammatory effect on colitis may be mediated by central mechanisms that involve the interaction of CRF and 5-HT, with the participation of the sympathetic system and HPA axis.

Key Words: Colitis, serotonin, stress, HPA axis, sympathetic nervous system

Introduction

A growing body of evidence indicates that stress plays a prominent role in the pathophysiology and/or clinical presentation of gastrointestinal conditions including inflammatory bowel diseases (1-3). Although the complex brain-gut interactions in stress-related gastrointestinal disturbances are not yet clearly known, it is possible to say that any physical or psychological stressor that threatens the homeostasis of an organism can initiate a set of behavioral and neuro-endocrinological responses that help the organism to adapt to the altered situation. In conducting the neuro-endocrine responses, the hypothalo-pituitary (HPA) axis and sympatheto-adrenal axis (SAA) are 2 major pathways mediating the major components of the stress response (4-6).

The duration of stress and its interaction with several central neurotransmitters including corticotropin-releasing factor (CRF), serotonin (5-HT) and cholecystokinin (CCK) influence the final outcome. It has been reported that acute stress causes (a) hyperactivation in many central CRF-containing neurons, particularly those found in paraventricular nuclei (PVN) of the hypothalamus and amygdala, and (b) stimulation in most of the central 5-HTergic and CCKergic systems (7-12). Regarding the peripheral actions of stress-induced central modulation, the net result is either hypo- or

hyperactivation of the HPA axis and/or autonomic nervous system with an altered release of adrenocorticotropin (ACTH), glucocorticoids and catecholamines (4,13).

Among the many central stress mediators that modulate responses of the HPA axis and sympathetic nervous system (SNS), CRF is regarded as the main neuropeptide involved in both physical and emotional stress. The results of a number of studies have suggested that the central CRF exerts a protective role on experimental colitis, possibly through the activation of the HPA axis (13,14). Recent studies have also shown that the 5-HTergic activity also takes part in the modulation of the HPA axis and SNS. Ascending 5-HTergic fibers increase the activities of the HPA axis and SAA by the release of CRF (15,16). However, the role of central 5-HT in stress-related modulation of colitis has not been studied yet.

The first aim of the study was to examine the impact of acute controllable emotional stress and the possible interactions of central CRF and 5-HT$_3$ receptors on the course and modulation of colitis pathogenesis. The second aim was to evaluate the effects of exogenous 5-HT on the course of colitis and the involvement of central CRF and 5-HT$_3$ receptors in the modulation of colitis severity. The third aim was to examine the participation of the SNS and HPA axis in mediating the inflammatory response of the colon to stress or to central 5-HT administration.

Materials and Methods

Experiments were performed on adult male Sprague-Dawley rats, weighing 200-270 g. The rats were anesthetized with ketamine (100 mg/kg; intraperitoneally, ip) and chlorpromazine (30 mg/kg; ip) and following the atlas of Paxinos and Watson, stainless steel cerebroventricular guide cannulas (22-gauge; Plastic Products, Roanoke, VAUSA) were inserted into the right lateral cerebral ventricles (17). Experiments were performed at least 1 week after cannulation. At the end of each experiment, methylene blue was injected to verify the correct placement of the cannulas, and the animals were then decapitated. All studies including the stress models were approved by the Marmara University, School of Medicine, Animal Care and Use Committee.

Induction and assessment of experimental colitis

Rats were deprived of food, but not water, for 16 h and were lightly anesthetized with ether, and a polyethylene catheter was inserted rectally into the colon so that the tip was 8 cm proximal to the anus. The induction of colitis was performed by intracolonic administration of 0.5 ml of 38% ethanol containing 15 mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma). On the 3rd day of colitis induction, the rats were decapitated and the colonic tissue was assessed by macroscopic damage scoring, microscopic evaluation and the degree of granulocyte infiltration estimated by the measurement of tissue myeloperoxidase (MPO) activity. The macroscopic scoring of the damaged segment was performed using criteria considering the amount and length of ulceration and inflammation with a maximum score of 10 (18). To avoid observer bias, light microscopic assessment of the colonic segment, stained with hematoxylin and eosin, was performed by the two observer who were unaware of the treatments, by using the following criteria: score 0, no damage; score 1, mild; score 2, moderate; and score 3, severe damage in the following parameters: (a) decrease in the size of the epithelial layer, (b) mucosal damage, (c) mucosal hemorrhage, (d) interstitial edema, and (e) inflammatory cell infiltration (Modified from Ref. 14). The maximum score that any colonic segment could achieve was 3. The measurement of MPO activity was assessed by measuring the H$_2$O$_2$-dependent oxidation of o-dianizidine.2HCL and was expressed as units per gram of wet tissue weight. One unit of enzyme activity was defined as the amount of the MPO present that produced a change in absorbance of 1.0 ml/min at 460 nm and 37 °C (19).

Experimental Design

Experiments were performed at least a week after intracerebroventricular (icv) cannulation following a recovery and acclimatization period. This study was designed to consist of 2 main parts: stress groups and central agonist groups. In each subgroup, at least 6 animals (6-15) were used and, to minimize any diurnal variation in their response, all procedures in these groups were performed between 10:00 and 12:00 *a.m* and 2:00 and 4:00 *p.m*. Identical doses of the central agonist or antagonists were also administered intraperitoneally to determine whether the effects were central or peripheral. Colitis was induced by the intracolonic administration of TNBS on the 4th hour after the previous stress exposure

or after the previous injection of the agonist. On the 3rd day of colitis induction, the rats were decapitated, the colonic segments were removed and damage was assessed using tissue MPO activity, and macroscopic and microscopic evaluations.

Stress group: Controllable psychological electric shock (ES) stress models were used. In acute ES, each rat was placed in a Plexiglas chamber for 30 min, where a serioes of 20 random electric foot shocks (within the range of 0.3-0.6 mA for 5 s) were supplied to the grid floor by a pulse-generated scrambler (Northel, İstanbul) (20).

Central agonist group: In the preliminary experiments, different treatment protocols with different doses of 5-HT (20, 200, 400 µg/kg) were performed to find the effective doses. Among these, icv injection of 5-HT at doses of 400 µg/rat, administered at 10:00 *a.m.* and 03:00 *p.m.* on the 1st day and at 10:00 *a.m.* on the 2nd day, significantly changed stress-induced colitis damage score.

Antagonist groups: In order to determine the central and peripheral mechanisms involved in stress- or central 5-HT (400 µg/kg)-induced modulation of experimental colitis, 10 min before an acute ES session or icv 5-HT injection, rats were injected (icv; 5 µl) with CRF receptor antagonist (astressin; 10 µg/kg) or $5-HT_3$ receptor antagonist (ramosetron; 40 µg/kg). In another group of rats, either hexamethonium (15 mg/kg; ip, 30 min before and 24 h after) or RU-486 (10 mg/kg; ip, 12 and 1 hour before and 24 h after) were given before and after stress or central 5-HT application. These dosage regimens were selected based on previous reports (13,21-23).

Administration of drugs: All icv injections were administered using a Hamilton syringe, in a 5-µl volume over a period of 1 min at least a week after the icv cannulation. The solutions of 5-HT (Sigma), ramosetron (Sigma) and astressin (kindly provided by Jean Rivier, The Clayton Foundation Laboratory for Peptide Biology, San Diego, California) were prepared in saline. A 10-min interval was given between the injection of the antagonists or the vehicle (saline) and icv agonist injection or stress induction.

In the last series of experiments, to determine the participation of the SNS and HPA axis in mediating the colonic inflammatory effects of stress and central agonist, some of the animals received an ip injection of 10 mg/kg (in a 1 ml volume) of RU-486 (the glucocorticoid antagonist, Sigma) dissolved in 25% ethanol. The others were treated with the ganglion blocker hexamethonium (15 mg/kg; ip in a volume of 0.3 ml/rat, Sigma) dissolved in saline.

Statistical Analysis: The results are expressed as means ± SE. Both parametric and non-parametric tests were used. For comparison of paired results, Student's t-test or the Mann-Whitney U test were used and for multiple comparisons, one-way ANOVA or the Kruskal-Wallis test were used. Differences were considered statistically significant if $P < 0.05$.

Results

Evaluation of TNBS-induced tissue injury: Intracolonic application of TNBS induced a typical colonic injury characterized by hyperemia, inflammation and ulceration extending up to 2 cm in length. When compared with the macroscopic damage score (2.6 ± 0.49) and tissue MPO activity (102.06 ± 17.24 u/g) of the vehicle (ethanol) group, administration of TNBS significantly increased both parameters (4.75 ± 0.52 and 162.62 ± 10.49 u/g, respectively; $P < 0.05$-0.01) (Figures 1a,b). In contrast to the nearly normal appearance seen in the vehicle group with a minute microscopic damage score (0.15 ± 0.05), histological observation in the colitis group showed severe epithelial and glandular damage accompanied by interstitial edema, severe hemorrhage, and inflammatory cell infiltration extending to the submucosa, and the microscopic damage score was significantly higher (2.9 ± 0.01, $P < 0.01$).

Stress group: When compared with the non-stressed colitis group, acute ES reduced MPO activity and macroscopic scores ($P < 0.05$, Figures 1a,b). Histological examination of colonic tissues taken from the acute ES group revealed reduced hemorrhage and damage in epithelial and glandular structures with a microscopic score of 2.31 ± 0.53 ($P < 0.05$). In the acute ES group, the reductions in each of the above-mentioned parameters were eliminated by the treatments with ramosetron or astressin ($P < 0.05$-0.001, Figures 1a,b). The degrees of epithelial and glandular damage, hemorrhage, edema and leukocyte infiltration in all of the antagonist-treated groups were similar to those seen in the non-stressed colitis group (extensive epithelial degeneration and submucosal leukocyte infiltration; 2.96
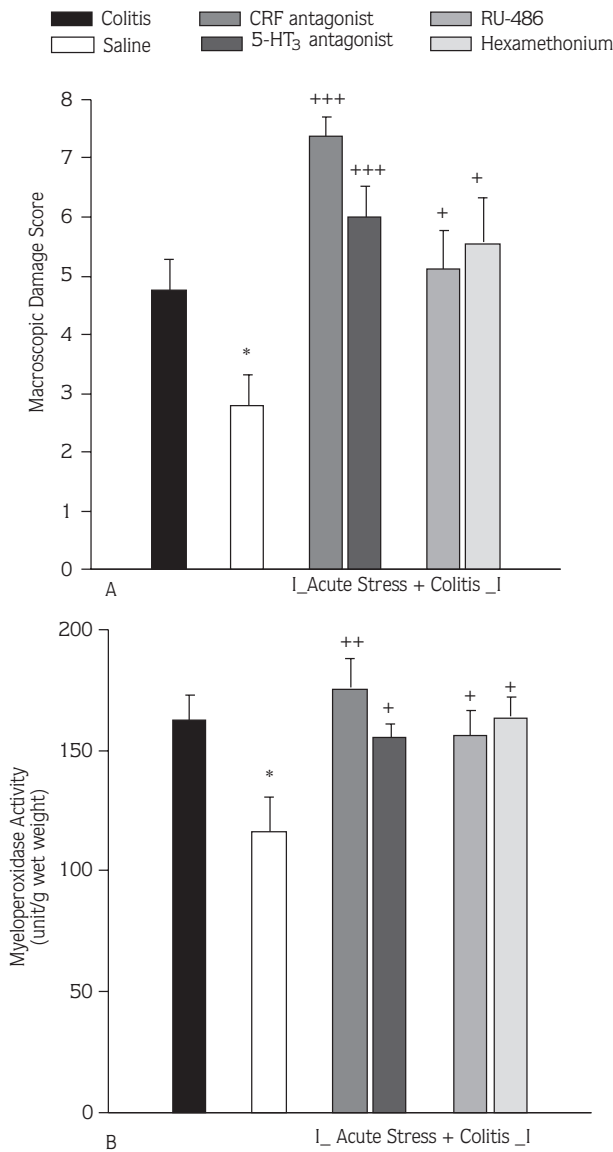
Figure 1. The effects of serotonin (5-HT$_3$; 40 µg/kg, icv), corticotropin (CRF; 10 µg/kg, icv) and glucocorticoid (RU-486; 10 mg/kg, ip) receptor antagonists and ganglion blocker hexamethonium (15 mg/kg, ip) on acute electrical shock-induced improvement of experimental colitis, as assessed by macroscopic damage score (A) and tissue myeloperoxidase activity (B).

*$P < 0.05$, compared with the non-stressed colitis group.

+$P < 0.05$, ++$P < 0.01$ and +++$P < 0.001$, compared to acute stress group.

± 0.04, $P < 0.001$). However, ip injection of 5-HT$_3$ receptor antagonist at the same doses did not significantly change acute ES-induced increases in macroscopic scores (ramosetron, $4.14 ± 0.94$) or MPO activity (ramosetron; $133.45 ± 6.12$ u/g).

Central agonist group: When compared with the non-stressed colitis group, in rats injected icv with 5-HT (400 µg/kg), both macroscopic score and tissue MPO activity decreased ($P < 0.05$, Figures 2a,b), whereas peripheral (ip) administration of 5-HT at the same doses had no significant effect on macroscopic score ($4.10 ± 0.60$) or MPO activity ($151.37 ± 12.86$ u/g). After icv 5-HT injection, microscopic damage score ($2.73 ± 0.13$) was not diminished. Treatment with ramosetron (40 µg/kg; icv) or astressin (10 µg/kg; icv) eliminated the reduction in colitis damage scores and tissue MPO activities of 5-HT-injected rats ($P < 0.05-0.01$, Figures 2a,b).

The effect of glucocorticoid receptor and sympathetic ganglion blockade: Considering the macroscopic damage score and MPO activity, blockade of the sympathetic ganglia by the ip injection of hexamethonium or RU-486 reversed the anti-inflammatory effects provided by acute ES or central 5-HT injection ($P < 0.05-0.01$, Figures 1,2). While ip injection of hexamethonium alone (without stress induction or icv agonist injection) had no effect on TNBS-induced colitis, ip administration of RU-486 alone reduced both macroscopic score ($2.21 ± 0.48$, $P < 0.01$) and tissue MPO activity ($120.82 ± 14.48$ u/g, $P < 0.05$).

Discussion

Our findings showed that controllable acute ES reduced the severity of TNBS-induced colitis, as evidenced by a decreased macroscopic score, an improvement in histological appearance, and decreased granulocyte recruitment. These data also provide evidence that, in addition to the known effects of CRF receptors, 5-HT$_3$ receptors may also participate in the stress-induced modulation of experimental colitis.

Previous studies regarding the relationship between different stress models and experimental colitis provide evidence to suggest the participation of CRF and the HPA axis in the pathophysiology of colitis. (13,14,20,24,25). Corticotropin, which is widely distributed in many brain regions, including the PVN, brain stem, limbic system and cortex, is involved in many pathophysiological responses to stress, including activation of the HPA and the sympathetic nervous system (4,6,26). A number of reports provide evidence that CRF exerts beneficial effects on inflammatory events. In 2 of those studies,
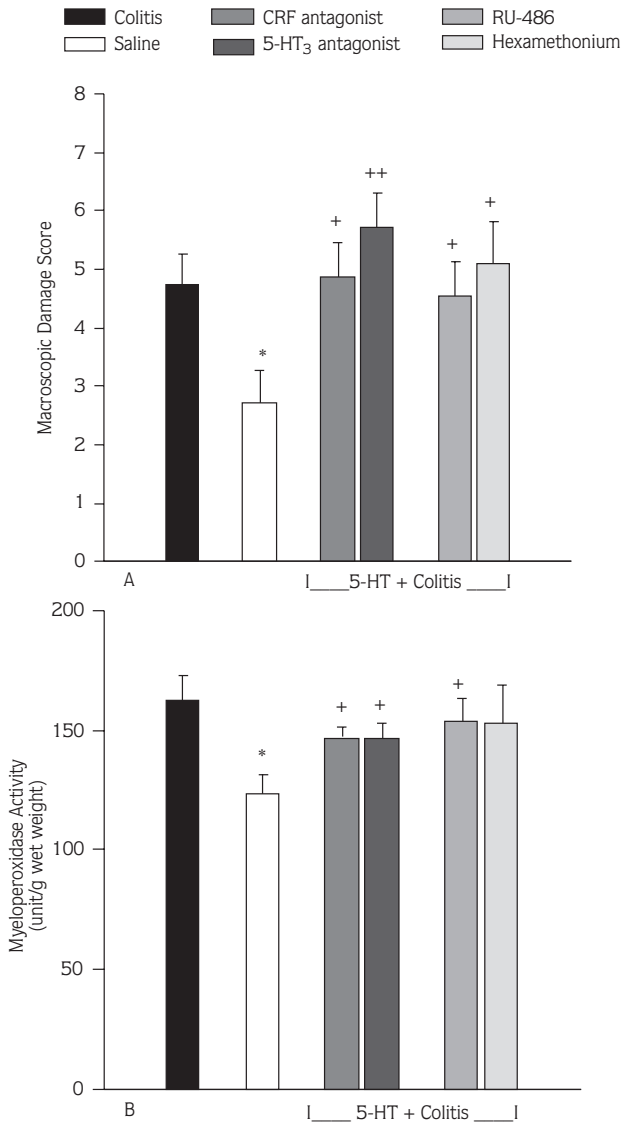
Figure 2. The effects of serotonin (5-HT$_3$; 40 µg/kg, icv), corticotropin (CRF; 10 µg/kg, icv) and glucocorticoid (RU-486; 10 mg/kg, ip) receptor antagonists and ganglion blocker hexamethonium (15 mg/kg, ip) on exogenous 5-HT-induced (400 µg/kg; icv) improvement of experimental colitis, as assessed by macroscopic damage score (A) and tissue myeloperoxidase activity (B).

*P < 0.05, compared with the colitis group.

$^+$P < 0.05 and $^{++}$P < 0.01, compared with the 5-HT group.

central CRF exerted a protective role against TNBS-colitis and experimental gastric injury induced by acute stress (13,27,28). In another study, central injection of CRF abrogated lipolysaccharide-induced expression of ICAM-1 expression and increased leukocyte recruitment measured by leukocyte rolling, adhesion and emigration, which were reversed by the blockade of endogenous glucocorticoids (22). In the present study, acute controllable ES caused anti-inflammatory effects on TNBS-induced colitis as demonstrated by reduced MPO activity, and decreased macroscopic and microscopic scores, which were reversed by the blockade of central CRF receptors and by peripheral glucocorticoid receptors.

Although the CRF and HPA axis dependent mechanism offers some possible explanation, our previous study showed that other central neurotransmitters/ neuropeptides such as CCK and SNS-dependent mechanisms may participate in the central modulation of colitis (20). Moreover, the results of this study demonstrate that, in addition to central CRF and CCK$_B$ receptors, the central 5-HT$_3$ receptor may also participate in stress-induced central regulation of experimental colitis. Central administration of 5-HT$_3$ antagonist eliminated the acute ES-induced reduction in MPO activity, and in macroscopic and microscopic damage scores.

Furthermore, in rats injected with icv 5-HT (400 µg/kg), both macroscopic score and tissue MPO activity, but not microscopic score, decreased, while peripheral administrations of these agonists had no significant effect. Use of a relatively narrow scale for microscopic analysis (0 to 3) probably masked the anti-inflammatory effect of 5-HT observed by the other 2 parameters. In the groups designed to study the interactions between central CRF and 5-HT, 5-HT$_3$ or CRF receptor antagonists eliminated the reduction in colitis damage scores of 5-HT-injected rats. These results may suggest that central 5-HT modulates experimental colitis through the release of CRF. Ascending 5-HTergic neurons originating from raphe nuclei make synapses with CRF neurons in PVN and also innervate some of the limbic structures including the hippocampus and amygdala. The stimulation of these fibers causes activation in the corticotropic axis, resulting in an increased CRF release into the hypophyseal portal system. CRF release capacity is also controlled by the periventricular 5-HTergic system (15).

There are 2 well-known pathways involved in the transportation of central response to effectors of the periphery. The central activation of the HPA axis and SNS either by neuronal stimulation or stress induction - including the used stress models, electric shock - is

accompanied by increased plasma ACTH, corticosterone and catecholamine levels and these may contribute to stress-induced modulation of colonic inflammation (4-7,13,29). As we already know, CRF-containing neurons originating from PVN innervate both the HPA axis and autonomic center in the brain stem and the stimulation of these neurons increases the plasma levels of stress hormones: glucocorticoids and catecholamines. Recent studies showed that, in addition to CRF, the central 5-HTergic system also modulates the HPA axis and SNS. Stress was shown to accelerate central 5-HT synthesis and metabolism together with an increase in plasma stress hormone levels. No stress-induced increase in the levels of plasma hormones was observed following the hypothalamic lesion of 5-HTergic neurons (15). In accordance with this assumption, the present study demonstrates that ip injection of RU-486 or hexamethonium eliminated acute ES-induced reductions in the macroscopic damage score and leukocyte infiltration in colitis. Moreover, 5-HT-induced anti-inflammatory effects were reversed by both RU-486 and

hexamethonium injections. These observations provide strong support for the hypothesis that, during stress - at least in the acute controllable ES - in addition to the central CRF and CCK, central 5-HT acts to minimize the influence of colitis through the stimulation of the HPA axis and/or SNS (20). In summary, controllable acute stress and central 5-HT agonist caused anti-inflammatory effects on TNBS-induced colitis. This protective action was provided by the central CRF and 5-HT and mediated by the SNS and HPA axis.

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