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Heightened Responses to Stressors in Patients with Inflammatory Bowel Disease

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OBJECTIVES: Several studies suggest that stressful situations (stressors) worsen the course of inflammatory bowel disease (IBD), but the mechanism is not known. Based on several lines of evidence, we hypothesized that psychosocial stress activates the brain-gut axis (BGA) and mucosal mast cells (MC), and activated MC produce proinflammatory cytokines. To test this hypothesis, we determined whether stressor-induced activation of BGA is exaggerated in IBD patients.

METHODS: Stress was induced in 15 IBD patients who were in remission (inactive IBD) and in seven controls by a widely used stressor, the cold pressor test (CPT), daily for five consecutive days. Induction of stress was confirmed objectively by measurement of stress hormones (serum cortisol and ACTH), and hemodynamic parameters and subjectively by questionnaire. Activation of the BGA by this stressor was assessed by evaluating colonic mucosal MC histology and degranulation, using electron microscopy (EM). The effects of the stressor on the intestinal mucosa were assessed by changes in inflammatory cell histology, epithelial mitochondria (EM), and oxidative tissue injury (assays for protein oxidation).

RESULTS: In both study groups, the stressor resulted in (1) increased levels of stress hormones, (2) the expected changes in hemodynamic parameters, (3) activation and degranulation of MC, (4) mitochondrial damage to epithelial cells, and (5) mucosal protein oxidation. These changes were more marked in IBD patients.

CONCLUSIONS: The heightened response to the stressors and the greater epithelial damage in IBD patients suggests that stress-induced activation of the BGA and of mucosal MC is important in the initiation and/or flare up of IBD.

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INTRODUCTION

Several studies have suggested that psychological stress can contribute to the initiation of or aggravate the symptoms of inflammation in inflammatory bowel disease (IBD) (1, 2). Specifically, stressful events (stressors) are associated with a more severe course of IBD, and those patients with a higher frequency of stressful life events require more potent medications. However, the mechanisms by which stressful events aggravate the pathogenesis of IBD initiation and flare-up are not clear. One mechanism through which stressors might contribute to inflammation and/or initiate flare-up is the activation of the brain-gut axis (BGA) leading to degranulation of mast cells (MC) and increase in proinflammatory cytokines in the gut mucosa (3–5).

The BGA begins in regulatory centers in the central nervous system, involves the autonomic and enteric nervous systems, and ends in target cells in the intestinal mucosa such as MC and neuroendocrine cells. Activated mucosal MC secrete neuropeptides and proinflammatory products (*e.g.*, cytokines, tryptase) (6–8). These proinflammatory products will increase intestinal permeability and initiate an immuno-inflammatory cascade in the mucosa (3, 4, 8–17), two major contributing factors to IBD flare-ups (18–24). Indeed, recent animal studies demonstrated that stressors increased intestinal permeability and worsened experimental colitis (6, 7, 9, 10, 25–29). This stress-induced leaky gut was MC-dependent since MC-deficient rodents did not develop intestinal leakiness after stress (30). Furthermore, human studies using healthy subjects showed that the cold pressor stress test

activated jejunal MC and released proinflammatory mediators into the intestine (31). These animal and human studies suggest that stress-induced aggravation of IBD course could be mediated by activation of the BGA and MC degranulation. However, direct evidence for this mechanism in IBD is lacking. Therefore, the aims of our study were to determine (1) whether stress causes greater activation of the BGA and more pronounced MC degranulation and activation in patients with IBD and (2) whether this degranulation is associated with oxidative damage and tissue injury in the colon, outcomes which might contribute to IBD flare-up.

MATERIALS AND METHODS

Our study was conducted at Rush University Medical Center and was approved by the Rush IRB. Informed consent was obtained.

Subject Recruitment

We recruited seven healthy controls, seven ulcerative colitis patients in remission (inactive UC), and eight patients with Crohn's disease in remission (inactive CD; four of whom had been diagnosed and treated for Crohn's colitis and four for Crohn's ileitis). Of these 22, 12 were male, 10 female; the average age was 43 ± 2.2 (mean $\pm$ SEM; range: 25–63). The diagnosis of IBD was based on classical endoscopic and histological findings with at least one documented flare-up within the last 12 months. Patients had inactive disease for at least 4 wk (both clinically and endoscopically, and by a CDAI $<$ 150 for CD patients. We chose to study patients with inactive rather than active IBD to avoid confounding factors associated with active inflammation such as MC degranulation and mucosal ulcer. Patients could be taking mesalamine or sulfasalazine, but these medications were stopped 24 h before sigmoidoscopy. None of the IBD patients had used medications for the 4 wk prior to enrollment that might affect MC population size or activation such as aspirin, NSAIDs, H1 and H2 blockers, antidepressants, cromolyn, immunomodulators, prednisone, TNF antibody infusion, and antibiotics. Those with a concomitant systemic disorder, organic GI disease other than IBD, food allergy, severe malnutrition, or alcoholism were excluded. One healthy control subject withdrew on day 2 of CPT after a severe vasovagal reaction; one CD subject withdrew after the first CPT due to intolerable pain.

The Stress Test

THE COLD PRESSOR TEST. Patients were administered the cold pressor test (CPT) at 8:30 AM after an overnight fast. Each subject had an intravenous catheter in the nondominant arm and sat in a comfortable chair in a semisitting position in a quiet room for 30 min. The nondominant hand was then immersed in cold water. The container was placed at a level such that the hand could be easily immersed and withdrawn. The temperature of the water was kept between 1°C and 4°C. The subject immersed his hand into cold water for 50 s and was then allowed to remove it for 10 s. This alternation, which prevents cold habituation and possible cold-induced injury, was administered for 15 min on day 1 and for longer times—20, 25, 30, and 30 min—on days 2, 3, 4, and 5, respectively.

SUBJECTIVE AND HEMODYNAMIC RESPONSES TO STRESSORS. Subjective perception to stressors was evaluated using a visual analogue scale (VAS) questionnaire, which evaluated the degree of pain and overall stress of the procedure on a scale of 0 to 10 for each domain. It was filled out by the subject after completion of each CPT. Blood pressure and pulse rate were monitored using the dominant arm before and throughout the CPT.

HORMONAL RESPONSES TO STRESSORS. Plasma cortisol and ACTH were measured by radioimmunoassay in the samples acquired before and after the first and the fifth CPT.

Sigmoidoscopy

The first sigmoidoscopy and biopsy were performed on the day of recruitment after an interview. The second sigmoidoscopy and biopsy were done just after the last CPT. Sigmoid biopsies were performed at 20 cm from the anal verge during unprepared sigmoidoscopy and samples were preserved for histology, electron microscopy, and biochemical assessment. Three of our control subjects underwent the third sigmoidoscopy and biopsy per protocol 10 days after CPT.

Histological Assessment

SAMPLE PREPARATION AND STAINING. Formalin-fixed samples obtained during sigmoidoscopy were stained using standard immunohistochemical techniques. Each slide contained three to four sections of the same tissue at different depths. MC granule staining was performed using mouse monoclonal antibodies against human tryptase (Chemicon, Temecula, CA) (Fig. 1).

HISTOLOGICAL PARAMETERS. Slides were characterized using $\times$400 high power field (HPF) magnification by an Axiovert 100 light microscope (Carl Zeiss, Germany). A total of nine distinct fields that were covered completely by transversely cut crypts (circular crypts) were evaluated for MC and inflammatory cells. The fields were not necessarily adjacent; all three to four tissue pieces on the slide were surveyed for suitable fields. Each slide was counted by two independent blinded observers and twice by one of them for calculation of inter- and intra-observer reproducibility. To determine the degree of cellular infiltration, we made a photograph and counted the number of intercryptic cells located between adjacent crypts in the field. To do this, line segments ($n = 20$) were drawn between adjacent crypt centers and the number of cells overlaying these intercryptic lines was counted. The average number of extracryptal cells per line was calculated for each field. The cellularity for each slide was calculated using the average of nine fields. Slides prepared with immunohistochemical techniques for staining MC granules

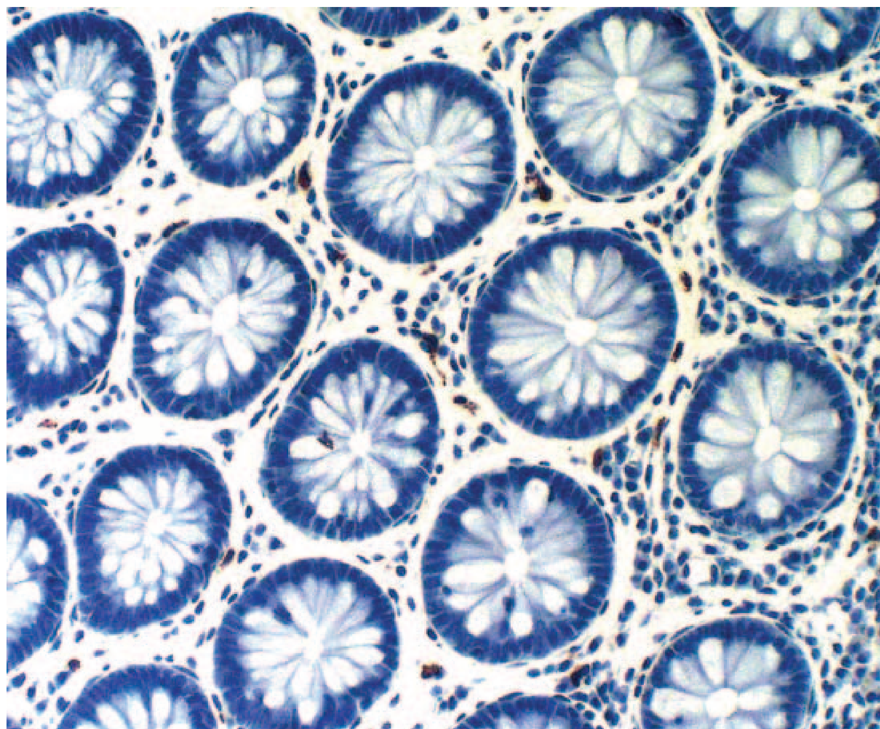

Figure 1. GI mast cells were assessed by immunohistochemistry for staining of mast cells granules using mouse monoclonal antibodies directed against human tryptase.

were used to assess the MC number in each field. In any given field, any accumulation of brown pigment that was associated with a cell nucleus was counted as one MC. Again, nine fields were selected for counting and the number of MC per HPF was calculated using the average MC count of the nine fields.

Electron Microscopy

SAMPLE PREPARATION. Fresh biopsy tissues obtained during sigmoidoscopy were fixed in 0.1 M Na Cacodylate buffer in 2% glutaraldehyde and kept refrigerated for the preparation and evaluation by EM. Tissue samples were placed in a 25% glutaraldehyde solution buffered with 0.2 M sodium cacodylate, dehydrated in ethanol and propylene, and embedded in 50/50 propylene plastic. Tissue "thick sections" (1 μm) were cut and stained with methylene blue and used for light microscopy and orientation. The block was then manually sliced into ultrathin sections (1 mm × 1 mm × 600 Å). Approximately five ultrathin sections were obtained per biopsy. These were transferred to a grid and stained with uranyl acetate and lead citrate. The stained sections were placed in a GEOL 100CX electron microscope for evaluation. The entire ultrathin section was evaluated and all MC within the tissue sample photographed (Fig. 2A). Each photograph was assigned a random number to keep investigators blinded to subjects identification. Two independent blinded observers interpreted the results and both observers reevaluated dissimilar reports.

MC ACTIVATION/DEGRANULATION. MC were classified as activated if piecemeal degranulation, granular halo, different color granules was observed (Fig. 2B). MC were regarded as degranulated if they only had evidence of anaphylactic degranulation (Fig. 2C).

MITOCHONDRIAL ASSESSMENT. Epithelial mitochondria were evaluated for structural damage (Fig. 3A). Rupture or swelling of mitochondrial cristae was considered an early sign of mitochondrial damage (Fig. 3B).

Oxidative Damage

SAMPLE PREPARATION. Fresh pinch biopsies obtained during sigmoidoscopy were snap frozen in liquid nitrogen and preserved in a -70°C freezer for analysis. One hundred microliters of 85.7 mM Tris-EDTA, pH 8.5 was added to each tube and 5 μg of protein was isolated for analysis.

PROTEIN OXIDATION. We used a monoclonal anti-carbonyl slot-immunoblot to assess tissue oxidation (protein carbonylation). For quantifying oxidation, we used two-dimensional densitometry of the blots. Carbonyl formation was expressed as percent of carbonyl formation in subject tissue compared to standard tissue.

Statistics

SAMPLE SIZE. The question of primary interest was whether IBD patients had different MC hyperplastic responses compared to controls after being challenged by stressors. This was calculated assuming the median MC number per HPF would increase by 10 when subjects were

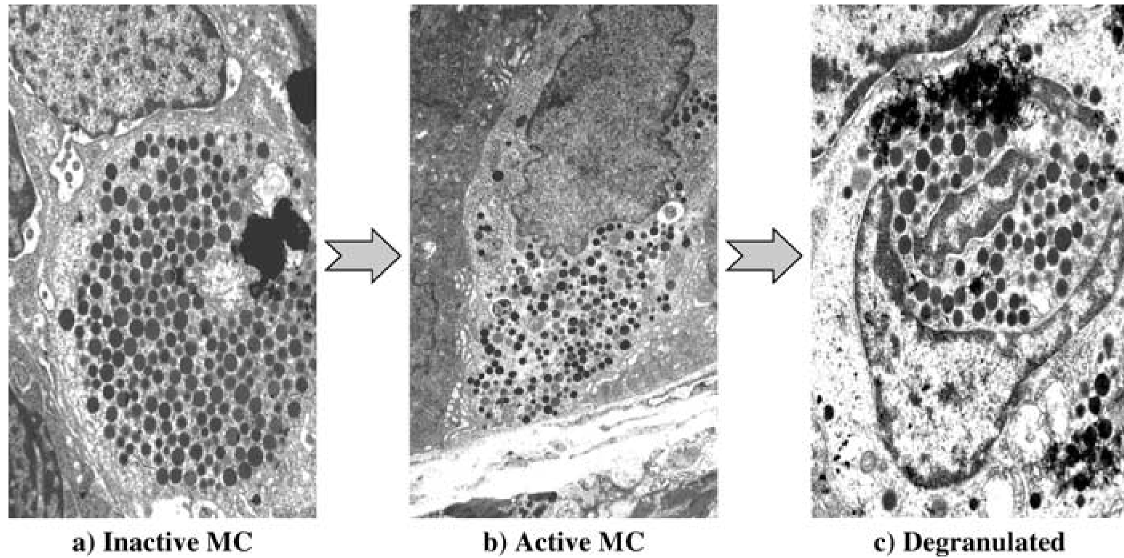

Figure 2. The effect of stress on mucosal MC activation and degranulation. All the granules were homogenous and confined to the cell membrane in inactive MC (*A*). Active MC showed evidence of different color granules which indicated various stages of granule synthesis, granular halo, or piecemeal degranulation (*B*). Degranulated MC showed evidence of release of granules in the form of anaphylactic degranulation with wide opening of the membrane and release of the granule materials across cell boundaries (*C*).

challenged by a stressor. Sample size was calculated for 80% power and a 5% type I error rate.

DATA ANALYSIS. Data were expressed as median and 25th and 75th percentiles (quartiles). Baseline and postchallenge data were compared as paired parameters using the Wilcoxon-signed rank test for nonparametric comparison. Comparisons between subjects with colitis and controls were done using a Mann-Whitney U test. $p < 0.05$ was regarded as significant.

RESULTS

The Effects of the Stressor on Markers of Stress

The CPT caused a clear stress response in all subjects as judged by significant increases in stress hormones (ACTH and cortisol; Fig. 4, left panel). The CPT caused similar levels of somatic pain and overall stress in control and IBD subjects (p = NS). All subjects developed tolerance to the rise in cortisol over the 5 days of testing (Fig. 4, Right Panel). There was a positive correlation between somatic discomfort and

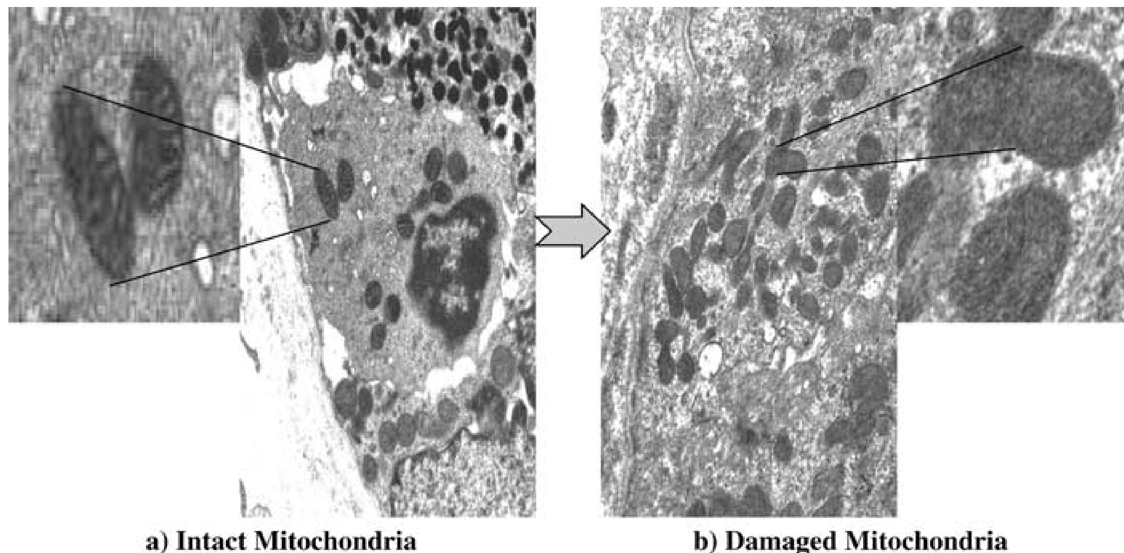

Figure 3. Mucosal epithelial mitochondria shows intact structure and cristae architecture (*A*). Swollen mitochondria with cristae rupture was considered an early sign of mitochondrial damage (*B*).

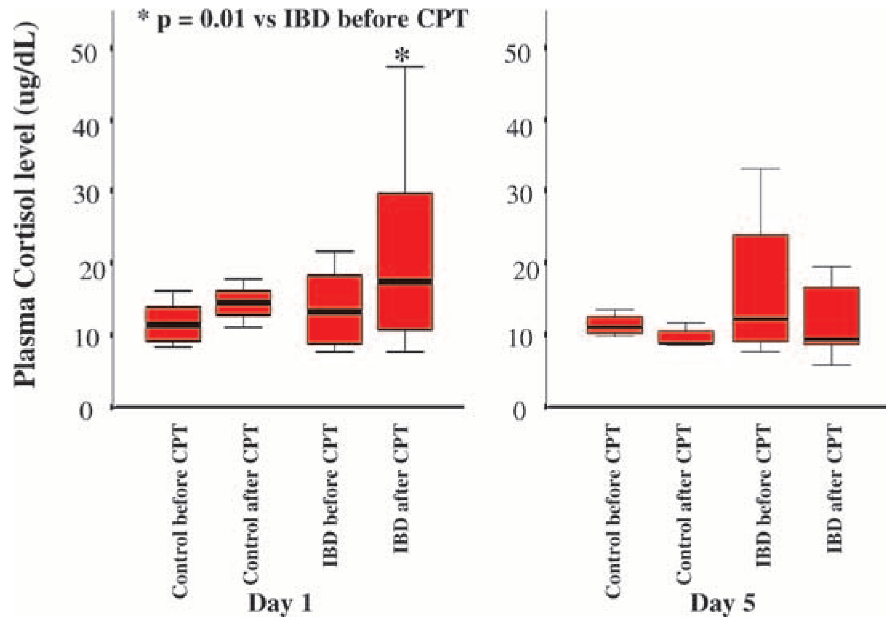

Figure 4. The effect of stress on plasma cortisol showed the first CPT was associated with significant rise in serum cortisol after CPT in IBD patients. It is interesting to note that there is significant difference in response to stress between day 1 and 5 in healthy and IBD subjects, suggestive of tolerance.

the rise of stress hormone levels in all subjects ($p = 0.01$, $r^2 = 0.6$).

In contrast to the similarity in cortisol response and in perception of somatic discomfort, IBD patients had more hemodynamic variability during stress. For example, IBD patients had less of a rise in pulse rate compared to controls (Table 1), and had more vasovagal fainting on the first test day ($p = 0.02$). Both controls and IBD subjects developed tolerance and none had vasovagal fainting on subsequent days. Tolerance to the stressor was confirmed by lack of a significant rise in cortisol on the fifth day of the CPT. Together, these data indicate that our stressor elicited a stress response in all of our study subjects.

The Effect of the Stressor on Mast Cells and Mast Cell Activation

MUCOSAL MAST CELLS. Controls and inactive IBD subjects had similar average (median) numbers of MC per HPF in baseline samples (Table 2). After the CPT, the number of MC per HPF did not significantly change in either control or IBD subjects. Poststress, the number of MC was significantly lower in IBD subjects than in controls ($p = 0.02$).

MAST CELL ACTIVATION/DEGRANULATION. Before stress, the proportions of activated MC in IBD patients and controls were similar (Fig. 5). Stress caused MC activation ($p = 0.012$) and degranulation ($p = 0.009$) in all subjects. For within group changes, the magnitude of MC activation and degranulation after stress reached statistical significance only for IBD patients ($p = 0.025$ and 0.011, respectively; Figs. 5 and 6).

The Effects of the Stressor on Field Cellularity and Markers of Epithelial Injury

FIELD CELLULARITY. Field cellularity rose in both IBD and control subjects, but neither change was significant (Table 2). IBD subjects had higher absolute levels of cellularity both pre- and post-CPT.

MITOCHONDRIAL DAMAGE. Stress caused significant mitochondrial injury in all subjects ($p = 0.04$). Thirty percent of controls and 50% of IBD patients had mitochondrial injury after stress. This difference was not statistically significant.

Table 1. The Median (Quartile Range) of the Visual Analogue Scale of Pain, Overall Stressfulness and Hemodynamic Parameters After CPT at Day 1 and Day 5 Assessed by the Subjects After Each Test

	CPT day 1				CPT day 5			
Assessed	Control		IBD		Control		IBD	
Pain	7.5 (4.7–9.3)		7.0 (2.8–8.0)		7.5 (6.3–9.3)		5.0 (3.0–8.0)	
Overall stress	6.3 (4.5–8.9)		6.2 (2.4–7.9)		6.9 (5.5–8.3)		4.9 (2.7–8.0)	
Relation to CPT	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Pulse rate/min	75 (67–80)	90 (76–107)	74 (62–84)	83 (69–94)	79 (70–88)	90 (75–97)	78 (66–91)	86 (71–97)
Systolic BP (mm)	111 (107–124)	131 (126–138)	122 (113–146)	152 (130–169)	108 (104–118)	132 (116–144)	120 (110–134)	146 (130–155)
ΔPR/ΔSBP	0.92		0.27		0.39		0.10	

Table 2. The Median (Quartile Range) of Field Cellularity (Cell per Intercryptic Line) and MC (per HPF) in the Sigmoid Tissue Before the First Cold Pressor Test (CPT) and After the Fifth CPT. Three of Our Control Subjects Underwent the Third Sigmoidoscopy and Biopsy per Protocol 10 Days After CPT

Assessed	Control			IBD	
Parameter	Before CPT	After CPT	10 days after CPT$	Before CPT	After CPT
Field cellularity	0.8 (0.7–1.3)	1.4 (0.7–1.6)	1.3 (1.2–1.5)	1.4 (1.2–2.1)#	1.8 (1.2–3.1)
Mast cells	22 (15–26)	28 (22–34)	22 (20–26)	20 (16–28)	19 (12–25)*

#$p = 0.022$ *versus* control before CPT.
*$p = 0.020$ *versus* control after CPT.
$Only in three cases.

OXIDATIVE STRESS. Stress increased mucosal protein oxidation in all subjects (median baseline protein carbonylation = 22% (range: 19–30%); median poststress = 28% (25–43%, $p = 0.022$). Increased tissue oxidation after stress was observed in 66% of controls and 86% of IBD patients. This was statistically significant only for IBD patients ($p = 0.043$; Fig. 7).

DISCUSSION

Several lines of evidence in our study suggest that subjects with IBD have heightened responses to stressful experiences (stressors) and greater associated epithelial damage, both of which could be important factors contributing to the initiation and/or flare-up of IBD. Several points bear further discussion.

The CPT appears to reliably create a stressful situation. It reproducibly induced submaximal pain and hemodynamic changes for five consecutive days, which were associated with significant apprehension, anxiety, and other pain-related psychological responses. It also increased cortisol, a marker of short-term stress. These findings are compatible with prior reports. Thus, stress paradigm in our study subjects appears to reliably model the stress paradigm defined by Hans Selye.

Our study suggests that patients with inactive IBD have exaggerated vagal responses to stress since more IBD patients experienced vasovagal fainting than controls. The exaggerated vagal response in IBD patients supports the notion of heightened BGA activity in response to stress in IBD patients. The vagus nerve is an important component of the BGA, which connects the CNS to the enteric nervous system and particularly to MC in the gastrointestinal mucosa (32). Santos *et al*. showed that TRH mediated vagal stimulation activates intestinal MC through muscarinic receptors and substance P and prostaglandin modulate the afferent pathway of this axis (33). In addition, the vagus nerve provides a major chemosensory pathway that connects immune cells and their mediators such as cytokines, to vagal sensory ganglia (34). Vagal afferent activation can even initiate local gastrointestinal reflexes that result in a variety of secretory, motor, and immune system alterations. Thus, higher vagal discharge in response to stressors in patients with IBD might contribute to the pathogenesis of IBD flare-up as predicted by our model (Fig. 8)

Our study showed that stress causes mild proinflammatory changes in colonic mucosa such as increased inflammatory cells in mucosa. These changes were noted in both controls and IBD patients. Our findings are consistent with prior

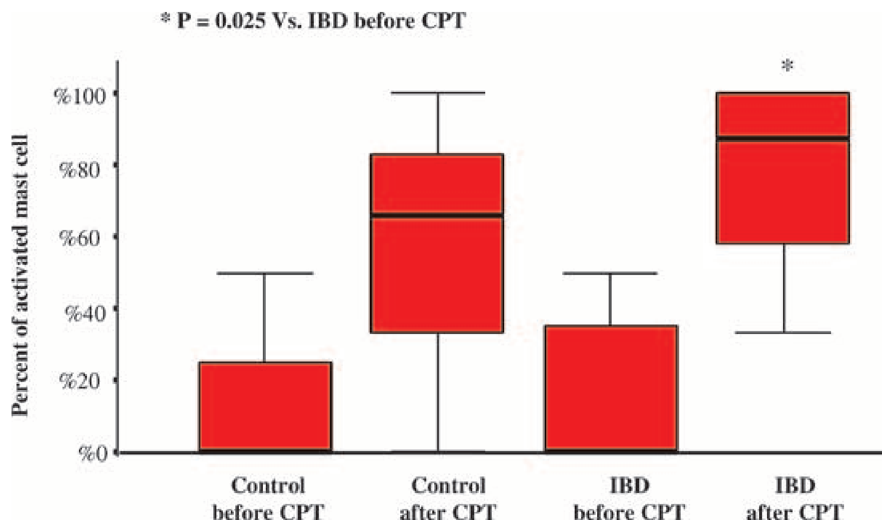

Figure 5. Mast cell (MC) activation assessed by EM showed that MC are significantly more activated after CPT in subjects with IBD than in healthy controls ($p = 0.012$).

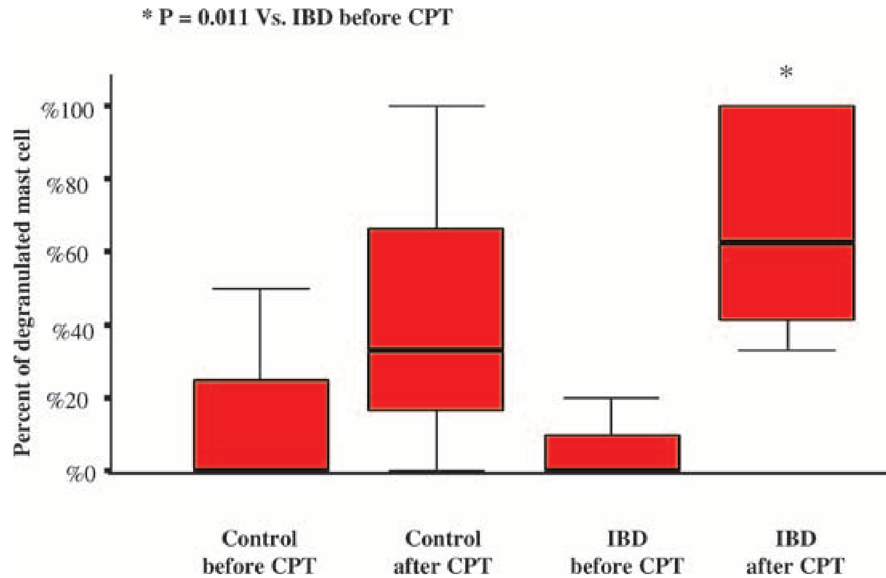

Figure 6. Mast cell (MC) degranulation assessed by EM showed that MC are significantly more degranulated after CPT in subjects with IBD compared to healthy controls ($p = 0.009$).

studies that used a rat model and showed that a stressful situation increased the number of inflammatory cells in the colonic mucosa (30).

We showed that stress caused mucosal oxidative injury in all subjects, more so in IBD patients. Increased inflammatory cells and tissue oxidative injury could be the result of release of proinflammatory cytokines in the mucosa. Several studies have shown that stress causes increased levels of proinflammatory cytokines (6–8). Indeed, our study showed that stress caused activation and degranulation of mucosal mast cells in both controls and patients with inactive IBD. However, the stressor caused more mast cell activation in IBD patients than in controls, which may result in initiation of an immunoinflammatory cascade and subsequent mucosal injury (stress-induced flare-up).

Our finding that stress increased MC activation but not MC number is consistent with prior reports. For example, Bischoff *et al*. (35) and King *et al.* (36, 37) reported that MC number was not different between controls and subjects with inactive IBD. Additionally, Santos *et al.* showed that the cold pressor stress test activates human jejunal MC, and he detected the biochemical evidence of MC activation by measuring MC mediators in the intestinal lumen (31). Santos *et al.* also showed EM evidence of MC activation in a rat model after forced swimming in cold water (30).

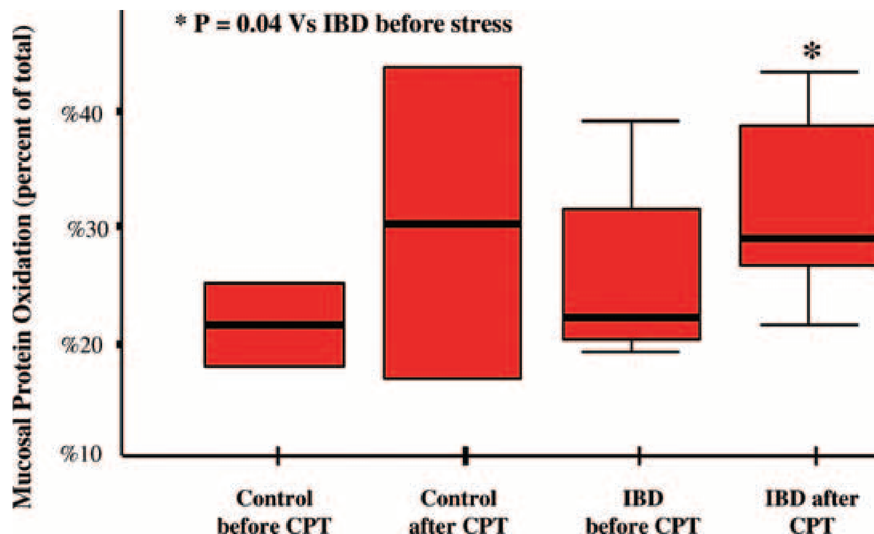

Figure 7. The effect of stress on mucosal protein oxidation. Overall stress induced oxidation of mucosal protein in all patients. The degree of oxidation reached significance only in patients with IBD.

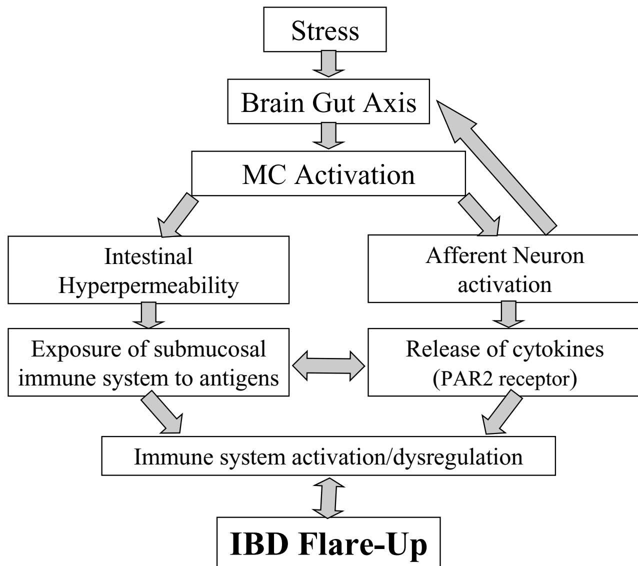

Figure 8. Proposed mechanism for the effects of stress in the pathogenesis of IBD.

Our study is the first human study that provides ultrastructural evidence of stress-induced colonic mucosal MC activation and degranulation. This is yet another line of evidence supporting the notion of heightened BGA response to stress in IBD patients.

Nevertheless, the mechanism of mast cell-induced mucosal inflammation and injury is not well established. One possible mechanism is activation of proteinase-activated receptor (PAR-2) mediated proinflammatory cascade. Indeed, MC mediators such as tryptase can activate PAR-2, and PAR2-induced release of proinflammatory cytokines from afferent neurons may contribute in part to further dysregulation of the mucosal immune system (38, 39). The latter can close the loop of a vicious circle in which the BGA is further activated (Fig. 8).

Our finding that stress results in mitochondrial swelling and damage in intestinal epithelial cells suggests that stress-induced activation of the BGA has biological significance. Prior animal studies also found that stressful experience (forced swimming) in rats causes mitochondria swelling and damage in the intestinal epithelial cells (30). It is highly plausible that activated mucosal mast cells are responsible for this mitochondrial injury. However, further studies are needed to determine the role of mast cells in this stress induced mitochondrial damage. There was no difference in the magnitude of mitochondrial damage in IBD subjects and healthy controls. Considering that there are higher baseline levels of mitochondrial damage in epithelial cells of IBD subjects, it is possible that the additional damage to mitochondria due to stressors might overload these cells such that they are no longer able to repair the damage and they fail to protect epithelial cells and intestinal barrier integrity. Mitochondrial injury and mucosal protein oxidative injury might contribute to the intestinal barrier dysfunction that is known to be induced by stressors. Indeed, we have shown that oxidative stress in monolayers of intestinal epithelial cells results in loss of intestinal barrier integrity (40–42). Thus, stress-induced oxidative injury and mitochondrial damage may also contribute to loss of intestinal barrier integrity, a crucial factor in the pathogenesis of IBD flare-up.

The number of cases in our study was designed to be able to show (with a power of 80%) differences in the number of mast cells in IBD. However, our study was not sufficiently powered to show a significant difference in all of our variables, particularly for those outcomes where we saw a trend. In subgroup analysis we compared IBD patients with colitis to those without colitis and subjects with ulcerative colitis with Crohn's disease. Our data suggested that there was no difference in stress response between these subgroups. However, the number of subjects in each subgroup is too small to confidently reach to this conclusion. In addition, adding both UC and CD subjects together in comparison with control group might increase the risk of not finding a significant difference in control subjects. Despite all these caveats, our findings that subjects with IBD respond differently to stress compared to healthy controls is a novel pilot finding and provides a rationale for future studies in this field. Further studies are needed to see if other stressors can also cause barrier disruption in IBD patients and whether interventions (*e.g.*, stress management, meditation, and hypnosis) can prevent stress-induced changes in intestinal epithelium and intestinal barrier. Such a strategy could benefit those IBD patients who experience flare-up after stressful events.

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