ENDOGENOUS CANNABINOID SYSTEM REGULATES INTESTINAL BARRIER FUNCTION IN VIVO THROUGH CANNABINOID TYPE 1 RECEPTOR ACTIVATION

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ABSTRACT

The deleterious effects of stress on the gastrointestinal tract seem to be mainly mediated by the induction of intestinal barrier dysfunction and subsequent subtle mucosal inflammation. Cannabinoid 1 receptor (CB1R) is expressed in the mammalian gut under physiological circumstances. The aim of this investigation is to study the possible role of CB1R in the maintenance of mucosal homeostasis after stress exposure. CB1R knockout mice (CB1R⁻/⁻) and their wild-type (WT) counterparts were exposed to immobilisation and acoustic (IA) stress for 2 hours per day during 4 consecutive days. Colonic protein expression of the inducible forms of the nitric oxide synthase and cyclooxygenase (NOS2 and COX2), IgA production, permeability to ⁵¹Cr-EDTA and bacterial translocation to mesenteric lymph nodes were evaluated. Stress exposure induced greater expression of pro-inflammatory enzymes NOS2 and COX2 in colonic mucosa of CB1R⁻/⁻ mice when compared with WT animals. These changes were related with a greater degree of colonic barrier dysfunction in CB1R⁻/⁻ animals determined by 1) a significantly lower IgA secretion, 2) higher paracellular permeability to ⁵¹Cr-EDTA, and 3) higher bacterial translocation, both under basal conditions and after IA stress exposure. Pharmacological antagonism with rimonabant reproduced stress-induced increase of proinflammatory enzymes in the colon described in CB1R⁻/⁻ mice. In conclusion, CB1R exerts a protective role in the colon in vivo through the regulation of intestinal secretion of IgA and paracellular permeability. Pharmacological modulation of cannabinoid system within the gastrointestinal tract might be
therapeutically useful in conditions on which intestinal inflammation and barrier
dysfunction takes place after exposure to stress.
INTRODUCTION

Besides its essential digestive function, the gastrointestinal tract represents the main interplay between the host and the environment, exerting an effective but also selective barrier function between the gastrointestinal mucosal immune system and the virtually infinite microbial and alimentary antigens on the mucosal surface. Intestinal epithelial cells constitute the main element of this barrier and exert pivotal roles both in the generation of tolerance towards alimentary antigens and commensal microbiota, and in the activation and orchestration of effective innate and adaptive immune responses (7, 16, 46, 47). However, intestinal barrier is a dynamic structure constituted not only by cellular components but also by an array of non-cellular elements such as mucin, antimicrobial peptides, secretory immunoglobulin A (IgA) as well as apical tight junctions between adjacent epithelial cells. Tight junctions are dynamic molecular structures that constitute the rate-limiting seal of the intestinal epithelial barrier paracellular pathway (30). A huge number of proteins take part in the structure of the tight junctions, including zonula occludens (ZO) family proteins, occludin, and the numerous proteins of the claudin family (53); furthermore, the junctional complex is closely related to a ring of actin microfilaments which contraction seems to directly regulate paracellular permeability.

Inflammatory cytokines such as interferon gamma (IFN\(\gamma\)) and tumor necrosis factor alpha (TNF\(\alpha\)) are capable of regulating tight junction barrier function (31). Intestinal barrier dysfunction leads to the translocation to the lamina propria, lymphatic vessels and portal circulation, of luminal bacteria.
capable of triggering and perpetuating local and even systemic inflammation. It occurs, for example, in acute pancreatitis and advanced liver cirrhosis; moreover, an adequate transcellular absorption process depends on the presence of an intact tight junction barrier, in order to maintain transepithelial concentration gradients. Indeed, increased intestinal permeability directly related to tight junction dysfunction is a characteristic feature of ulcerative colitis, Crohn’s disease, celiac disease and food allergies (2, 12). Also, it has been shown intestinal tight junction disruption in experimental models of stress, assuming their direct responsibility on the increased intestinal permeability that characterizes acute stress in laboratory animals (11, 33, 37). In this sense, exposure to physical and psychological stress triggers and / or modifies the clinical course of a variety of gastrointestinal disorders such as irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD) (6, 49).

Growing evidence from experimental studies supports the ability of psychosocial stress to induce biochemical and histological inflammatory changes in the intestinal mucosa. Indeed, animal stress models represent an excellent tool to assess intestinal barrier physiology and pathophysiology. A common finding observed in several models of stress-induced intestinal inflammation is an increased expression and activity of the inducible isoforms of nitric oxide synthase (NOS2) and cyclooxygenase (COX2) in intestinal tissue homogenates (9). The resulting high concentrations of NO and other reactive oxygen and nitrogen species produced by NOS2 as well as COX2-derived prostaglandin E2 (PGE2) have been involved in barrier dysfunction (3) and water / chloride secretion (48), respectively associated with intestinal inflammatory conditions. A wide variety of results suggest that such intestinal
inflammatory response triggered by psychological and physical stress could be mediated, at least in part, by the induction of intestinal barrier dysfunction resulting in bacterial translocation and enhanced uptake of luminal antigens (9).

The endogenous cannabinoid system regulates many different functions in the gastrointestinal system of vertebrates. The two types of cannabinoid receptors (CBR) that have been discovered and cloned, CB1R and CB2R (20), are differentially expressed in the human colon: whereas CB1R is expressed in intestinal epithelial cells, smooth muscle, myenteric plexus and lamina propria plasma cells, CB2R is mainly expressed under physiological circumstances in plasma cells and macrophages (51), but has also been recently found in myenteric and submucosal neurons of rodent (13) and human bowel samples (52). The effects of CBR activation and the physiological roles for endocannabinoids in the gastrointestinal tract have been extensively reviewed (23): briefly, CB1R activation acts mostly via brain-gut axis to reduce gastrointestinal motility, diarrhea, pain or hyperalgesia, transient lower esophageal sphincter relaxations, emesis and gastric acid secretion, as well as to promote eating; CB2R activation acts mostly via immune cells to reduce inflammation (39) through, at least, an inhibitory effect on interleukin-8 release in human colonic epithelial cells (21). It has been also suggested a role for CBR in gastrointestinal carcinogenesis as down-regulation of CB1R and up-regulation of CB2R have been observed in intestinal samples of colon cancer patients (22). However, the role of the endocannabinoid system, and in particular CB1R, in intestinal barrier function and mucosal homeostasis is still largely unknown; however, and although the exact mechanisms are poorly understood, some findings support the notion of an endogenous anti-
inflammatory activity of CB1R, since mice lacking CB1R show enhanced colitis compared to their wild-type littermates (32, 42). Consistent with this observation, administration of CB receptor agonists (26) or targeting endocannabinoid degradation (43) has been shown to protect against various forms of experimental colitis in animal models. Nevertheless, the role of the endocannabinoid system in intestinal barrier function has not been previously explored.

Therefore, in the present study we aim to investigate whether CB1R modulates intestinal barrier function in mice exposed to IA stress; for this purpose we took the advantage of the use of genetically modified mice lacking CB1R as well as pharmacological manipulation of CB1R. We report herein that stress-induced changes were related with a greater colonic barrier permeability and inflammation, lower IgA secretion and higher bacterial translocation when CB1R is absent.
MATERIAL AND METHODS

Animals. Adult male CB1R double mutant (CB1R^{-/-}) mice and wild type littermates (WT, CD1 mice) were used in all the experiments. A total of 94 animals were used. The generation of mice lacking the CB1R was previously described by Ledent and colleagues (27). WT and CB1R^{-/-} mice used in a given experiment originated from the same breeding series and were matched for age and weight (age = 10-12 weeks; weight = 25-30 g). Mice were maintained at a constant temperature of 23 ± 2 ºC and under a 12:12 h light–dark cycle (light from 8:00 to 20:00 h), with free access to food and water. All animals were maintained under constant conditions for 4 days before stress. All experimental procedures were carried out between 10:00 and 13:00 h. All experiments were performed following the highest standards of animal care, monitoring health and minimizing pain and suffering, in accordance with the European Communities (86/609/EEC) and Spanish Laws for the Care and Use of Laboratory Animals.

Experimental groups. The following groups (n = 6-9 in each group) were used: a WT and CB1R^{-/-} mice control groups (animals were handled for few seconds once at 11:00 h); and a WT and CB1R^{-/-} subacute stress groups (see below) sacrificed immediately after the last stress exposure. On the other hand various groups of WT animals were subcutaneously injected with the CB1 antagonist rimonabant (SR141716A, kindly supplied by Sanofi-Aventis) or vehicle, both under control conditions and subacute stress. Rimonabant (3 mg/kg body weight) was dissolved in vehicle solution (one drop of Tween-80 in 3 ml 2.5% dimethylsulfoxide in saline) (32) and injected at the onset of the daily stress session. Drug was injected subcutaneously (20 ml/kg body weight) under
light isofluorane anesthesia. To modulate glucocorticoid effects CB1R-/− mice (n=8) were ip injected, before each stress session, with the glucocorticoid synthesis blocker metyrapone [2-methyl-1, 2-di-3-pyridyl-1-propanone (MET)] (100 mg/kg).

**Stress protocol.** Mice were exposed to immobilisation and acoustic (IA) stress using an ultrasound bath (2h during 4 days) as previously described (25), using 50ml Corning® tubes (11.5 cm in length) with modified caps, each with a small hole to accommodate the mouse’s tail. Adequate ventilation was provided by holes drilled into the conical end of the tube and at the sides of the tubes. The tubes did not allow for forward, backward, or rotational movement. Because of the circadian rhythm inherent in corticosterone production, restraint was applied at 10:00 am for all experiments and all animals were killed using sodium pentobarbital (320 mg/kg ip; Vetoquinol, Spain) at the same point in time.

**Western blot analysis.** After determination and adjusting protein levels, homogenates of tissues, once centrifuged (12000 g, 20 min at 4 ºC) were mixed with Laemmli sample buffer (Bio Rad, Hercules, CA, USA) with beta-mercaptoethanol (50 μL per mL of Laemmli) and 20 μg were loaded into an SDS-acrilamide electrophoresis gel (90 mV). Once separated by molecular weight, proteins from the gels were blotted onto a polyvinylidene difluoride membrane (Amersham Biosciences Europe GmbH, Friburg, Germany) by semidry transfer system (Bio Rad) and, after blocking for 1 hour RT with 5% skimmed milk in TBST they were incubated with specific antibodies: rabbit polyclonal COX2 (Santa Cruz, 1:1000), rabbit polyclonal NOS2 (Santa Cruz, 1:500), CCL28 (R&D, 0.2 μg/mL) and rabbit polyclonal occludin (Santa Cruz, 1:1000). Proteins recognized by the antibody were visualized on X-ray film by
chemiluminiscence (ECL) following manufacturers instructions (Amersham Ibérica, Madrid, Spain). Autoradiographs were quantified by densitometry (Image J, NIH), and several time expositions were analysed to ensure the linearity of the band intensities.

**Colonic permeability assessment.** Mice were slightly anesthetized with isofluorane. For stressed groups, experiments were performed just after the last stress session. To measure colonic paracellular permeability, a catheter (OD 1 mm) was inserted rectally at 2 cm from the anus. 1.5 µCi $^{51}$Cr-ethylenediaminetetraacetic acid (EDTA) (Perkin Elmer España, Madrid, Spain) in 0.3 mL NaCl 0.9% was slowly perfused into the colon. After 2 hours, mice were sacrificed using sodium pentobarbital and blood was collected by cardiac puncture and anti-coagulated in the presence of tri-sodium citrate (3.15% w:v, 1 vol. citrate per 9 vol. blood). The $[^{51}\text{Cr}]$-bound radioactivity was counted using a gamma counter was used to measure radioactivity of the samples. The permeability was expressed as the ratio between blood and total $^{51}$Cr instilled and reported as percentage.

**Bacterial translocation.** Mice were anesthetized, and the abdominal skin was shaved and sterilized with an iodine solution. After death, mesenteric lymph nodes (MLN), liver, and spleen were removed under sterile conditions. After weighing and homogenization, aliquots (2 mL) of serial 10-fold dilutions of the suspension were plated onto 5% blood and MacConkey's agar plates for recovery of aerobic bacteria and Brucella blood agar plates supplemented with vitamin K$_1$ and haemin for anaerobic bacteria. After 24 and 48 h of incubation at 37 °C, for aerobic and anaerobic cultures respectively, colonies were counted. Quantitative culture results were expressed as the number of colony-forming
units (CFU) per gram. Any positive MLN, liver or spleen cultures were
considered indicative of bacterial translocation from the intestinal lumen.
Bacterial strains were identified by Gram stain, biochemical tests and standard
biochemical identification systems.

**Immunoglobulin A (IgA) determination.** Colonic IgA levels were
determined by using commercially available ELISA kit (Bethyl Laboratories,
Montgomery, USA). Colonic samples were homogenized (glass/glass) at 4°C in
4 volumes of homogenization buffer containing 320 mmol/L sucrose, 1 mmol/L
DL-dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2
μg/mL aprotinin, 0.2% Nonidet® P40, and 50 mmol/L Tris brought to pH 7.0 with
HCl (homogenization buffer). The homogenates were centrifuged (13,000g for
20 min at 4°C) and supernatants were used for determinations according to the
manufacturer’s instructions.

**Plasma corticosterone assay.** Plasma was obtained within 1 h of
obtaining blood samples by using a commercially available enzyme
immunoassay (EIA) kit of corticosterone (Enzo, Plymouth, PA, USA). The
values obtained in control animals (25.3 ± 8.1pg/mL) match expected values in
adult male CD1 mice at the time of blood extraction (= 12.00 h) (29).

**Chemicals and statistical analysis.** Chemicals were from Sigma
(Spain) or as indicated. Results are expressed as mean ± SE of the indicated
number of experiments, and statistical comparisons were made using Mann-
Whitney and one way ANOVA followed by the Newman-Keuls test, as
appropriate. P < 0.05 was considered statistically significant.
RESULTS

1. The role of CB1R on colonic paracellular epithelial permeability and bacterial translocation after stress exposure. IA stress induced a significant increase in colonic permeability to $^{51}$Cr-EDTA (figure 1) both in wild type and CB1R$^{-/-}$ animals. As shown, the degree of stress-induced intestinal barrier dysfunction was significantly higher in CB1R$^{-/-}$ animals. In parallel, stress induced translocation of different bacterial species to MLN, as demonstrated by the significant increase in CFU/g cultured in those tissues from mice exposed to stress compared with control animals (figure 2). Characterization of aerobic and anaerobic isolated species showed usual colonic commensal flora: Bacteroides fragilis, Peptostreptococcus spp, Enterococcus spp, Escherichia coli, Propionibacterium acnes, and Micrococcus spp, among others. In the same way as it occurred in the permeability experiments described above, significantly higher CFU/g were observed in the MLN, liver and spleen of CB1R$^{-/-}$ animals exposed to stress compared to WT mice. Analysis of the expression of occludin – one of the essential proteins of the tight junction complex – in colonic mucosal tissue did not show differences between experimental groups (data not shown).

2. The role of CB1R on the expression of proinflammatory enzymes in the colon after stress exposure. To clarify the possible role of CB1R on the expression of the major proinflammatory enzymes NOS2 and COX2, their protein expression in colonic samples was assessed. Repeated IA stress during 4 consecutive days resulted in higher NOS2 and COX2 expression in colonic
mucosa of CB1R Knockout mice compared to WT animals (figures 3A and B). These results were reproduced using a pharmacological approach with the CB1R antagonist, rimonabant (figures 3C and D).

3. The role of CB1R on the colonic secretion of IgA after stress exposure. To clarify possible local homeostatic mechanisms involved in CB1R anti-inflammatory after stress-induced colonic dysfunction and bacterial crossing to abdominal organs, levels of one of the major mucosal defense mechanisms, IgA, were assessed in colon. IA stress induced a significant decrease in colonic IgA production both in WT animals and CB1R Knockout mice (figure 4A). On the other side, rimonabant did not significantly decrease colonic IgA levels both in control and stressed WT-CD1 mice (Figure 4B). In order to explore mechanisms implicated in decreased colonic IgA levels observed in CB1R Knockout mice, the mucosal expression of intestinal epithelial cell-derived chemokine CCL28 (35) – involved in the recruitment of IgA-producing plasma cells to the gut mucosa – were analyzed. Stress induced a marked increase of CCL28 colonic expression only in CB1R Knockout mice (figure 5).

4. Effects of IA stress on plasma corticosterone levels in CB1R Knockout and wild-type mice. The quantification by EIA of plasma corticosterone levels at the time of blood extraction (approximately 12:00) revealed an expected increase in corticosterone in all groups of stressed animals compared with their respective controls (figure 6A); however, this increase was statistically higher in CB1R Knockout mice than in WT animals. In order to analyze whether the observed effects on intestinal barrier function may be due to elevated corticosterone
levels in the mice lacking CB1 receptor, a complete set of CB1R-/- mice (n=8) were ip injected, before each stress session, with the glucocorticoid synthesis blocker metyrapone [2-methyl-1, 2-di-3-pyridyl-1-propanone (MET)] (100 mg/kg). The dose of MET used was chosen based on previous studies showing that this dose is sufficient to block glucocorticoid synthesis during 24 h after injection (38). Indeed, by using this particular protocol, MET inhibited the stress-induced increase in corticosterone levels in CB1R-/- mice: control: 14.5±3.3; control+MET: 14.4±4.2; stress: 61.16±6.1 –p<0.05 vs control and control+MET-; stress+MET: 26.61±8.8 pg/mL, p<0.05 vs stress. As shown in figure 6B, pharmacological inhibition of glucocorticoid synthesis did not significantly modify colonic permeability to $^{51}$Cr-EDTA in stressed CB1R-/- mice.
DISCUSSION

It is well established that experimental stress leads to an acute intestinal inflammatory response and barrier dysfunction resulting in bacterial translocation and enhanced uptake of luminal antigens. These phenomena are associated with maintained stimulation of lamina propria and submucosa immune cells, and finally – under certain circumstances – with chronic inflammation. Besides its consequences on barrier function, stress also stimulates water and chloride secretion in the gut, increases mucin release from goblet cells, inhibits gastric emptying and small bowel motility and stimulates colonic motility and defecation (9); all these phenomena resemble some of the pathophysiological changes that characterize human IBS and contribute to the understanding of the mechanisms involved in stress-induced relapses of IBD. In this sense, repeated immobilization stress in rodents represents an easy, reproducible, and widely used model to study pathophysiological aspects of the stress response and to study possible pharmacological manipulations to decrease the negative effects of stress exposure on gastrointestinal tract (36). The results presented herein corroborate previous findings of our (37) and other groups (5, 40, 41) demonstrating that experimental stressful conditions induce intestinal barrier dysfunction – determined as increased paracellular permeability to macromolecules and a decreased secretory IgA secretion – leading to bacterial translocation to MLN, liver and spleen. The role of the endogenous cannabinoid system in this process is shown here for the first time, demonstrating that CB1R plays a homeostatic function in the colon in vivo. The results of the present study show, both by genetic and pharmacological
approaches, that animals lacking CB1R signaling show increased proinflammatory enzymes expression in the colonic mucosa after stress exposure. In our experimental setting, CB1R seems to exert this homeostatic and anti-inflammatory role through the reinforcement of colonic epithelial permeability (measured by permeability to $^{51}$Cr-EDTA and colonic bacterial translocation), and the control of mucosal IgA production. Our results are in agreement – in a different experimental setting but with several common features – with previously published data that elegantly demonstrated CB1R protects against intestinal inflammation in two classical models of chemical-induced colitis (32).

The role of proinflammatory enzyme NOS2 in stress-induced mucosal inflammation and barrier dysfunction has been previously outlined. NO produced by NOS2 activation increases intestinal epithelial permeability both in vitro (3) and in vivo (17), in an effect that seems to be related to NO-induced cytoskeleton rearrangement and subsequent tight junction’s dysfunction. In this sense, increased colonic permeability observed after acute stress has been recently associated with down-regulation of ZO-2 and occludin mRNA (11), although we did not find significant differences in occludin expression assessed by Western blot. Therefore, NOS2 derived NO postulates as a good candidate to mediate the stress-induced barrier dysfunction described in wild type, but in a greater degree in CB1R$^{-/-}$ mice. In fact, previous studies demonstrated that $\Delta^{9}$-tetrahydrocannabinol attenuates NOS2 expression and activity in macrophages, in a process mediated through the CB2R-dependent inhibition of the transcription factor nuclear factor kappa B (NFκB) binding activity (24); similar results have been communicated by other investigators using specific CB1R
agonists in rat glioma cells (14). These data support our results that indirectly suggest that CB1R activation down-regulates NOS2 expression in the colonic mucosa. But on the other hand, increased epithelial permeability and subsequent bacterial translocation to lamina propria could lead to the activation of the expression of these enzymes both in intestinal epithelial cells and macrophages, in a process mediated mainly by pattern recognition receptors – mediated NFκB activation. Therefore, whether increased NOS2 mucosal expression observed in our model is a cause or a consequence – or even both – of enhanced epithelial permeability in CB1R−/− stressed animals deserves further investigation.

Our results show that genetic CB1R blockade not only induced proinflammatory mechanisms in the colon but also decreased the production of protective, homeostatic molecules such as IgA. Secretory IgA represents a first line defence in the mucosal surfaces against pathogens, through its ability to neutralize virus, to bind toxins and food antigens, and to agglutinate bacteria, preventing them from binding to intestinal epithelial cells (50). Thus, a significant decrease in the amount of IgA in the colonic lumen observed in a greater degree in stressed CB1R−/− mice could contribute, together with the increased colonic paracellular permeability, to the bacterial translocation to MLN, spleen and liver. Nevertheless, we have failed in demonstrating the same effect using pharmacological CB1R blockade with a dose of rimonabant (3 mg/kg). In a further attempt to explore mechanisms of CB1R-dependent IgA production, the expression of the epithelial chemokine CCL28, involved in the recruitment of plasma cells to the colonic lamina propria (18, 35) was analysed in colonic homogenates; the stress protocol used in our study induced a marked
increase of CCL28 colonic expression only in CB1R−/− mice. But this result seems to be logical since CCL28 expression in colonic epithelium is regulated, at least in part, by the transcription factor NFκB (35), in the same way as NOS2 and COX2 mucosal expression. Thus, if the stress-induced diminished IgA levels in the colonic mucosa results from additional mechanisms such as a decrease of IgA synthesis by lamina propria plasma cells, or if there is a lack of transportation to the colonic lumen due to, for example, a down-regulation of the polymeric Ig receptor (pIgR) expression in CB1R−/− mice, is a subject of further investigation.

Previous findings from our group demonstrated an analog protective role of the peroxisome proliferator-activated receptor gamma (PPARγ) in the colonic mucosa of rats exposed to immobilisation stress: in those experiments pharmacological PPARγ activation abrogated mucosal inflammation, barrier dysfunction and decreased IgA production induced by experimental stress (37). Although PPARγ is primarily involved on adipocyte differentiation and glucose homeostasis, significant anti-inflammatory properties have been elucidated during the last years, and as previously outlined by other authors (1), there are close functional similarities between effects mediated by PPAR and CBR activation; indeed, it has been described how certain natural and synthetic cannabinoids bind to the ligand-binding domain of PPARγ and activate it (1, 34). The main endocannabinoids, anandamide and 2-arachidonoylglycerol and also ajulemic acid, a THC structural analogue, have anti-inflammatory properties mediated by PPARγ (45). Other authors have proposed alternative mechanisms by which cannabinoids can potentially lead to PPARs activation. For example, CB1 binding could stimulate MAPK pathways (28), which regulates PPARγ
transcriptional activity by direct phosphorylation (8). If there is any kind of cross-talking between both molecules in intestinal epithelial cells seems to be worthy of further research.

Some studies have showed a dysregulated HPA axis activity in basal and after stress exposure in CB1 KO mice (4). In the present study, basal response do not change, but stress response is higher in CB1 KO than in WT mice, further supporting a homeostatic role of CB1R in conditions of stress exposure, as we have recently demonstrated to occur in brain (54). Our results show that pharmacological inhibition of glucocorticoid synthesis with MET did not significantly modify colonic permeability to $^{51}$Cr-EDTA in stressed CB1-/- mice, suggesting that the lack of CB1R in the colonic epithelial cells accounts, at least in part, for the observed intestinal barrier dysfunction in these mice. On the other hand, there is a possibility that altered neural responses due to the lack of CB1 receptor in the neurons of the enteric nervous system could impact the mucosal inflammatory response and intestinal barrier dysfunction; this point should be a subject of further investigation.

Intestinal barrier dysfunction is a hallmark of a variety of gastrointestinal and systemic diseases – celiac disease, IBD, food allergies, advanced liver cirrhosis, acute pancreatitis, severe burns – and it is involved in their clinical course and some of their severe complications (16, 46); moreover, altered barrier function is proposed to be also involved in visceral hypersensitivity observed in animal models of stress and that characterize human post-infectious IBS (10, 15). Activation of CB1R leads to the inhibition of excitatory cholinergic pathways and subsequently small bowel and colonic motility, and its role in visceral nociception in the gut has been already proposed (19); those
effects constituted up to date the rationale for the proposed use of cannabinoids in the treatment of patients with IBS (44); the effects of CB1R activation on the intestinal barrier function that we describe herein represents a novel mechanism involved in, and suggest that CB1 activation might be an attractive therapeutic strategy against diverse digestive pathologies related with stress exposure.
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FIGURE LEGENDS

Figure 1. Colonic permeability to $^{51}$Cr-EDTA (expressed as the ratio between blood and total $^{51}$Cr instilled and reported as percentage) in wild type (wt) and CB1R$^{-/-}$ mice, under control conditions or exposed to IA stress. The data represent the mean ± SEM of 8 mice. *$P < 0.05$ vs control, non-stressed animals. #$P < 0.05$ vs stressed wt mice.

Figure 2. Bacterial translocation to mesenteric lymph nodes in wild type (wt) and CB1R$^{-/-}$ mice, under control conditions or exposed to IA stress. The data represent the mean ± SEM of 6 mice. No translocation were observed in wt and CB1R-/- non-stressed mice. *$P < 0.05$ vs control, non-stressed animals. #$P < 0.05$ vs stressed wt mice.

Figure 3. (A) NOS2 and (B) COX2 protein expression (representative gel and densitometric analysis of n=3) in colon from wild type (wt) and CB1R$^{-/-}$ mice, under control conditions or exposed to IA stress; mean density was calculated, plotted and compared for statistical significance. *$P < 0.05$ vs control, non-stressed animals. #$P < 0.05$ vs stressed wt mice. (C) NOS2 and (D) COX2 protein expression (representative gel and densitometric analysis of n=3) in colon from wild type mice treated with vehicle or CB1R pharmacological antagonist, rimonabant (RIMO), under control conditions or exposed to IA stress; mean density was calculated, plotted and compared for statistical significance. *$P < 0.05$ vs control, non-stressed animals. #$P < 0.05$ vs stressed wt mice.
Figure 4. (A) Colonic IgA production in colon from wild type (wt) and CB1R−/− mice, under control conditions or exposed to IA stress; the data represent the mean ± SEM of 8 mice. *P < 0.05 vs control, non-stressed animals. #P < 0.05 vs stressed wt mice. (B) Colonic IgA production in colon from wild type mice treated with vehicle or CB1R pharmacological antagonist, rimonabant (RIMO), under control conditions or exposed to IA stress; the data represent the mean ± SEM of 8 mice. *P < 0.05 vs control, non-stressed animals.

Figure 5. CCL28 protein expression (representative gel and densitometric analysis of n=3) in colon from wild type (wt) and CB1R−/− mice, under control conditions or exposed to IA stress; mean density was calculated, plotted and compared for statistical significance. **P < 0.01 vs all of the groups studied.

Figure 6. (A) Plasma corticosterone levels (ng/mL) at the time of blood extraction (12:00h) from wild type (wt) and CB1R−/− mice, under control conditions or exposed to IA stress. *P < 0.05 vs control, non-stressed animals. #P < 0.05 vs stressed wt mice. (B) Colonic permeability to 51Cr-EDTA (expressed as the ratio between blood and total 51Cr instilled and reported as percentage) in CB1R−/− mice exposed to IA stress treated with vehicle or glucocorticoid synthesis blocker metyrapone (MET) (100 mg/kg). The data represent the mean ± SEM of 8 mice. No significant differences were found between two groups.
FIGURE 3

A

B

C

D

A.U.  

wt control  CB1-/- control  wt stress  CB1-/- stress

A.U.  

wt control  CB1-/- control  wt stress  CB1-/- stress

A.U.  

wt control  CB1-/- control  wt stress  CB1-/- stress

A.U.  

wt control  RIMD  wt stress  stress+RIMD