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Ileal iNOS expression in response to stress is modified in Sprague-Dawley rats exposed to a previous intestinal inflammation.

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Running title Stress and ileal iNOS expression

Abstract

The ability of stress to initiate or reactivate an inflammatory process seems to depend on the individual susceptibility to stressful stimuli. The aim of this study was to establish whether previous inflammation alters the response to stress in Sprague-Dawley rats, a strain not especially susceptible to stressful stimuli. Stress exposure was performed in indomethacin-treated rats during the inactive phase of intestinal inflammation. Both control and indomethacin-treated rats submitted to stress showed a decrease in body weight gain and blood leukocytes levels, as well as an increase in faecal pellet output. The increase of mast cell count induced by stress was similar in both groups of animals. Moreover, no differences were observed between control and indomethacin-treated rats in the degree of bacterial translocation and myeloperoxydase levels after stress exposure. Despite these similarities, differences between groups were observed in inducible nitric oxide synthase (iNOS) mRNA expression. Whereas ileal iNOS mRNA expression was inhibited in healthy rats submitted to stress, stress failed to modify this parameter in indomethacin-treated animals. As iNOS is another inflammatory marker, our results allow for the possibility that a previous intestinal inflammation could change the intestinal susceptibility to stress. Whether these differences in ileal iNOS expression can be indicative of a possible change in the predisposition to develop an intestinal inflammatory reaction in response to stress in Sprague-Dawley rats remains to be elucidated.

Introduction

The gastrointestinal tract is a well-known target for physiological changes that occur during stressful life events (Bhatia and Tandon, 2005). The main effects related to stress are motility disturbances (Cao et al., 2005; Morrow and Garrick, 1997; Taché et al., 2001) and epithelial barrier dysfunction (Santos et al., 2000; Soderholm and Perdue, 2001).

Several animal studies have been addressed to assess the role of stress as a trigger of intestinal inflammation. The results obtained clearly show that, per se, both acute (Santos et al., 1999; Saunders et al., 1994) and chronic (Santos et al., 2000) stress increase intestinal permeability, an effect that can allow the penetration of luminal bacteria into the epithelium (Kiliaan et al., 1998; Kuge et al., 2006). This bacterial translocation can cause an overstimulation of the immune system and hence, initiate the inflammatory process (Soderholm et al., 2002). However, most of these studies have been performed in high-stress responsive strains that show an increased susceptibility to develop intestinal inflammation under stress conditions (Mawdsley and Rampton, 2005; Soderholm and Perdue, 2001). For instance, Wistar-Kyoto rats show an enhanced hypothalamic-pituitary-adrenal (HPA) reactivity in addition to a blunted noradrenergic stress response (Pardon et al., 2002), and these alterations can contribute to the intestinal inflammation observed in these animals after stress exposure. Recently we have reported that stress induces mucosal mast cell hyperplasia and bacterial translocation in Sprague-Dawley rats (a strain not especially susceptible to stressful stimuli), similarly to that observed in Wistar-Kyoto rats. However, unlike to that described in Wistar-Kyoto strain, no clear signs of intestinal inflammation were observed in these animals (Jorge et al., 2010). Thus, the ability of stress to trigger an inflammatory response seems to depend upon the stress susceptibility showed by an individual in a given moment. Therefore, factors that can

modify the stress susceptibility could facilitate the development of intestinal inflammation.

It is well known that immune stimuli can activate the HPA axis, causing similar effects to those observed after stressful conditions (Dunn et al., 2005; Shanks et al., 1998). However, prolonged exposure to inflammatory cytokines can alter both basal and stress-induced HPA activity. Indeed, a HPA axis dysfunction with blunted glucocorticoids response has been found in several inflammatory chronic diseases such as rheumatoid arthritis (Chikanza et al., 1992), systemic lupus erythematosus (Gutierrez et al., 1998), as well as inflammatory bowel disease (IBD) (Mawdsley and Rampton, 2005). In addition, previous enteric infection may lead to the condition of so-called post-infectious irritable bowel syndrome (PI-IBS) (Spiller and Garsed, 2009). In this chronic gastrointestinal disease, as well as in IBD (where bacteria seems to play a fundamental role), stress seems to be a contributing factor modulating the time-course of the illness (Bitton et al., 2003; Mardini et al., 2004; Monnikes et al., 2001).

These observations, consequently, prompt the question whether previous inflammation can alter the intestinal response to stress, and hence, modify the predisposition to develop an inflammatory process. Thus, the main goal of this study was to elucidate whether, in absence of a genetic predisposition for stress vulnerability, a previously inflamed intestine would show an altered response to stress that could favour the appearance of an inflammatory reaction. For this purpose we used Sprague-Dawley rats, a strain not especially susceptible to stress, treated with indomethacin to induce intestinal inflammation. As previously reported (Porras et al., 2004), two subcutaneous injections of 7.5 mg/kg indomethacin 48 hours apart induced a characteristic cyclic oscillation of active and quiescent phases of inflammation. Active phases were characterized by intestinal hypomotility and increased paracellular and transcellular permeability associated with bacterial translocation and an increase of inflammatory markers. By contrast, intestinal hypermotility and only enhanced paracellular

permeability (without alterations in the transcellular pathway) were observed during the quiescent phases, correlating with a decrease in the incidence of bacterial translocation and a return to basal levels of inflammatory markers (Porras et al., 2006; Porras et al., 2004). In the present study, stress was applied during the quiescent phase, when no clear signs of intestinal inflammation were present, in order to assess whether stressful stimuli were able to trigger an inflammatory response. Our hypothesis was that a previous intestinal inflammation can facilitate bacterial translocation under stress conditions and hence, trigger an inflammatory reaction. To test this hypothesis, we compared the stress responses between a healthy intestine and a previously inflamed one by assessing the following parameters: bacterial translocation, mucosal mast cell count, iNOS mRNA expression and myeloperoxidase (MPO) concentration in ileum samples.

Materials and methods

Animals

Male Sprague-Dawley rats (Charles River, Lyon, France), 8-10 weeks old and weighing 300-350 g were used. They were kept singly-housed under conventional conditions in an environmentally controlled room (20-21ºC, 60% humidity, 12:12-h light-dark cycle, lights on at 07:00) with tap water and standard laboratory rat chow ad libitum. Animals were handled daily by the same investigator who performed the experiments for one week before the study, in order to minimize the stress of unusual contact with humans. All experimental protocols were approved by the Ethical Committee of the *Universitat Autònoma de Barcelona*.

Experimental model of enteritis

Intestinal inflammation was induced by administration of two subcutaneous injections of indomethacin (7.5 mg/kg) 48 hours apart, as previously described (Porras et al., 2004). As mentioned in the introduction section, this model shows a spontaneous cyclic oscillation between active and quiescent phases of inflammation, which can be identified by determining the time-course of Blood leukocytes (BL) levels (150 μl blood samples were taken every two days) through the duration of the experiment. In this study BL levels reached higher values during the active phase (22 954 ± 925.1 cells/mm^3), BL levels during the quiescent phase were similar to that observed in control rats (14 900 ± 462.5 cells/mm^3 in indomethacin-treated rats; 12 957 ± 194.2 cells/mm^3 in control rats). Figure 1 (panel A) shows this typical oscillatory pattern of BL levels in an individual rat maintained for up 60 days after indomethacin administration. In this study, exposure to the chronic stress protocol was performed during the quiescent phase following the second peak of high BL levels (two active phases were recorded before starting stress exposure). Quiescent phase was identified when two consecutive BL counting were inferior to the maximal leukocyte value reached during the second active phase (Figure 1, panel B)

Chronic stress protocol

Chronic stress was induced by submitting animals to a heterotypic chronic intermittent stress paradigm involving the physico-psychological stress of wrap restraint (WR) and the psychological stress of water avoidance stress (WAS). During the WR procedure the limbs and the body of animals were wrapped in a cloth harness to restrict, but not to prevent, body movements. Water avoidance procedure (WAS) consisted of placing the rat on a plastic platform (7x7x10 cm) located in the middle of a plastic container (55 cm diameter) filled with warm water (25ºC) to 1 cm below the platform . The chronic stress protocol was adapted from the previously described method (Million et al., 1999), and consisted of submitting animals to 1h WR or 1 h WAS alternatively for 5 consecutive days (WR-WAS-WR-WAS-WR). Control animals were kept in their home cages during the procedure. All the experiments were performed between 9:00 and 12:00 AM to minimize the effect of diurnal circadian rhythm.

Experimental design

Rats were randomized in four groups:

1) Control-non stressed group (n=16) that received saline subcutaneously and were kept in their own home cage during the stress procedure,
2) Control-chronic stress group (n=16) that received saline subcutaneously and were submitted to the chronic stress protocol,
3) Indomethacin-non stressed group (n=13) that received indomethacin subcutaneously to induce a cyclical intestinal inflammation disease and were kept in their own home cage during the stress procedure, and
4) Indomethacin-chronic stress group (n=15) that received indomethacin subcutaneously to induce intestinal inflammation and were submitted to the stress protocol. Exposure to the chronic stress paradigm was performed during the quiescent period following the second active phase of the BL oscillatory

pattern. The stress protocol was started when two consecutive BL counting were inferior to the maximal leukocyte value reached during the active phase.

Figure 2 shows the study time-course. Stress exposure was started in indomethacin-treated rats immediately after the quiescent phase had been identified (indomethacin-chronic stress rat). Simultaneously, the stress protocol was applied in one control animal (control-stress rat). A similar procedure was performed between indomethacin-non stressed and control-non-stressed rats. In all cases, animals were submitted to the stress/control protocol around 23 ± 6 days after indomethacin or saline administration. Animals were euthanized 1h after the last stress/control session by isofluorane inhalatory anesthesia and exsanguinated by heart puncture, to obtain intestinal tissue samples.

Animal monitoring

Several parameters were evaluated through the entire experimental period in all groups of rats. These parameters led us to assess the effectiveness of both enteritis induction by indomethacin and the chronic stress protocol, but also to evaluate the putative differences in the responses to stress between groups.

Growth: Rats were weighed daily. Body weight change was expressed as % of weight gain in relation to the first day of the stress protocol.

Food intake: Food consumption was recorded daily through the stress protocol period and results were expressed as mean g/day.

Defecation: Defecation, as an indirect index of colonic propulsive activity (Maillot et al., 2000) was determined at each session during the stress protocol. Pellets expelled during 1 hour stress/sham session were counted and expressed as the number of fecal pellets/1h.

Blood leukocytes: Blood leukocytes levels (BL) were monitored every two days through the study. Blood samples were taken by the tail-nick procedure. This procedure consisted of gently wrapping the animals with a cloth, making a 2mm incision at the end of the tail veins and then massaging the tail while collecting 150 μl of blood into EDTA capillary tubes (Starsted, Granollers, Spain). Once the stress/control protocol was started, blood samples were obtained 30 minutes after the stress/control session. BL were counted using a Neubauer chamber and expressed as cells/mm^3. BL change was expressed as percent (%) of BL count rise comparing to the first day of the stress protocol.

Bacterial Translocation

Bacterial translocation from the lumen of the intestine was determined by detection of viable enteric bacteria in mesenteric lymph nodes (MLN). MLN from the ileocaecal region of each rat were removed aseptically immediately after being euthanized. Specimens were frozen in liquid nitrogen, powdered in a mortar and weighed. Samples were homogenized in ten parts of milk (Difco, Detroit, MI) and incubated under aerobic conditions onto blood agar and McConkey agar for 48 h at 37ºC. After incubation, colonies were identified by studying their morphological and biochemical properties. The incidence of bacterial translocation was expressed as the number of positive cultures of the total number of samples in each group.

RMCP II immunochemistry and mucosal mast cell count

Distal ileum samples were fixed in 10% buffered formalin and embedded in paraffin wax. Transverse sections were cut (3 μm) and immunodetection of rat mast cell protease II (RMCP II) was carried out using a monoclonal antibody (1:500; Moredun Animal Health, Edinburgh, UK). Detection was performed with avidin/peroxidase (Vectastain ABC kit; Vector Laboratories, Burlingame CA, USA). Sections were counterstained with haematoxylin and counted at x 400 magnification. Positively

stained mast cells were counted in three to five sections per animal. Seven to ten well-oriented villus-crypt units (VCU) were examined per section. Analysis of all morphological data was performed blinded to prevent observer bias. Estimation of mast cell numbers was expressed as cells per VCU.

RT-PCR studies

Distal ileum samples of each rat were taken immediately after being euthanized. Tissue segments were frozen in liquid nitrogen and stored at -80ºC until use.

RNA extraction: Isolation of total RNA was performed using TRI Reagent (Ambion, Madison, WI, USA) according to the manufacturer's instructions and treated with DNA-free (Ambion) for 30 min at 37ºC to remove any genomic DNA contamination.

RT-PCR*:* First-strand cDNA was synthesized from 5 µg total RNA in a reaction mixture of 50 µL containing 0.5 µg of oligo18 (dT) primer (Ambion), 2mM dNTP (Ecogen, Barcelona, Spain), and 10 units Moloney murine leukemia virus (Ambion). The resultant cDNA was amplified in a total volume of 50 µL with 1 unit of *taq*DNA (Ecogen), 1mM dNTP mixture, and 1.2 µM primers (Proligo). The sequences of sense and antisense primers for rat iNOS and GAPDH (a housekeeping gene used as an internal control) are listed in Table 1. Thermal cycling conditions were as follows: denaturation for 4 min at 95 ºC, then 35 cycles PCR with denaturation at 95ºC for 1 minute, annealing at 62ºC (iNOS) or 50 ºC (GAPDH) for 1 minute, and extension at 72ºC for 1 minute, and a final extension of 72ºC for 5 minutes. Amplified products were electrophoresed on 1.5% agarose gel in TAE buffer, stained with ethidium bromide, photographed under ultraviolet light, and quantified using image-analyzing software (PC-BAS 2.0). For semiquantification, the ratio of the optical density of each PCR product and GAPDH was determined.

Tissue Myeloperoxidase (MPO) Determination

Proteins were extracted from the ileal tissue of each animal using a lysis buffer containing protease inhibitors (Minicomplete tablet, Roche Diagnostics, Mannheim, Germany). MPO concentration was determined using a specific enzyme-linked immunosorbent assay (HyCult Biotechnology, Uden, The Netherlands), with a minimal detectable concentration of 1 ng/mL.

Statistical Analysis

Data are expressed as means ± SEM. Statistics were performed using Minitab 15 Statistical Software (Coventry, UK). Differences between groups were compared using a Two-way ANOVA (factors: inflammation and stress) and Tukey post hoc analysis. Results from bacterial translocation were analyzed using Chi-square test. In all cases, results were considered to be statistically significant when $p<0.05$.

Results

Effects of stress on animal monitoring parameters

As seen in figure 3 showing the percent of body weight (BW) gain during the first 10 days after indomethacin administration, rats with induced intestinal inflammation showed a transient loss of BW gain, whereas a linear increase in this parameter was observed in the control group . This loss of BW gain induced by indomethacin together with the higher BL levels recorded in these animals (19 264 ± 791 cells/mm^3 and 20 975 ± 729.2 cells/mm^3 2 and 4 days after indomethacin administration [all indomethacin-treated animals n= 28] respectively versus 12 957 ± 194.2 cells/mm^3 in control rats [all control animals n= 32]) are consistent with the development of intestinal inflammation. Exposure to stress during the quiescent phase of inflammation induced a significant decrease of BW gain, which was similar to that observed in stressed control rats ($F_{1,55}$ =13.47 $p < 0.001$) (Fig. 4). This stress-induced effect in BW gain rate was not related with differences in food intake, either in healthy (24.71 g/day ± 0.51 [n= 16] vs. 26.07 ± 0.56 in control non-stressed group [n= 16]) or indomethacin treated rats (25.09 g/day ± 0.58 [n= 15] vs. 26.84 ± 0.6 in inflamed non-stressed group [n= 13]).

Regarding the defecation frequency, the inflammatory process was associated with an increase of pellet output when compared to the healthy conditions ($F_{1,55}$=8.81, p = 0.004). Moreover, stress exposure also caused a significant increase of defecation frequency, independent of whether intestine had been previously inflamed or not ($F_{1,55}$ =118.48, p <0.0001) (Fig. 5)

In accordance with previously reported data (Porras et al., 2004), non-stressed indomethacin-treated rats showed the oscillatory BL level pattern characteristic of this experimental model, associated to the active and quiescent phases of chronic intestinal inflammation. Chronic stress was applied during the remission phase of intestinal inflammation, when BL levels (15 435 ± 502.1 cells/mm^3 , n= 28) were similar to that observed in control animals (12 727 ± 410 cells/mm^3, n= 32). Exposure to stress

resulted in a significant decrease of BL count both in inflamed (n= 15) and control groups (n= 16) when compared to their respective counterparts (inflamed-non stressed group n= 13; control-non stressed group n=16) ($F_{1,55}$=78.39, p <0.001). This decrease was not related to the inflammatory state in indomethacin-treated animals ($F_{1,55}$ =2.60, p=0.1125). These results are shown in figure 6.

Effects of stress on bacterial translocation

Bacterial translocation is defined as the migration of bacteria from the intestinal lumen to mesenteric lymph nodes or other extra-intestinal sites (Berg, 1995).

As expected, no viable enteric bacteria were found in mesenteric lymph nodes (MLN) of non-stressed control rats (n= 14). By contrast, unstressed animals with intestinal inflammation (n= 13), even though they were in the quiescent phase of the inflammatory process, showed bacterial translocation.

As previously reported (Jorge et al., 2010), a significant bacterial translocation was found in control rats submitted to stress (n= 16). By contrast, exposure to stress did not significantly increase the total bacterial translocation in the inflamed group (n= 15). However, it is important to note the remarkable translocation of *Enterobacteriaceae*, which was present in 53% of MLN of indomethacin-treated rats exposed to chronic stress. This result was statistically significant when compared to the control group . All results are summarized in table 2.

Effects of stress on mucosal mast cells

The immunochemical detection of RMCP II, a chymase predominantly expressed by rat intestinal mucosal mast cells, is commonly used to determine mucosal mast cell count.

Animals with small intestinal inflammatory disease selected during the remission phase showed a mild, but statistically significant, increase of mucosal mast cell count ($F_{1,50}$ 5.16, p = 0.0274). Stress exposure for 5 consecutive days increased this parameter in control (n = 12) as well as in inflamed animals (n = 13) when compared to their

respective counterparts (control-non stressed group n= 14; inflamed-non stressed group n=15) ($F_{1,50}$ =46.01, $p < 0.001$). The magnitude of the stress-induced increment in mast cell count was similar in both groups. (Fig. 7).

Effects of stress on ileal iNOS mRNA expression

As seen in Figure 8, healthy rats exposed to chronic stress (n=16) showed a significant decrease of iNOS mRNA expression, when compared to the non-stressed animals (n= 15) (Student t-test $p<0.05$) This decrease in ileal iNOS mRNA expression induced by stress was not observed in rats with intestinal inflammation (inflamed-non stressed group n=13; inflamed-stressed group n=13).

Effects of stress on tissue Myeloperoxidase (MPO)

MPO, an enzyme found in granulated cells, has been widely used as a reliable index of inflammatory activity (Smith and Castro, 1978). As previously reported (Porras et al., 2006), MPO levels in unstressed indomethacin-treated rats (1.16×10^{-3} % of total protein, n=13) were similar to those observed in unstressed control group (0.97×10^{-3} % of total protein n=13), confirming that they were in the quiescent phase of the inflammatory process. Exposure to stress did not modify MPO levels neither in control (0.92×10^{-3} % of total protein, n=13) nor in inflamed animals (1.35×10^{-3} % of total protein, n=12) ($F_{1,47}$ =0.11, p= 0.7450)

Discussion

The rationale of this experimental approach was to establish whether previous inflammation alters the response to stress in Sprague-Dawley rats, a strain not especially susceptible to stressful stimuli, and to assess whether these putative changes can trigger an inflammatory reaction.

Besides the role of stress as a trigger of intestinal inflammation, several studies have been performed to evaluate the involvement of stress in the exacerbation of the inflammatory process. It has been described that stress increases the severity of subsequent colitis induced by dextran sulphate sodium (DSS) as well as by 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Israeli et al., 2008; Reber et al., 2008). Moreover, exposure to stress during the acute phase of inflammation also enhances the inflammatory response (Gué et al., 1997; Million et al., 1999). However, few studies exist to assess whether stress can induce or reactivate an inflammatory response in an intestine that has apparently recovered from a previous inflammation. To address this question, the experimental model of indomethacin-induced small intestinal inflammation was used. It has been postulated that depletion of endogenous prostaglandins (Fang, 1997), increased activity of iNOS (Konaka et al., 1999b) and vascular dysfunction (Piepoli et al., 2005) may be involved in the inflammation induced by indomethacin. Moreover, the initial inflammatory reaction has been associated with bacterial overgrowth and wall invasion by luminal bacteria (Porras et al., 2004; Yamada et al., 1993). In a previous study we demonstrated that two injections of 7.5 mg/Kg indomethacin 48 hours apart induce an oscillatory pattern with active and inactive phases of inflammation that can be observed for up 60 days after drug administration (Porras et al., 2004). Although no clear signs of intestinal inflammation were present during the inactive phases in this model, enhanced paracellular permeability and low-grade bacterial translocation were still present (Porras et al., 2006). The recurrent appearance of active phases in this model must be attributed to these remaining

alterations rather than to a direct effect of indomethacin, which was administrated several days before. Thus, this experimental model can represent a useful tool to study the pathophysiological mechanisms of gastrointestinal illnesses in which the equilibrium between microbial flora and host response seem to be deranged, as in IBD and PI-IBS. In both cases, persistent enteric infection associated with increased gut permeability have been proposed as factors contributing to the development of the disease (Kalischuk and Buret, 2010; Spiller and Garsed, 2009).

As previously reported, chronic stress induced a decrease in body weight gain (Velin et al., 2004; Yang et al., 2006), increased colonic motor activity (Monnikes et al., 2001) and resulted in a decrease in blood leukocytes level in control animals (Dhabhar and Mcewen, 1997). Similar effects were observed in indomethacin-rats exposed to stress during the quiescent phase of inflammation, indicating that the effectiveness of the stress protocol used was similar in both experimental groups. To note, as described in IBS and IBD patients during the remission phase of inflammation (Ansari et al., 2008; Simren et al., 2002), unstressed indomethacin-treated rats showed an increased defecation frequency when compared to the unstressed control group, suggesting that the motility disturbances induced by the inflammatory process were still present.

In keeping with similar results (Velin et al., 2004; Zareie et al., 2006), stress induced translocation in control animals. While the results obtained in inflamed rats exposed to stress did not reach statistical significance when compared to their unstressed counterparts (probably due to the small number of data used in the Chi-square test), some observations can be made. Whereas bacterial translocation was observed in 46% of unstressed indomethacin-treated rats, the incidence after stress exposure was around 73% of inflamed animals. Recently, we have reported that the changes induced by stress in the small intestinal motor activity can facilitate bacterial translocation in control Sprague-Dawley rats, even in the absence of clear signs of villous epithelial permeability dysfunction (Jorge et al., 2010). Thus, the effects of stress exposure can

be added to the motor and intestinal permeability disturbances caused by the inflammatory process and hence, increase the passage of bacteria to the MLN. Another observation can be made in relation to the specific bacterial species found in MLN. It has been reported that host-mediated inflammatory response induced by an infecting agent and/or a chemical trigger can disturb the intestinal microbiota balance and allow an overgrowth of Enterobacteriaceae (Lupp et al., 2007). In agreement, an increase in luminal Enterobacteria occurred during the active phases of inflammation in our experimental model (Porras et al., 2004). This can be related to our findings showing that Enterobacteria were isolated from MLN in 53% of stressed indomethacin-treated rats, whereas its incidence in the stressed control group was 19%. More studies are needed to really elucidate whether these observations can represent a difference in the stress response between a previously inflamed intestine and a healthy one.

It is well known that mast cells are involved in stress-induced intestinal barrier function disturbances (Soderholm et al., 2002; Yang et al., 2006) that can allow bacterial translocation. Although exposure to stress of control SD rats increased mast cell counts, in a previous study, we demonstrated that this increase was not associated to villus epithelial (VE) barrier dysfunction (Jorge et al., 2010). However, other studies suggest that mast cells are also involved in the stress-induced barrier disruption of follicle associated epithelium (FAE) (Keita et al., 2010), providing a possible explanation for the bacterial translocation seen in these animals in our study. Also, in our study the bacterial translocation observed in unstressed inflamed rats was accompanied by an increase of ileal mast cells. In this case, this rise in mast cell number might be involved in the paracellular permeability increase observed during the quiescent phase of inflammation in this experimental model as reported previously (Porras et al., 2006). Exposure to stress further increased mast cell number in indomethacin-treated rats, an event that could have led to a worsening of the

permeability disturbances and hence to have facilitated the passage of bacteria to the MLN.

The differences observed in iNOS mRNA expression after stress exposure supports the hypothesis that a previous inflammation alters the intestinal response to stressful stimuli. Under non-stress conditions, control and indomethacin-treated rats showed similar levels of iNOS mRNA expression. However, the decrease of iNOS mRNA expression induced by stress in control rats was not observed in indomethacin-treated animals. It has been reported that bacterial translocation is the first step required for activation of various factors such as neutrophils and iNOS (Konaka et al., 1999a), and that prolonged exposure to high NO levels can cause a breakdown in the gut barrier function (Nadler et al., 1999). On the other hand, glucocorticoids exert an inhibitory action on iNOS expression (Hamalainen et al., 2008; Korhonen et al., 2002). It can be hypothesized that the iNOS decrease observed in control animals exposed to stress could represent a defensive mechanism triggered by the release of corticosteroids in order to avoid NO overproduction and hence, to limit the degree of bacterial translocation by maintaining the intestinal barrier integrity. In keeping with the findings that prolonged exposure to inflammatory cytokines can alter the activity of the HPA axis (Shanks et al., 1998), the failure of stress to decrease iNOS expression in indomethacin-treated rats could be due to a change in stress susceptibility due to a previous intestinal inflammatory process. Another possibility could be related to the ability of Enterobacteria to directly induce iNOS expression (Kolios et al., 2004). In this way, the iNOS expression induced by the persistent bacterial translocation that occurs in this experimental model could counterbalance the inhibitory effects exerted by glucocorticoids, explaining why iNOS levels remained unchanged in stressed indomethacin-treated rats.

As we have previously reported, stress in control animals is not enough to trigger intestinal inflammation (Jorge et al., 2010). Exposure to stress of indomethacin-treated

rats did not modify MPO levels. However, taking into account that iNOS is another inflammatory marker and that stress in indomethacin-treated rats failed to decrease iNOS expression as it did in control animals, we cannot conclude that stressful stimuli were able to trigger an inflammatory reaction. It is well known that the effects of stress depend on the quality and duration of the stressor. The stress protocol used in the present study can be considered as a mild-stressful stimulus when compared with other chronic stress protocols such as chronic subordinate colony housing (Reber et al., 2008) or 10 days of WAS stress exposure (Soderholm et al., 2002). In a previous study performed in a murine model of DSS-induced colitis, a trend towards a reactivation of the inflammatory process was observed after 7 days of WAS exposure, that was enhanced by administration of a sub threshold dose of DSS (Melgar et al., 2008). Collins et al. (Collins et al., 1996) found some changes associated with inflammation after stress exposure in TNBS-induced colitis in rats, although they failed to observe clinically significant colitis. Thus, differences between studies can be related to both the stress protocol and the experimental model used to induce inflammation.

In summary, our results show differences in ileal iNOS expression after exposure to stress between a healthy intestine and a previously inflamed one. Whereas a decrease in this parameter was observed in control animals submitted to stress, stressful stimuli did not change ileal iNOS expression in indomethacin-treated rats. Although no clear signs of inflammatory response were observed in these animals, the failure of stress to inhibit ileal iNOS expression opens the possibility that a previous intestinal inflammation alters the “intestinal susceptibility” to trigger an inflammatory reaction in Sprague-Dawley rats after stress exposure. More studies are needed to assess to what extent these differences in ileal iNOS expression are indicative of a change in the predisposition to develop an intestinal inflammatory reaction in response to stress.

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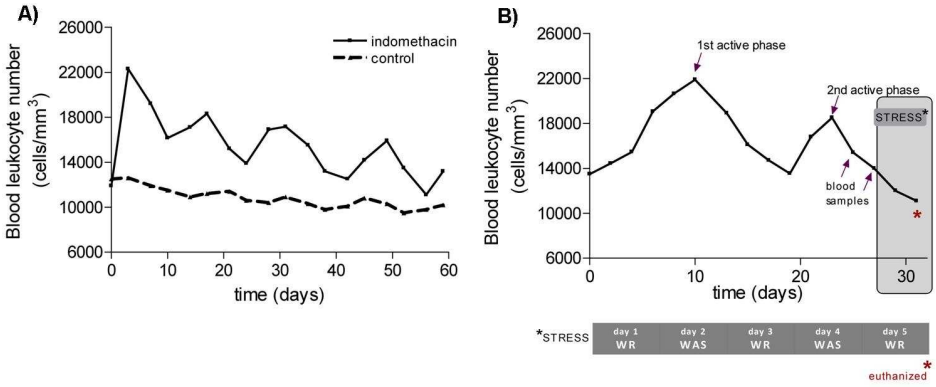

Figure 1. Representative graphs showing the cyclic oscillation of BL levels associated with active and inactive phases of intestinal inflammation induced by indomethacin administration. (A) Representative graph of BL evolution in an indomethacin-treated rat and in a control animal. The cyclic oscillation of BL was maintained for up 60 days after drug administration. (B) Representative graph showing the moment to start the stress protocol. Stress exposure was performed during the inactive phase of inflammation following the second peak of high BL levels. The inactive phase was identified when two consecutive BL counting were inferior to the maximal leukocyte value reached during the active phase. The grey zone of the graph corresponds to the 5 days of stress exposure using the protocol illustrated by the * Stress scheme.
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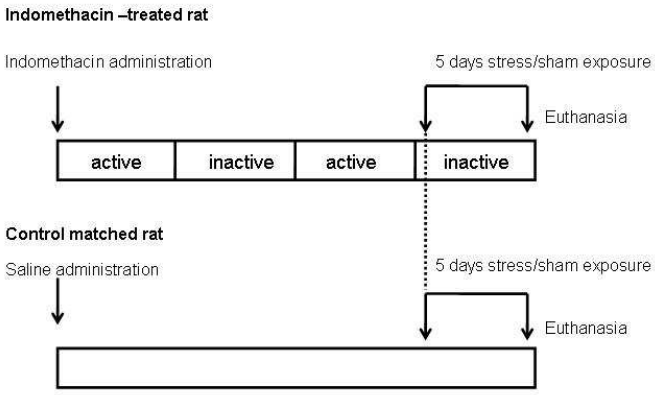

Figure 2. Overview of the experimental design used in the study. Each indomethacin-treated animal was matched to a control rat. The stress/sham sessions start when the inactive phase following the second active phase of inflammation was identified in an indomethacin-treated animal. Simultaneously, the stress/sham sessions were started in their matched control individual. After 5 days of stress/sham exposure, both indomethacin and control animals were euthanized.
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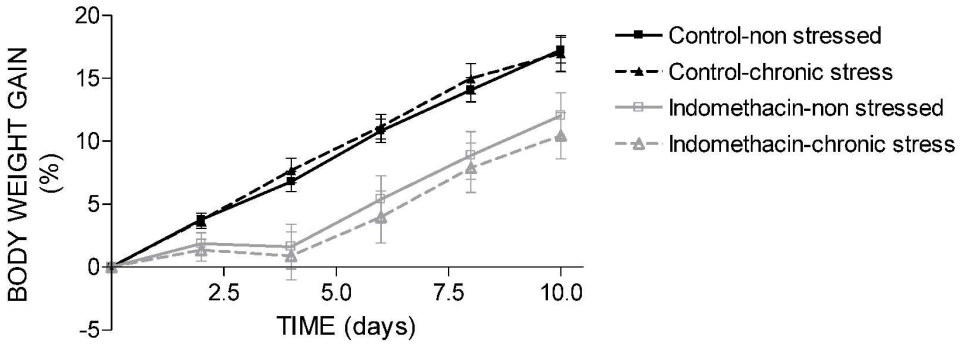

Figure 3. Effects of indomethacin on the evolution of the percent of body weight gain in relation to the first day of drug administration. Data are means ± SEM, n=13-16 animals/group. A decrease in body weight gain was observed after indomethacin administration, whereas control rats showed a linear increase of this parameter.
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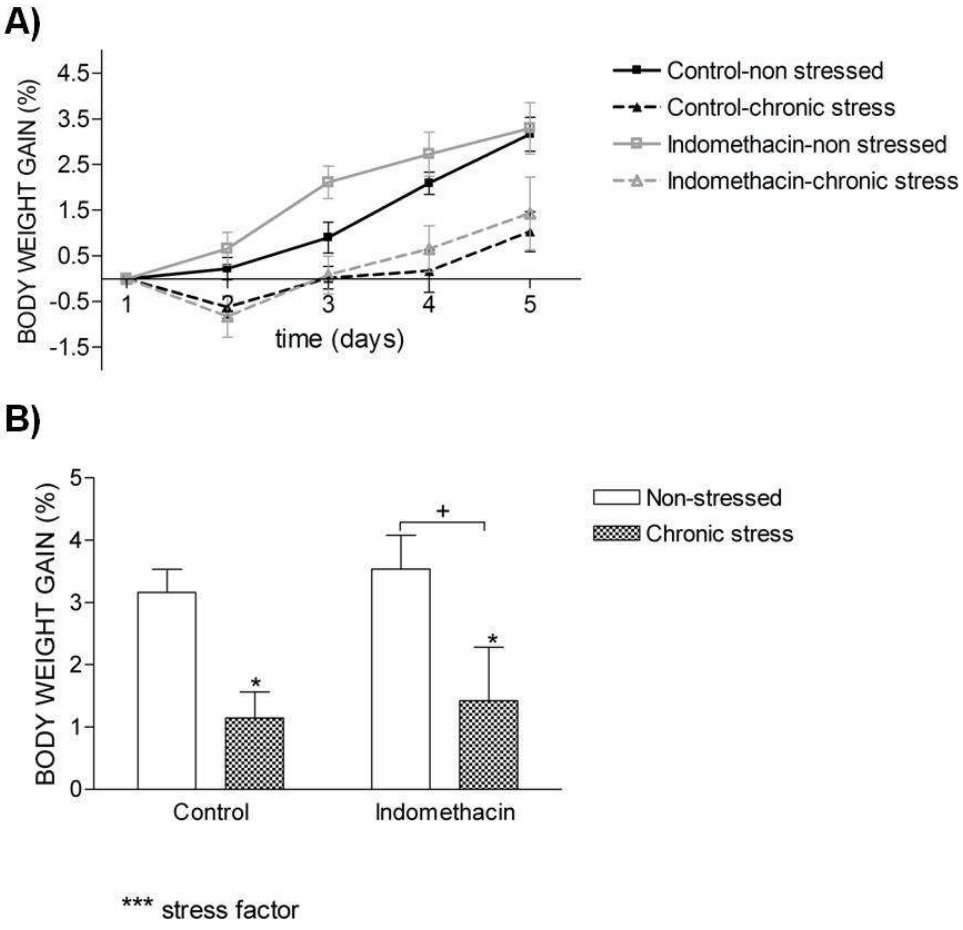

Figure 4. Effects of stress on body weight gain in control and indomethacin-treated animals. Data are means ± SEM, n=13-16 animals/group (A) Evolution of the percent of weight gain in relation to the first day of the stress/sham protocol in control and indomethacin-treated rats. (B) Percent of body weight gain in control and indomethacin groups 5 days after stress exposure. Stress significantly decreased body weight gain (Two-way ANOVA, p = 0.0005). * p<0.05 vs control-non stressed group; + p< 0.05 vs indomethacin-non stressed group.
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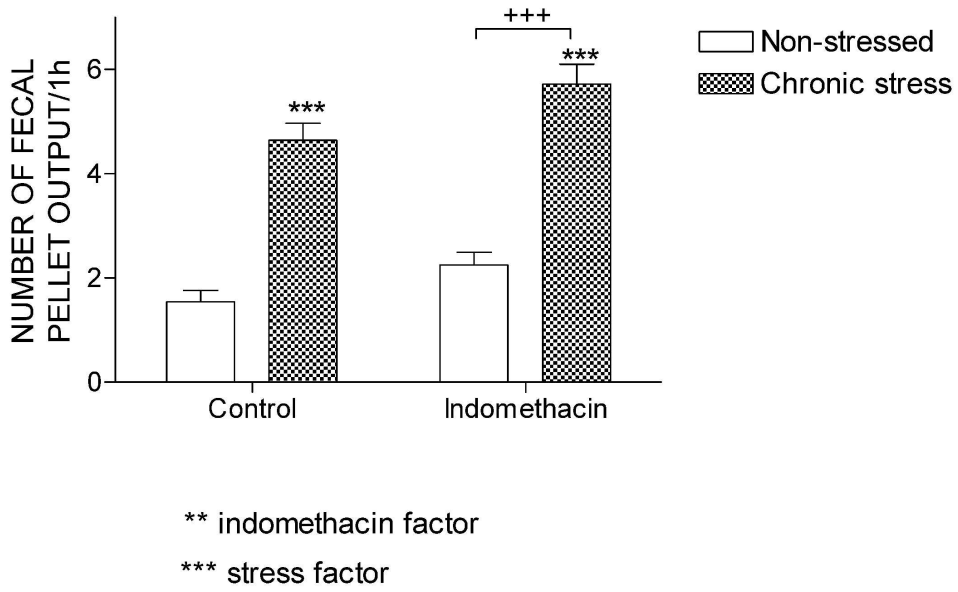

Fig. 5. Effect of stress exposure on fecal pellet output in control and indomethacin-treated animals. Date are means ± SEM, n=13-16 animals/group. Indomethacin-treated rats showed an increase of defecation frequency when compared to the healthy animals (Two-way ANOVA p = 0,004). Moreover, stress significantly increase pellet output when compared to the unstressed animals (Two-way ANOVA p <0,0001). *** p<0.001 vs control non-stressed group: +++ p< 0.001 vs indomethacin non-stressed rats.
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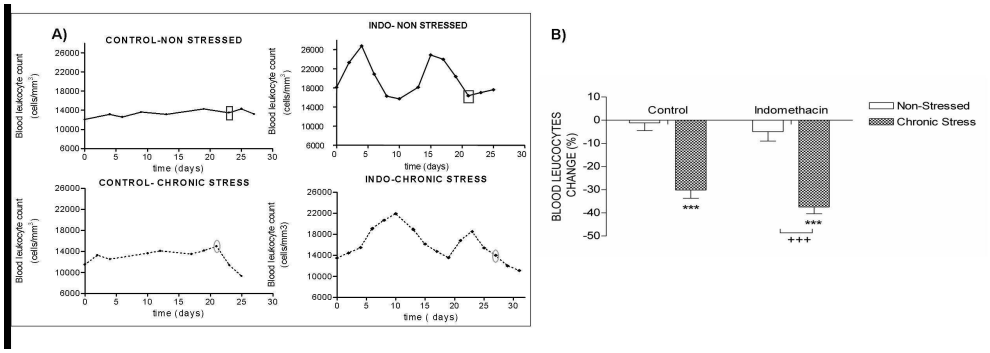

Fig. 6. Effect of stress on BL levels in both control and indomethacin-treated rats. (A) Representative graph showing the evolution of BL in an individual animal of each group. In each graph was indicated the moment in that stress/sham exposure was initiated (square in unstressed rats; circle in stressed rats). (B) Change in BL count 5 days after stress exposure expressed as percent of BL count of recorded the first day of the stress/sham protocol. Data are means ± SEM, n=13-16 animals/group. Stress significantly decreased BL levels (Two-way ANOVA, $p < 0.0001$). This decrease was not related to the inflammatory state in indomethacin-treated animals (Two-way ANOVA p=0,1125). *** $p<0.001$ vs control non-stressed group; +++ $p<0.001$ vs indomethacin non-stressed group.
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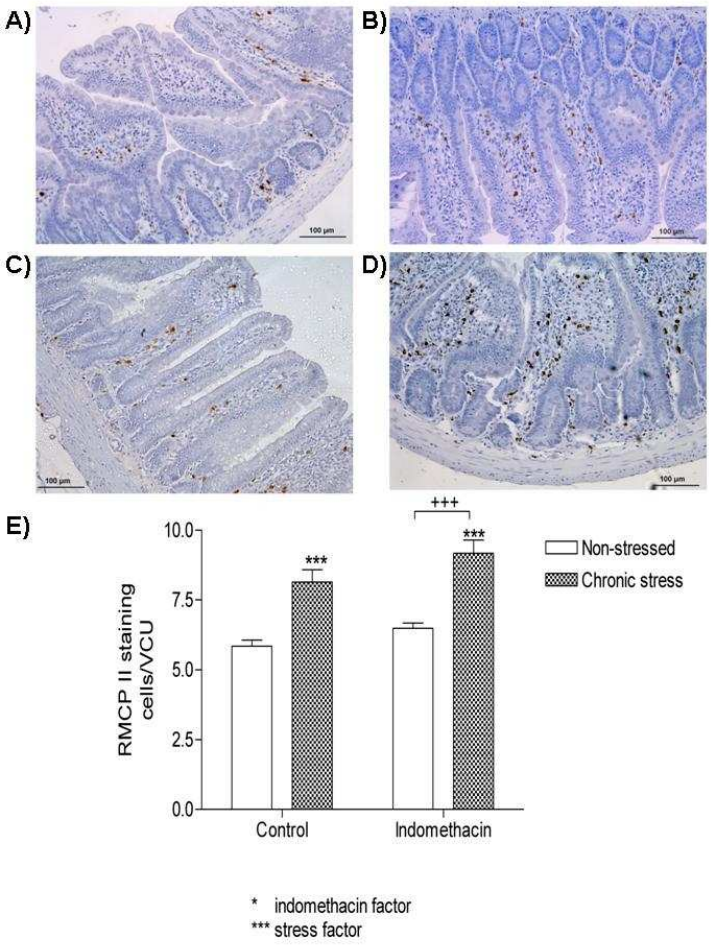

Figure 7. Effect of stress in the number of mucosal mast cells per villus-crypt unit (VCU). Microphotographs showing rat mast cell protease II (RMCPII) immunopositive cells (mucosal mast cells) in the intestinal ileum mucosa of (A) control non-stressed, (B) control-stressed, (C) indomethacin non-stressed and (D) indomethacin stressed animals. (E) Bar diagram representing the number of mucosal mast cells per VCU in ileum mucosa of each group. Three to five sections were counted per rat, 7-10 units well-oriented VCU were examinated per section. Data are means ± SEM, n=13-16 animals/group. Indomethacin-treated rats showed an increase of mast cell count per VCU compared to the control group (Two-way ANOVA p = 0,0274). In addition, stress also increases mast cell count per VCU both in control and indomethacin-treated animals (Two-way ANOVA p < 0,001). ***p<0.001 vs control non-stressed group; +++p<0.001 vs indomethacin non-stressed group.
254x247mm (96 x 96 DPI)

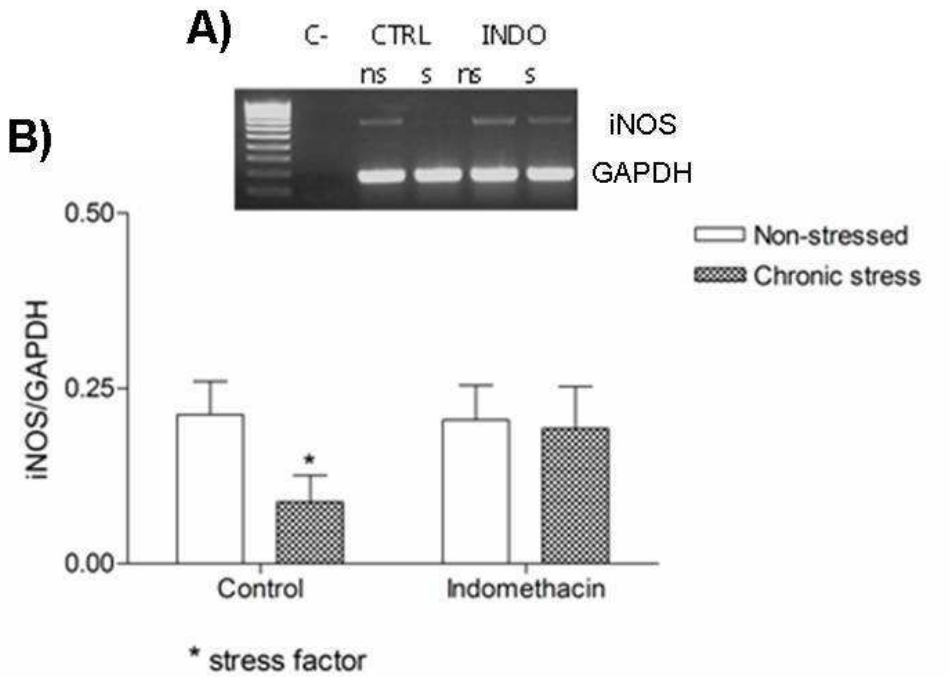

Fig 8. Effect on iNOS mRNA expression in both control and indomethacin-treated rats. (A) Representative photograph of agarose gel showing RT-PCR products for inducible isoform of nitric oxide synthase (iNOS) mRNA in ileum. C-: negative PCR control; CTRL (ns: control-non stressed animal; s: control-stressed animal),and INDO (ns: indomethacin-non stressed animal; s: indomethacin-stressed animal). (B) Bar diagram showing semiquantitative analysis by RT-PCR of iNOS mRNA expression. Data are means ± SEM, n=13-16 animals/group. Stress induced a decrease in iNOS mRNA expression in control animals exposed to stressful stimuli. Student t-test *p<0.05 vs control non-stressed group.

122x85mm (150 x 150 DPI)

Table 1. Primer sequences for RT-PCR

Gene	Primer Sequence	Amplicon lenght (bp)
iNOS		
Sense	5'-ACAACAGGAACCTACCAGCTCA-3'	651
Antisense	5'-GATGTTGTAGCGCTGTGTGTCA-3'	
GAPDH		
Sense	5'-CCGCCCCTTCCGCTGATGCC-3'	140
Antisense	5'-ATGAGCCCTTCCACGATGCC-3'	

Table 2. Number of animals with bacterial translocation and incidence for a specific microorganism.

	Enterobacteriaceae	*Enterococcus/ Streptococcus spp.*	*Lactobacillus spp.*	Bacterial translocation
Control-Non stressed	0/14	0/14	0/14	0/14
Control-Stressed	3/16	3/16	3/16	8/16**
Indomethacin-Non-stressed	3/13	3/13	1/13	6/13**
Indomethacin-*Stressed*	8/15**	5/15*	0/15	11/15**

Data are expressed as number of positive cultures of the total animals of each group. *,**,*** $p<0.05$, 0.01 and 0.001 respectively, compared to control-non stressed group (Chi-square test).