Implication of Nerve Growth Factor in intestinal mucosal mast cell activity and colonic motor alterations in a model of ovalbumin-induced gut dysfunction in rats

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Research report presented by Ferran Jardí Pujol to complete the Pharmacology PhD Program (UAB)

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Hereby declare:

That the research work entitled "Implication of Nerve Growth Factor in intestinal mucosal mast cell activity and colonic motor alterations in a model of ovalbumin-induced gut dysfunction in rats" presented by FERRAN JARDÍ PUJOL to complete the PhD training program in Pharmacology from the Universitat Autònoma de Barcelona, has been performed under our supervision and that we authorize its presentation to be evaluated by the corresponding examination board.


Patrocinio Vergara Esteras  
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ABSTRACT

**Background:** Nerve Growth Factor (NGF) and its receptors (TrkA) have been implicated in the neuronal remodeling present in patients with irritable bowel syndrome and in animal models of the disease. **Aim:** To determine the involvement of NGF in mast cell function and colonic motor alterations in a model of ovalbumin (OVA)-induced gut dysfunction in rats. **Methods:** Rats were exposed to OVA for 6 weeks. After the third week, animals received the TrkA antagonist K252a. Colonic mucosal mast cell (MMC) density and tissue levels of rat mast cell protease II (RMCPII) were measured. Colonic strips were obtained to assess contractile activity *in vitro*. **Results:** OVA exposure increased the number of MMCs and colonic RMCPPII concentration. Spontaneous colonic contractility was similar in vehicle- and OVA-treated animals and was inhibited in similar proportion by K252a. Responses to carbachol were increased in OVA-treated rats in a K252a-independent manner. The NO-synthase inhibitor LNNA increased spontaneous activity in OVA-treated animals and this response was completely prevented by K252a. **Conclusions:** These observations support an involvement of NGF in the functional changes observed in this animal model. NGF receptors may represent a potential therapeutic target for the treatment of gastrointestinal disorders characterized by the presence of motor alterations.
INTRODUCTION

Irritable Bowel Syndrome (IBS) is a functional gastrointestinal disorder characterized by abdominal pain, alterations of the bowel habits and visceral hypersensitivity, in the absence of apparent organic alterations (16). IBS is highly prevalent in industrialized countries and may affect up to one quarter of the population (26). The underlying pathophysiology of IBS remains unclear. Furthermore, as there are no specific diagnostic tests its identification relies on the application of symptom-based criteria (Rome III criteria) (17), leading to potential diagnostic confusion. As a result, IBS therapeutics has a limited development. Therefore, the process has a tremendously important economic burden on health care resources for direct (e.g., diagnosis, therapy) and indirect (e.g., work absenteeism) costs (16).

Many factors, such as external stressors, dietary constituents, genetics or intestinal infections have been suggested to play a role in IBS pathogenesis. Regarding the involvement of food in the pathophysiology of the disease, a large number of patients have described the exacerbation of their symptoms immediately following food ingestion (45). Although this onset of symptomatology could be just due to the normal increase of secretion and motor activity characteristic of the digestive process, in some patients could be related to food intolerance or alimentary allergy. While food intolerance is an adverse non-immune mediated reaction (e.g., an enzyme deficiency), alimentary allergy is an abnormal immunological response to food constituents. The best characterized food allergy reactions are those mediated primarily by immunoglobulin E (IgE) antibodies, although cell-mediated mechanisms may also be involved. For instance, Bischoff et al. observed that, in patients with abdominal symptoms suspected to be related to food allergy, some food antigens caused intestinal weal and flare reactions, although specific IgEs in serum were low (10). Evidence has been reviewed regarding the potential role of adverse food reactions in IBS (38). For example, as it concerns to food allergy, it has been demonstrated that there is an increase in the prevalence of atopic conditions in diarrhea-predominant IBS patients (50). Moreover, the prevalence of IBS is higher in patients with bronchial asthma compared to patients with other pulmonary disorders (41). Finally, several studies demonstrate usefulness of oral disodium cromoglycate, an inhibitor of mast cell
degranulation, and elimination diets (50; 51) in diarrhea-predominant IBS patients, thus suggesting that part of the symptomatology observed could be related to food allergy or food intolerance.

To better understand IBS pathophysiology, results from human and animal studies have to be integrated in a comprehensive manner. However, the selection of animal models for IBS is limited, in part because of the large, undefined, spectrum of underlying mechanisms of the disease. While some of the described models focus on the development of intestinal motor alterations characteristic of IBS, others emphasize visceral hypersensitivity or the stress component of the disease (37; 52). As it relates to models of altered motility, several strategies have been used to mimic the changes of colonic motor activity associated with IBS. For instance, either colonic irritation, experimental infections or acute and chronic stress have been used in rats and mice to induce colonic motor alterations reminiscent of those observed in humans with IBS (1; 8; 29; 52).

Recently, mast cells have been considered as potential key players in IBS pathophysiology. Mast cells are ubiquitous in the body, although they are especially bound within tissues that interface with the external environment, such as the skin or the respiratory/intestinal mucosa. They originate from precursors of the hematopoietic lineage and circulate in blood and the lymphatic system before entering specific tissues, where they mature under microenvironmental influences. As a consequence, mast cells in different tissues exhibit heterogeneity due to locally produced growth and differentiation factors. In rodents, mast cells can be classified into two subtypes: connective tissue mast cells (CTMCs) and mucosal mast cells (MMCs). As their name indicate, CTMCs are mainly located in connective tissues (around blood vessels and in the peritoneal cavity) while MMCs are found in the intestinal and pulmonary mucosa.

Mast cells produce an impressively broad array of mediators that can be subdivided into two classes: mediators that are preformed and stored in secretory granules (mainly histamine, serotonin, serine proteases, proteoglycans and some cytokines) and mediators that are rapidly synthesized de novo when mast cells are activated (mainly arachidonic acid metabolites, platelet activating factor, chemokines and some cytokines). Activation of mast cells occurs mainly by interaction of a multivalent agent
with specific IgE antibodies bound to the cell membrane. Therefore, mast cells represent critical effectors cells in allergic disorders and others IgE-mediated acquired immune responses (9). However, they are activated not only by IgE-dependent mechanisms, but also by several non-immunological stimuli, including neurotransmitters and neuropeptides (58).

In the gastrointestinal mucosa, there is evidence that MMCs are closely apposed to nerves fibers, likely forming membrane-to-membrane contacts (48; 49). This relationship becomes more intimate when there is an increase in the MMC infiltrate, such as during IBS (61) or intestinal nematode infection in rats (49). The mechanisms that regulate this anatomical relationship are still unclear. It is hypothesized that sprouting of nerves towards mast cells is initiated by the release of mediators from mast cells. Blennerhassett et al. used time-lapse photomicroscopy to show that growing neurons were deflected towards RBL cells (a MMC-like cell line) in an apparent chemotactic response (11). However, it cannot be ruled out that mast cells might be attracted to nerves by neuropeptides. Both hypotheses could be possible as there is evidence that mast cells express receptors for neurotransmitters (57) and that peripheral nervous system neurons express receptors for mast cell-derived mediators (44). Several observations in vitro and in vivo evidence this bidirectional cross-talk between MMCs and neurons. For instance, De Jonge et al. observed that degranulation of MMCs by the compound C48/80 caused neuronal activation in vitro (13). Moreover, MMCs in primary culture were degranulated by the application of substance P and calcitonin gene-related peptide (CGRP) (13). Furthermore, Gottwald et al. demonstrated that the stimulation of the vagus nerve in the rat resulted in an increase in histamine content in MMCs (21). In addition, the same group observed that truncal vagotomy and sensitive deafferentiation by neonatal treatment with capsaicin decreased intestinal MMC population (22). These results suggest that, apart from an anatomical relationship, it exits a functional link between MMCs and the enteric nervous system (ENS).

Several observations support an involvement of mast cells in the pathophysiology of IBS. First, many studies have evidenced a mast cell infiltration in the colon of IBS patients and high levels of their mediators (mainly tryptase and histamine) in supernatants from colonic biopsies (4). Second, the number of activated mast cells in close proximity to colonic nerve terminals is enhanced in IBS patients compared with
healthy controls (3). More interestingly, the severity and the frequency of abdominal pain in these patients correlate positively with these close appositions (3). Finally, supernatants from colonic biopsies of IBS patients, containing a variety of mast cell mediators, elicit functional responses (visceral hypersensitivity and alterations in colonic barrier function) in animals (12; 20), thus supporting an involvement in symptom generation in IBS patients. Moreover, murine sensory neurons in culture are sensitized by the same supernatants. Overall, these data point towards an important role of mast cell-derived mediators and the interaction mast cells-nerve fibers on the disturbed secretomotor and sensory functions that characterize IBS.

The role of the neurotrophin nerve growth factor (NGF) and its relationship with MMCs in the pathophysiology of IBS is receiving increasing attention. NGF regulates the survival, differentiation, development and functional maintenance of both peripheral and central neurons. In addition, NGF also influences the development and activation of many hematopoietic cell types, including mast cells. The biological actions of NGF are mediated through two classes of cell surface receptors: the TrkA high-affinity, NGF-specific, receptor and the p75 low-affinity receptor, which presumably binds to all neurotrophins (18). Although TrkA is responsible for most of the neuronal effects of NGF, it has been reported that p75 acts as a coreceptor for TrkA, increasing its affinity for NGF (24).

Within the gut, immunoreactivity for NGF and TrkA receptors is found in the ENS (25) and, probably, associated with MMCs (15). NGF content is low in the normal gut, but inflammation results in an up-regulation of NGF mRNA (40). Although it has been demonstrated that several gut-resident cell types, such as epithelial cells (59), lymphocytes (31) and mast cells (46) can produce NGF, the source of NGF in the gastrointestinal tract is still unclear.

Apart from its physiological functions, NGF has been implicated in the neuroimmune alterations that characterize inflammatory and functional disorders of the gut (47). In animal models of IBS, NGF has been associated to neuronal remodeling and recruitment of MMCs. In particular, anti-NGF treatment completely blocked the intestinal hypermotility induced by *T. spiralis* infection in rats (53) and abolished the increase in visceral sensitivity to colorectal distension observed in the maternal
separation model in rats (5). In the same model, treatment with anti-NGF prevented the increase of mast cells in close proximity to nerve fibers (7). In the colonic mucosa of IBS patients, it has been observed an enhancement of NGF immunoreactivity associated to MMCs (2). The same study also showed that mucosal supernatants from IBS patients induced a significantly higher neuronal sprouting on cultured neuronal cell lines than those from healthy controls, an effect that could be reduced partly by NGF neutralization. From these observations, the authors suggested that mast cell-derived NGF may contribute to the pathophysiology of IBS (2).

During the last years, our group has characterized an animal model of IBS in rats based on the chronic exposure to oral ovalbumin (OVA). In this model, Sprague Dawley (SD) rats exposed to oral OVA, without any adjuvant, for a six-week period develop an intestinal MMC hyperplasia together with motility alterations, reminiscent of some of the IBS symptoms in humans (55). Interestingly, these responses are not mediated by classical Th2 components like IgE, IgG, eosinophils or IL-4. This is somehow unexpected since SD rats have been widely used as a model of OVA-induced allergic response. However, in most of these models, OVA sensitization is done by parenteral injection of the peptide and only the final challenge is given orally. Therefore, the chronic oral exposure to OVA represents a valid approach to IBS non-atopic patients, whose symptoms are associated with the ingestion of particular foods.

In this model, we have also observed that oral exposure to OVA causes NGF overexpression in the colon, as assessed by conventional PCR (54). In addition, pretreatment with the mast cell stabilizer ketotifen prevents OVA-induced NGF overexpression. However, perhaps the most interesting observation is that there is a positive correlation between the number of colonic MMCs and the level of expression of NGF. All together, these data point towards a functional link between MMCs and NGF, thus supporting the observations derived from other animal models or from IBS patients.

From these observations, it was considered of interest to further explore the role of NGF and its receptors in the colonic motor changes observed during the chronic oral OVA exposure in rats.
HYPOTHESES AND AIMS

Taking into consideration the background exposed, the working HYPOTHESES of the present study are:

(i) Colonic MMCs are involved in the changes in NGF expression observed during the chronic oral exposure to OVA in rats and might represent a source of NGF.

NGF is increased in the colonic mucosa of IBS patients and part of the cells showing NGF immunoreactivity are MMCs (2). However, the observations made in animal models of the disease are disparate, showing both mast cell-dependent and -independent variations in the content of NGF (5; 47). The chronic oral OVA exposure model seems appropriate to investigate these questions since, as mentioned, it also shows changes in mast cells and NGF expression (54).

(ii) OVA-induced colonic motor alterations implicate NGF-dependent mechanisms.

Previous studies demonstrated functional and morphological effects of treatment with anti-NGF antibodies in relevant animal models of IBS. In particular, our group has demonstrated that immunoneutralization of NGF in Trichinella spiralis-infected rats prevented most of the intestinal motor abnormalities induced by the nematode (53).

From these hypotheses, the AIMS of the present study are:

(i) To further characterize the alterations of colonic motility associated to chronic oral OVA exposure in rats.
(ii) To investigate the implication of NGF in the development of OVA-induced motor changes by the use of the alkaloid K252a, an antagonist of the high-affinity NGF receptor TrkA.

(iii) To determine, in the same animal model, the colonic expression of NGF and its possible relation with MMCs.
MATERIALS AND METHODS

Animals

Adult (5 week-old at arrival), specific pathogen free (SPF), Sprague-Dawley (SD) male rats were used (Charles River, Les Oncins, France). Animals had free access to water and a standard pellet diet that did not contain any trace of OVA or any other egg derivative (A04; Safe, Augy, France). During all the experiment, rats were maintained under conventional conditions in a light (12h/12h light-dark cycle) and temperature controlled (20-22ºC) room, in groups of two per cage. Animals were acclimatized to the new environment for 1 week before starting any experimental procedure. All the experimental protocols were approved by the Ethical Committee of the Universitat Autònoma de Barcelona.

Experimental protocols

Oral exposure to ovalbumin (OVA) and treatment with K252a

Rats received OVA by oral gavage (1mg/mL, 1 mL/rat, n=16). Treatment was performed daily during a 6-week period (43). A group of rats receiving vehicle (1 mL/rat, n=13) was used as control (Unexposed). After the third week, six of the animals receiving OVA and five animals receiving vehicle were treated subcutaneously with K252a (50 µg/kg). Treatment with K252a was performed daily for 4 weeks. The remaining of the rats (10 exposed animals and 8 unexposed) were used as control groups in which the treatment protocol was the same but K252a was replaced by the corresponding vehicle (1 mL/kg, sc). Animals were euthanized by decapitation 10 days after the end of the OVA exposure period. Tissue samples from colon were removed and either fixed in 4 % paraformaldehyde for immunohistochemical studies or frozen in liquid nitrogen and stored at -80ºC until analysis.

Organ bath

Full thickness preparations were obtained from the mid portion of the colon, cut 1 cm long and 0.3 cm wide and hung to record circular muscle activity using a standard
organ bath system. Strips were mounted under 1 g tension in a 10-mL muscle bath containing carboxenated Krebs solution (95% O₂ – 5% CO₂) maintained at 37 ± 1°C. The composition of Krebs solution was (in mmol/L): 10.10 glucose, 115.48 NaCl, 21.90 NaHCO₃, 4.61 KCl, 1.14 NaH₂PO₄, 2.50 CaCl₂, and 1.16 MgSO₄ (pH 7.3–7.4). One strip edge was tied to the bottom of the muscle bath using suture silk and the other one to an isometric force transducer (Harvard VF-1 Harvard Apparatus Inc., Holliston, MA, USA). Output from the transducer was fed to a PC through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain). Strips were allowed to equilibrate for about 1h. After this period, contractile responses to carbachol (CCh; 0.1 - 10 µM) and the NO inhibitor NG-nitro-L-Arginine (LNNA; 1 mM) were assessed. For CCh, cumulative concentration-response curves, with a 5 min interval between consecutive doses, were constructed. For LNNA, spontaneous activity was recorded during a 10-minutes period after the addition of the drug.

To determine spontaneous contractile activity, the preparation tone was measured for 15 minutes and the mean value (in g) determined. To determine the effects of CCh, the maximum peak from the basal tone was measured after each concentration tested. To measure the response to LNNA, the 10 minutes mean of the strip tone before the drug administration was compared with the 10 minutes mean of the strip tone after the administration.

**Immunohistochemistry (IHC)**

Immunodetection of RMCP2 and NGF was carried out on paraformaldehyde-fixed colonic samples using a monoclonal antibody anti-RMCP2 (Moredun Animal Health, Edinburgh, UK) and a polyclonal rabbit anti-NGF (ab1526; CHEMICON International, Temecula, USA). Antigen retrieval for NGF was achieved by processing the slides in a pressure cooker at full pressure, for 10 minutes, in 10 mM citrate solution. The secondary antibodies included biotinylated horse antimouse IgG (BA-2000; Vector Laboratories, Burlingame, CA, USA) and biotinylated swine antirabbit Ig (E0353; DAKO, Carpinteria, CA, USA), as appropriate. Detection was performed with avidin/peroxidase kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) and counterstaining with haematoxylin. Specificity of the staining was confirmed by omission of the primary antibody. When performing IHC for NGF, mouse submaxillary
glands were used as a positive control. Slides were viewed with an Olympus BH-2 microscope. For MMC quantification, at least 20 non-adjacent X40 fields of colonic mucosa were randomly selected and the number of RMCPII-immunopositive cells counted manually. Procedures were carried out using coded slides to avoid bias.

**ELISA**

Protein was extracted from colonic tissue samples using lysis buffer (50 mM HEPES, 0.05 % Triton X-100, 0.0625 mM PMSF and the Mini Complete protease inhibitor Roche) and RMCPII concentration was determined by ELISA using a commercial kit (Moredun). Total protein was determined using the Bradford assay kit (BIO-RAD, Hercules, CA, USA).

**Chemicals**

OVA (OVA Grade V; A5503) was purchased from Sigma-Aldrich (St.Louis, MO, USA) and dissolved in saline solution. K252a (Tocris Bioscience, Ellisville, MO, USA) was reconstituted in 8.75 % ethanol in milli-q water. LNNA and CCh (Sigma-Aldrich) were dissolved in distilled water as stock solutions.

**Statistics**

All data are expressed as mean ± SEM. Results from the organ bath are presented as raw data (g of force). EC50 for CCh was calculated by non-linear regression to a sigmoidal equation (GraphPad Prism 4.01, San Diego, California, USA). Statistical analysis was performed with one-way or two-way ANOVA, as appropriate, followed, when necessary, by a post hoc Newman-Keuls test. Differences between groups were considered statistically significant when P < 0.05.
RESULTS

Colonic mucosal mast cell count

MMCIs were clearly identified as cells located in the mucosa and submucosa of the colon, with an irregular, brown-stained, cytoplasm (Fig. 1). No staining was observed in other areas of the colon. Omission of the primary antibody resulted in the total absence of immunoreactivity. The density of MMCIs was relatively low in control conditions (3.8±0.5 cells/field), in agreement with that previously described in similar experimental conditions (55). Oral exposure to OVA increased by 40% the number of colonic MMCIs, although statistical significance was not reached (Fig. 1). Treatment with K252a did not affect the density of MMCIs in control conditions or the increase observed during OVA exposure (Fig. 1).

Figure 1  A: Photomicrographs showing RMCPII-immunopositive cells in colonic mucosa (X400). A.1: Control rat. A.2: OVA-treated rat. Note the higher number of positive mast cells in the OVA-treated compared with the unexposed rat. B: Density of MMCIs in the different experimental groups. Data are mean ± SEM; n=2-8 per group (ANOVA).
Colonic RMCPII content

Colonic RMCPII content was low in control conditions and levels were not affected by treatment with K252a (Fig. 2). OVA exposure increased RMCPII levels by 65% when comparing the control-control and the OVA-control groups, and by 60% when comparing control-K252a and the OVA-K252a groups (Fig. 2). Overall, a two-way ANOVA analysis revealed an OVA effect (P=0.022), although statistical significance among individual groups was not achieved.

![Figure 2](image)

**Figure 2** RMCPII content in colonic extracts from the different experimental groups. Data are mean ± SEM; n=5-10 per group. #: P=0.075 vs. control-control (ANOVA).

Colonic spontaneous contractility

Spontaneous contractile activity was similar in vehicle- and OVA-exposed animals (control: 0.53±0.06 g; OVA: 0.51±0.03 g; P>0.05; Fig. 3). Treatment with K252a decreased spontaneous activity in OVA-exposed animals (0.35±0.05 g, P<0.05 vs. OVA) and similar tendency was observed in the vehicle-treated group (0.40±0.05 g; P=0.07 vs. control; Fig. 3).
Contractile responses to carbachol

In control conditions, CCh elicited a concentration-dependent contractile response with an estimated EC$_{50}$ of $8.7 \times 10^{-4} \pm 4.5 \times 10^{-4}$ mM (Fig. 4). OVA exposure significantly increased the responses to CCh, leading to a right-shift of the concentration-response curve and an estimated EC$_{50}$ of $1.7 \times 10^{-4} \pm 4.1 \times 10^{-5}$ mM (P<0.05 vs. control; Fig. 4). Treatment with K252a did not affect the responses to CCh, neither in vehicle- nor in OVA-exposed animals (Fig. 4).

Contractile responses to LNNA

Overall, LNNA showed a tendency to increase spontaneous activity in all experimental groups. However, significance was only achieved in the OVA-control (basal: $0.5 \pm 0.1$ g, LNNA: $0.9 \pm 0.1$ g, P<0.05; Fig. 5). Treatment with K252a completely prevented LNNA-induced hyperactivity in OVA-treated animals (Fig. 5). In these conditions, effects of LNNA were similar as those observed in the groups not exposed to OVA (Fig 5).
Figure 4  Contractile responses to carbachol. A: Concentration-response curves. Note that oral exposure to OVA leads to a right-shift of the concentration-response curve, an effect not modified by treatment with K252a. B: Estimated EC₅₀ for the different experimental groups. Data are mean ± SEM; n=5-10 per group. *: P<0.05 vs. respective control (ANOVA).
Figure 5  Effect of NO blockade with LNNA on spontaneous contractility in the different experimental groups. Data represent the change in spontaneous activity over the control period (before LNNA addition) and are mean ± SEM; n=5-10 per group. *: P<0.05 vs. other experimental groups (ANOVA).

Immunohistochemistry for NGF

Within the colon, immunoreactivity for NGF was detected in the submucosal and myenteric plexuses (Fig. 7). The epithelium and some crypts also showed a diffuse staining. Within de villi, there were scarce cells, of undetermined type, showing NGF-like immunoreactivity (Fig. 7). No labelling was detected in the muscle layers. No differences in the staining pattern or intensity were observed between OVA- and vehicle-treated animals or associated to the treatment with K252a (Fig 7). All immunoreactivity was absent in sections in which the primary antibody was omitted, thus confirming the specificity of the staining. Staining was intense and well localized in positive controls from mouse submaxillary glands.
Figure 6. Photomicrographs showing NGF-like immunoreactivity in colonic tissues. A: Control-control rat. B: OVA-control. C: Control-K252a. D: OVA-K252a. E: Negative control (omission of the primary antibody). Labelling for NGF is detected in the submucosal and myenteric plexuses, epithelium and crypts, and in scarce, unidentified cells within the villi. Note that no differences in the staining pattern or intensity are observed between OVA- and vehicle-treated animals or associated to the treatment with K252a.
DISCUSSION

This study shows that oral exposure to OVA in rats induces a moderate MMC infiltration in the colon, increases colonic MMC activity, as assessed by tissue levels of RMCPII, and causes colonic motor alterations. In addition, we also show evidence that NGF is implicated in colonic spontaneous motor activity and mediates some of the motor alterations induced by OVA.

As we have previously demonstrated, chronic exposure to oral OVA in rats leads to colonic dysfunction in the absence of any systemic allergic response, as revealed by the absence of circulating anti-OVA IgEs or IgGs (55). In addition, oral exposure to OVA does not affect the number of eosinophils in the mucosal intestine or IL-4 concentration in serum. Therefore, the mechanism by which OVA, or OVA digested fragments, is able to recruit and stimulate MMCs is Ig-independent. Hence, chronic oral exposure to OVA in rats represents a good model mimicking the condition of patients with symptoms of IBS who suffer from food hypersensitivity not mediated by intestinal mucosal mechanisms involving IgEs (23).

The results of this study confirm that OVA increases RMCPII levels within the colonic wall, as an indicator of MMC activity. This is in agreement with data obtained in colonic biopsies from IBS patients in which the number of degranulating MMCs was significantly increased with respect to healthy subjects (3). In parallel to the enhancement of RMCPII levels, we observed a moderate increase in the number of MMCs in the colon of rats exposed to oral OVA, similarly to that described previously in the same model (55). At this point, it should be mentioned that authors differ when it comes to MMC hyperplasia in the colon of IBS patients. While Barbara et al. observed a major MMC density in IBS patients with respect to healthy controls (3), Cenac et al. found no differences (12). However, both studies demonstrated an increase in the amount of tryptase, a MMC mediator, released from IBS biopsies, thus suggesting higher levels of MMC activation and degranulation. In animal models of the disease, and in particular in the neonatal maternal deprivation model, opposite results, as it relates to colonic MMC hyperplasia, have also been obtained in two different studies (5; 56). Nevertheless, both studies demonstrated the importance of MMC degranulation in
the onset of visceral hyperalgesia, one of the main symptoms of IBS. Our results support these observations. Although we did not observe a significant MMC hyperplasia associated to OVA exposure, tissue levels of RMCPII were increased, indicating a higher degree of mast cell activation in OVA-treated animals. Overall, these observations support a role for MMC mediators in the colonic secretomotor and sensory abnormalities that characterize IBS.

One of the most common symptoms that characterize IBS is altered colonic motility (27). The results of our study clearly show that rats exposed to OVA have colonic motor alterations. As previously described in this model (55), contractile responses to CCh were enhanced, thus indicating an increased excitability of the muscle to the cholinergic stimulation, which represents the major excitatory neurotransmitter within the ENS. Histological examination of the tissues excludes a muscle hypertrophy as a potential cause for this hyperresponse (data not shown). Interestingly, these increased excitatory responses are associated also to an enhanced NO-dependent inhibitory tone, as suggested by the increase in spontaneous contractility observed during the blockade of NO production with LNNA. Previous studies evidenced that rat’s colonic spontaneous motility \textit{in vivo} is mediated by neuronal release of acetylcholine and partially suppressed by the constitutive release of NO (32). Hence, seems feasible to speculate that the OVA-induced increase in the nitrergic inhibitory tone might represent a mechanism to compensate the enhancement of cholinergic excitatory responses.

A possible explanation for this OVA-induced increase in cholinergic excitability could be related to an excited-activated state of MMCs, as suggested by the higher tissue concentration of RMCPII observed in OVA-treated animals. In fact, a previous study found a significant positive correlation between the colonic response to CCh and RMCPII content (55). Following the same line, there is evidence that mast cell mediators can be released in close proximity to the ENS causing neuronal excitability. For example, in small intestine submucosal neurons of milk-sensitized guinea-pigs, exposure to \textit{β}-lactoglobulin increased excitability in nerve cells including cholinergic neurons, an event that could be significantly reduced by the mast cell stabilizer ketotifen (35). In that study, the authors identified histamine, prostaglandins and leukotrienes as the mast cell mediators involved in the excitatory responses to \textit{β}-lactoglobulin. This is in agreement with another study that demonstrated that histamine
caused acetylcholine release in the guinea-pig ileum (42). However, ENS-mediated motor responses may also be elicited by other MMC mediators. For instance, Gao et al. demonstrated that mast cell tryptase evoked protease-activated receptor 2 (PAR-2)-dependent excitatory responses in guinea pig enteric neurons (19). Moreover, they also observed that part of the PAR-2 sensitive neurons expressed immunoreactivity for nitric oxide synthase, suggesting a modulation of the nitrergic inhibitory tone. Therefore, MMC mediators, including proteases, might also mediate direct changes in the nitrergic system, an effect that can not be excluded in the present study.

In animals models of IBS, both mast cell degranulation and NGF have been implicated in gut dysfunction and, indeed, NGF has been suggested to be mast cell derived (5; 6). For instance, NGF immunoneutralization reverses colonic hypersensitivity in rats with colitis or that have experienced neonatal maternal deprivation (5; 14; 56). Along the same line, our group has described that colonic NGF expression is increased by OVA exposure (54). In addition, we have found a positive correlation between the number of MMCs and the expression of NGF, thus suggesting a functional link between MMCs and the neurotrophin within the colon. In order to expand these observations to the protein level and to elucidate the colonic source of NGF, IHQ for the neurotrophin was assessed in colonic samples. Although we were able to see specific NGF staining, with similar patterns of distribution as those previously reported (63), we did not find any obvious differences in staining, intensity or distribution among the different experimental groups. Colonic immunoreactivity for NGF was localized mainly in epithelial cells at the mucosal surface and in the submucosal and myenteric plexuses, in agreement with previous observations (25; 47; 63). In colonic biopsies of patients with functional and inflammatory gastrointestinal disorders, NGF immunoreactivity has been localized in MMCs (2; 15). In spite of that, we have been unable, so far, to detect NFG immunoreactivity in MMCs during double labeling studies (data not shown). These results might look unexpected given that we had previously found a positive correlation between the density of MMCs and NGF expression, as assessed by conventional PCR (54). However, a recent study assessing changes in colonic NGF content in a colitis model in rats found evidence for the presence of a higher molecular weight form of the neurotrophin, probably a proNGF, in isolates from cultured mast cells (47). The authors showed the expression of a 13 kDa NGF-like protein (corresponding to the mature form of the neurotrophin) in the inflamed mucosa and, by
PCR from laser microdissected cells, evidenced that NGF was principally synthesized by epithelial cells. However, neither lamina propria cells nor cultured MMCs expressed mRNA for NGF or produced the 13 kDa NGF form. Therefore, the authors suggested that MMCs could represent a source of the NGF precursor, although the conditions of its release and further processing to the final mature form are still unknown. Studies on cultured rat peritoneal mast cells support this hypothesis since cells extracts contained 73 kDa NGF immunoreactive-like species but not the mature form of the peptide, thus suggesting that mast cells are a source of NGF precursors (46). Hence, although not confirmed immunohistochemically, the positive correlation between the density of MMCs and the expression levels of NGF could represent a true functional link between the production of the neurotrophin in the gut and MMCs. Further studies are needed to clarify the potential role of MMCs on the enhanced NGF levels in OVA-exposed rats.

In this study, we aimed also to elucidate the functional implication of NGF in the colonic motility alterations characteristic of the model. A role for NGF on gastrointestinal motor alterations in IBS models has been previously described in rats infected with *Trichinella spiralis* in which anti-NGF treatment completely blocked the development of spontaneous hypermotility in the small intestine (53). In order to study the involvement of NGF in OVA-induced colonic motor dysfunction in rats, we used a pharmacological approach based on the blockade of NGF receptors with K252a. K252a is an inhibitor of protein kinase C and cyclic nucleotide-dependent kinases that functions as an antagonist of the high-affinity NGF receptor TrkA (28). For instance, K252a was effective inhibiting NGF-induced neuritic outgrowth in PC12 cells in vitro (30) and NGF-mediated effects in different in vivo models (39; 62).

In our conditions, treatment with K252a decreased spontaneous colonic motility and prevented the enhancement of the nitric inhibitory tone induced by oral exposure to OVA, thus indicating a role for the neurotrophin in the regulation of colonic motility. It is feasible to speculate that a tonic NGF-dependent stimulation might be necessary to maintain the ENS at optimal functional and morphological conditions. Therefore, the persistent blockade of NGF receptors, as performed here, might lead to changes in the regulatory mechanisms implicated in normal motor activity or the alterations induced by OVA. It has been demonstrated that NGF not only produces structural responses in neurons but also functional changes as there are substantial evidences that this
neurotrophin regulates neuropeptides synthesis and release. Rat myenteric neurons express the high-affinity receptor TrkA (33) and it has to be taken into consideration that NGF has been reported to significantly enhance neurite arborizations and neuronal sprouting in cell cultures (2; 64). Lindsay et al. reported that NGF regulated the expression of mRNAs encoding the precursors of substance P and calcitonin gene-related peptide (CGRP) in adult dorsal root ganglion neurons (34). Moreover, NGF immunoneutralization significantly reduced the colonic content of CGRP in rats (40). Therefore, we can hypothesize that K252a is binding to TrkA receptors of enteric neurons preventing NGF-mediated functional and/or structural effects within the ENS. However, it remains unclear why enhanced CCh responses during OVA exposure are not affected by K252a treatment since enteric cholinergic neurons also express TrkA receptors (33). A possible answer could be that OVA-induced dysmotility implies the recruitment of additional NGF/TrkA-independent mechanisms.

The dose of K252a used here is similar to that used in different studies showing biological effects in vivo, thus suggesting and effective blockade of the receptors (39; 62). A recent study demonstrated that treatment with K252a or with TrkA antisense oligonucleotides blocks in similar manner chronic stress-induced visceral hypersensitivity to colorectal distension in rats (63), indicating that the biological effects of K252a are associated to the selective blockade of TrkA receptors. However, K252a might bind to other Trk receptors present in the ENS (60). Therefore, it cannot be ruled out that the effects observed might be, at least partially, associated to the blockade of other neurotrophins receptors. Nevertheless, the similar effects obtained during the pharmacological blockade with K252a, the treatment with TrkA antisense oligonucleotides and the direct immunoneutralization of NGF (63) suggest that NGF-mediated effects within the gastrointestinal tract are predominantly mediated through TrkA-dependent mechanisms.

Expression of TrkA receptors within the gut is not only restricted to the ENS. Preliminary studies of our group suggest that lamina propria cells from rat colon express TrkA receptors and the majority of these cells are also RMCPII-immunoreactive, thus being positively identified as MMCs (data not shown). These data are in agreement with studies that demonstrate that NGF is able to degranulate mast cells (36). Therefore, another mechanism of action of K252a could be preventing
MMC degranulation. Since during the degranulation process mast cells might release potential precursor forms of NGF (46), treatment with K252a would diminish the neurotrophin availability within the colon. As a consequence, direct effects of K252a on TrkA receptors on enteric neurons would be boosted by the reduced NGF availability. In addition, apart from NGF, the release of other MMC mediators would also be reduced, leading to a state of diminished interaction with the ENS.

In summary, this study shows that oral exposure to OVA increases colonic MMC density and degranulation and causes alterations of colonic motor activity. Although we had previously demonstrated that OVA exposure results in an increase in colonic NGF expression, immunohistochemical studies did not reveal any effect of OVA exposure on NGF immunoreactivity. Nevertheless, pharmacological studies using the TrkA antagonist K252a suggest an involvement of NGF in OVA-induced colonic motor alterations. Overall, our study highlights a potentially important role for NGF-dependent mechanisms on colonic motor alterations associated to inflammatory and functional disorders of the gut. Therefore, NGF receptors could represent a therapeutic target for the treatment of gastrointestinal disorders characterized by altered motility.
CONCLUSIONS

In conclusion, the results from this study show that:

i) Colonic MMC density and activity, as assessed by RMCPPII content, is increased by oral exposure to OVA.

ii) Chronic exposure to oral OVA causes colonic motor alterations characterized by increased cholinergic responses and NO-dependent inhibitory tone. MMC mediators seem to play a pivotal role on these motor alterations.

iii) Spontaneous colonic contractile activity in the rat depends, at least partially, on NGF/TrkA pathways.

iv) NGF/TrkA-dependent mechanisms mediate some of the colonic motor alterations associated to the OVA model. The blockade of TrkA receptors might have a direct effect on enteric neurons and/or prevent the release of MMC mediators to the ENS, thus affecting colonic motility.
PERSPECTIVES

From the results obtained in this study, follow-up studies should further explore:

i) If OVA exposure is indeed associated to an increase in colonic NGF content. So far, at the mRNA level there seems to be an increase in the expression of colonic NGF. However, we have been unable to confirm this using immunohistochemistry. Other molecular tools, namely Western-blot, might be of help determining if mRNA changes are translated at the protein level. These techniques would also allow to assess the molecular forms of NGF (precursors vs. mature form) present in the colon.

ii) The potential role of MMCs as a cellular source of NGF in the colon. A combination of double immunofluorescence (for RMCPII and NGF) and PCR detection of mRNA for NGF in MMCs isolated through laser capture microdissection might be of help answering this question.

iii) The presence of phenotypic changes in the colonic innervation of OVA-treated rats. The expression of different neuronal markers, such as neuronal specific enolase (NSE) and growth-associated protein 43 (GAP-43), can be used to assess OVA-induced neuroplastic changes in the colon, particularly at the level of the mucosa (assessing the density of nerve terminals and their proximity to MMCs) and the myenteric plexus.

iv) If chronic oral OVA exposure results in other functional alterations besides dysmotility. It might be of particular interest to assess changes in visceral sensitivity. Visceral hyperalgesia to colorectal distension is one of the hallmarks of IBS and is a representative surrogate marker in most of the animal models of the disease. Both NGF and MMCs have been implicated in sensory responses leading to visceral hyperalgesia. These studies would increase the validity of the OVA exposure model and further characterize the involvement of NGF and MMCs in visceral sensitivity.
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