Effects of nerve growth factor antagonist K252a on peritoneal mast cell degranulation: implications for rat postoperative ileus

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Berdún S, Rychter J, Vergara P. Effects of nerve growth factor antagonist K252a on peritoneal mast cell degranulation: implications for rat postoperative ileus. Am J Physiol Gastrointest Liver Physiol 309: G801–G806, 2015. First published September 24, 2015; doi:10.1152/ajpgi.00152.2015.—Stabilization of mast cell (MC) degranulation has been proposed to prevent postoperative ileus (POI). Nerve growth factor (NGF) mediates MC degranulation. The aim of the study was to evaluate whether NGF receptor antagonist K252a acts as a MC stabilizer in vitro and in vivo model of POI. Peritoneal mast cells (PMCs) were obtained from Sprague-Dawley rats and were incubated with K252a and exposed to NGF or Compound 48/80 (C48/80). MC degranulation was assessed by β-hexosaminidase assay. POI was induced in rats by intestinal manipulation (IM). Rats were pretreated with K252a (100 μg/kg sc) 20 min prior to POI induction. At 20 min after IM, release of rat mast cell protease 6 (RMCP-6) was evaluated in peritoneal lavage. At 24 h, intestinal transit (IT) and gastric emptying (GE) were evaluated. Ileal inflammation was assessed by myeloperoxidase (MPO) activity, expression of IL-6, NGF, TrkA, RMCP-2 and 6, and MC density within the full-thickness ileum. C48/80 and NGF evoked degranulation of PMCs in a dose-dependent manner. K252a prevented NGF-evoked, but not C48/80-evoked, MC degranulation. IM evoked the release of peritoneal RMCP-6 and subsequently delayed IT and GE. IM increased MPO activity and expression of IL-6. In IM rats, K252a prevented upregulation of IL-6 expression and reduced TrkA, IT, GE, and inflammation were not affected by K252a. K252a inhibited NGF-evoked degranulation of PMCs in vitro. In vivo, K252a decreased IL-6 and PMC degranulation. This may be of relevance for the development of new therapeutic targets for POI.

postoperative ileus; nerve growth factor; mast cells; gastrointestinal motility

POSTOPERATIVE ILEUS (POI) is a motility disorder characterized by general hypomotility of the gastrointestinal (GI) tract evoked by intestinal manipulation (IM) during major abdominal surgery. POI leads to postoperative discomfort and prolonged hospitalization. Although many advances have been made in the perioperative management of patients, such as minimal invasive surgery or epidural local anesthetics, there is a lack of etiologic treatments and therapeutic targets (14).

A major event evoked by IM during abdominal surgery is the activation of peritoneal mast cells (PMCs), observed in mice and humans (2, 3, 7, 23, 26), suggesting a role of mast cells (MCs) in the pathogenesis of POI. In addition, mast cell-deficient knockout mice suffer from a less severe POI, and MC-stabilizing drugs such as ketotifen and doxantrazole prevent POI in mice (3). Moreover, ketotifen improves delayed gastric emptying in patients undergoing abdominal surgery, although it does not successfully ameliorate POI (27). In contrast, a recent study using new Cpa3Cre/+ mast cell-deficient mice reported that degranulation of PMCs plays no role in murine POI (7). Based on these studies, the importance of MCs in the development of POI is controversial and further research needs to be conducted to clarify whether new strategies to block MC activity might become useful to prevent POI.

Nerve growth factor (NGF) is a member of neurotrophin family exerting its actions mainly through the high-affinity NGF receptor, tropomyosin receptor kinase A (TrkA). TrkA activation by NGF leads to MC migration, activation, or degranulation, which can be antagonized by the specific TrkA receptor antagonist K252a (13, 17, 22, 24). Furthermore, inhibition of the NGF-TrkA pathways has been shown to prevent alterations of motility in models of inflammatory bowel syndrome (9, 28). Thus the TrkA receptor presents a potential target to modulate MC mediated inflammation and to normalize intestinal motility under inflammatory conditions.

We therefore investigated the effect of K252a as a potential antagonist of MC degranulation both in vitro and in an in vivo rat model of POI. The effect of treatment with K252a was evaluated by the following criteria: 1) intestinal transit and gastric emptying; 2) myeloperoxidase activity and interleukin-6 expression to determine intestinal inflammation in the manipulated intestine; 3) release of rat mast cell protease-6 (RMCP-6) in peritoneal lavage; 4) number and protease expression of intestinal, wall-resident MCs, and 5) gene expression of NGF and TrkA.

MATERIALS AND METHODS

Animals. Seven- to 8-wk-old (285–350 g) male Sprague-Dawley rats (from the Animal House facility of the Universitat Autònoma de Barcelona) were kept under conventional conditions in an environmentally controlled room (temperature: 20–22°C; photoperiod: 12:12-h light-dark cycle). Animals had free access to tap water and standard commercial pellet diet (2014 Harlan Maintenance). Animals were caged in groups of two to three upon arrival and allowed to acclimate to the new environment for 5 days. All experimental procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (code 2478) and the Generalitat de Catalunya (code 7974).

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**Collection of peritoneal mast cells and in vitro activation with NGF.** To obtain PMCs, animals were euthanized by CO₂ inhalation, and 15 ml of PBS was injected into the peritoneal cavity through a small incision in the abdominal wall. After 2 min of gentle abdominal massage, 10 ml of the peritoneal lavage was extracted with a syringe. The peritoneal lavage was centrifuged at 240 RCF (relative centrifugal force) for 10 min at 4°C. The pellet was resuspended in 1 ml of Hank’s balanced salt solution (H9394, Sigma-Aldrich, St. Louis, MO) supplemented with 11.25 μl of heparin (604754.0, Hospira, Madrid, Spain), pipetted on top of a 1-ml histopaque layer (11191, Sigma-Aldrich), and centrifuged at 240 RCF for 10 min. The resulting pellet was resuspended in 2 ml DMEM F-12 medium (12634-010, Invitrogen, Madrid, Spain) to obtain an enriched suspension of PMCs. Cells were prepared accordingly (21) and then incubated for 90 min with NGF (1 ng to 10 μg/ml, N = 3) or Compound 48/80 (C48/80; 0.1 to 100 μg/ml, N = 3) dissolved in DMEM F-12. To determine whether K252a affects the degranulatory response of MCs, cells were preincubated for 20 min with K252a (1.0 to 200 nm) or carrier (DMSO, final concentration 0.01%) added as 1 μl to each well. NGF (N2513) and C48/80 (C2313) were purchased from Sigma-Aldrich. K252a was purchased from Tocris Bioscience (1683, Ellisville, MO).

**Assessment of mast cell degranulation in vitro by β-hexosaminidase assay.** β-Hexosaminidase release was determined as previously described (21) by using 7.5 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (N9376, Sigma-Aldrich) diluted in 0.2 M citrate buffer (pH 4.5). After incubation, absorbance was read at 460 and 560 nm by using a Victor-2 plate reader (PerkinElmer; Waltham, MA).

**Prophylactic treatment of rats with K252a.** Rats received an injection of K252a (100 μg/kg, 1 ml sc) or vehicle (8% ethanol, 1 ml sc) 20 min prior induction of the POI model: Sham-vehicle (N = 5), IM-vehicle (N = 5), Sham-K252a (N = 5), IM-K252a (N = 5). Dose and route of administration have been previously shown to have biological effects through TrkA inhibition (18). Peritoneal lavage was collected 20 min after IM and 50 min after K252a administration, whereas GI motility test, euthanasia, and sampling were performed 24 h after induction of the model.

**Induction of postoperative ileus.** Rats were anesthetized by inhalation of isoflurane (4% for induction and maintenance, respectively; IsoFlo, Esteve Veterinaria, Barcelona, Spain). Animals were prepared for sterile surgery. Small bowel and cecum were carefully externalized onto soaked sterile gauzes. Distal jejunum, ileum, cecum, and proximal colon were manipulated for 10 min with sterile cotton swabs. Abdominal wall and skin were closed by performing a reverbid and subcuticular pattern, respectively (Safil 3/0, B. Braun Vetcare, Rubi, Spain). A single dose of buprenorphine (0.05 mg/kg sc, Buprex, Schering-Plough, Berkshire, UK) was used for analgesia. No mortality or signs of clinical infection were observed during the postoperative period.

**Collection of peritoneal lavage.** Peritoneal lavage was collected 20 min after IM as following: during surgery, a syringe was inserted through the laparotomy and 7 ml of sterile 0.9% NaCl solution was injected into the peritoneal cavity. After 20 min of exposure, 3.5 ml of the solution were extracted from the peritoneal cavity. A cocktail of protease inhibitors (P8340, Sigma-Aldrich) was added to the sample (1:100) to prevent protein degradation by endogenous proteases. Samples were then centrifuged at 240 RCF for 10 min at 4°C. Supernatants were stored at −80°C till further analysis.

**Assessment of mast cell mediator release in peritoneal lavage.** Mediator release of PMCs was measured by detecting extracellular RMCP-6 in surgical lavage. For this purpose, a colorimetric assay was developed by using a specific anti-RMCP-6 antibody. Appropriate controls omitting samples and primary and/or secondary antibodies were also performed to validate the assay. Peritoneal supernatant samples were diluted in carbonate coating buffer (1:2, Na₂CO₃ : NaHCO₃, pH 9.6) and incubated onto microplates (Nunc Maxisorp flat bottom 96-well plates, 300 μl maximum volume, 44-2404-21) overnight at 4°C. After nonspecific binding of antibody was blocked, wells were incubated with a 1:1,000 dilution of primary polyclonal antibody for rat RMCP-6 (32473, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 37°C. Donkey anti-goat IgG conjugated to horseradish peroxidase (1:200, 1 h at 37°C, 2020, Santa Cruz Biotechnology) was used as a secondary antibody. Colorimetric reaction was developed by using TMB substrate solution (3,3',5,5'-tetramethylbenzidine, T0565, Sigma-Aldrich). Absorbance was read at 450 nm by use of a microplate reader (Infinite F200, Tecan).

**Functional studies: gastrointestinal motility.** Food was restricted to 11 g the night before motility was assessed (restriction of 55%). The following day, 1.5 ml of semiliquid and noncaloric phenol red solution (dissolved in 0.5% carboxymethylcellulose/distilled water) was given by oral gavage (with a round-shaped-ending metallic feeding tube). Rats were killed by CO₂ inhalation 1 h later. Stomach and small intestine were removed from the abdominal cavity. Stomach and small intestine (SI) were separated and the later was divided into nine equal, longitudinal segments (S1–S9). The phenol red concentration was determined in the stomach and individual intestinal segments as previously described (16). The absorbance of phenol red was read at 560-nm wavelength in a plate reader (Ultracomp 2000, Pharmacia Biotech).

**Gastric emptying (GE).** was expressed as percentage of phenol red found in intestinal segments 1 to 9 referred to the total amount of phenol red found in the whole GI tract (including stomach).

**Intestinal transit was expressed as the geometric center (GC), indicating the intestinal segment where phenol red was mainly located (23).**

**Sampling.** Immediately after euthanasia (see previous section), full-thickness samples of distal ileum were removed and fixed in 4% paraformaldehyde in phosphate buffer overnight and paraffin embedded or snap frozen in liquid nitrogen and stored at −80°C until analysis.

**Assessment of mast cell density in the ileum.** Transverse tissue sections (5 μm) of distal ileum were cut on a microtome from paraffin blocks and routinely processed for staining.

**Toluidine blue was used for staining of connective tissue mast cells (CTMCs).** Tissue sections were stained with 1% toluidine blue (pH = 7) for 20 min. Samples were then dehydrated and mounted in DPX medium.

**Immunohistochemistry for RMCP-2 was used for identification of mucosal mast cells (MMCs) by using primary monoclonal anti-rat RMCP-2 (1:500, overnight at 4°C, Moredun Animal Health, Edinburgh, UK) and biotinylated horse anti-mouse IgG (1:200, 1 h at room temperature, BA-200; Vector Laboratories, Burlingame, CA) as a secondary antibody.** The technique employed has been previously described in a recent paper (2). RMCP-2-positive and toluidine blue-stained cells were quantified in a blinded fashion under light microscopy at ×40 magnification (Olympus CH 30, Tokyo, Japan).

**The number of positive cells was expressed as the mean of counted cells on 15 nonoverlapping high-power fields.**

**MPO assay.** Myeloperoxidase activity (MPO) activity was determined as previously described (15). Briefly, ileum samples were weighed and homogenized in hexadecyltrimethylammonium bromide buffer (1 ml/50 mg tissue) by using GentleMACS (130-093-235, Eflow, Esteve Veterinaria, Barcelona, Spain). Animals were prepared accordingly (21) and then incubated for 90 min with 1% H₂O₂. Absorbance of the reaction was read at 450 nm in a plate reader (Infinite F200, Tecan). Samples, blank and standard (M9698, Sigma), were assayed in triplicate. MPO activity was expressed as units of activity per milligram of tissue (U/mg).

**RT-qPCR.** For extraction of total RNA, full-thickness ileum samples were homogenized in TRI Reagent (Ambion, AM9738) using a homogenizer (IKA T10 Basic Ultra-Turrax). Sample homogenates were mixed with chloroform for RNA separation and then with isopropl alcohol for RNA
precipitation. After washing steps, RNA was quantified by NanoDrop (NanoDrop Technologies, Rockland, DE) and converted into 20-μl reaction volume of cDNA by using a High Capacity cDNA Reverse Transcription Kit according to manufacturer’s instructions (Applied Biosystems). Reverse transcription-quantitative real-time PCR (RT-qPCR) was performed by using validated TaqMan gene expression assays (Applied Biosystems) with hydrolysis probes: interleukin-6 (IL-6) (Rn01410330_m1), RMCP-2 (Rn01478347_m1), RMCP-6 (Rn00569857_g1), NGF (Rn01533872_m1), and TrkA (Rn00572130_m1). Gene expression was normalized to the reference expression of the housekeeping gene -actin (Rn00667869_m1).

Expression of -actin was not altered by IM or K252a treatment and was kept constant between the four experimental groups. PCR reaction mixture was incubated on the ABI 7900 HT Sequence Detection System (Applied Biosystems). Samples were assayed in duplicate. Data were analyzed by use of the Bio-Rad CFX Manager 2.1 software. Results were quantified by the 2^ΔΔCT method with respect to the expression of control groups and were expressed as fold change relative to the housekeeping gene expression.

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed with GraphPad Prism 6.0 software. Two-way ANOVA with Sidak’s multiple-comparison test was used for all groups. A Student’s t-test for independent samples comparing test dose with control group (incubation with DMEM F-12) was performed for data in Fig. 1. A P value less than 0.05 was considered significant.

RESULTS

Effects of NGF and K252a on activation of peritoneal mast cell in vitro. A 90-min incubation of PMCs with 10 μg/ml of NGF induced release of β-hexosaminidase (P < 0.05, Fig. 1A). C48/80 also induced a significant release of β-hexosaminidase at 10 and 100 μg/ml, respectively (P < 0.05, Fig. 1B). β-Hexosaminidase release evoked by 10 μg/ml of NGF was completely inhibited following preincubation with 200 nM K252a (P < 0.05, Fig. 1C). In contrast, K252a did not inhibit β-hexosaminidase release evoked by 100 μg/ml of C48/80 (Fig. 1D). K252a by itself did not induce any release of β-hexosaminidase (not shown).

Effect of IM and K252a on peritoneal mast cell activation in vivo. Rats undergoing IM presented higher peritoneal release of RMCP-6 compared with sham groups (P < 0.05, Fig. 2), thus indicating that IM induces mast cell degranulation in

Fig. 1. β-Hexosaminidase release from peritoneal mast cells incubated with NGF (1–10,000 ng/ml) (A) and C48/80 (0–100 μg/ml) (B) for 90 min. Preincubation with K252a (0–20 nM) for 40 min following incubation with 10,000 ng/ml NGF (C) or 100 μg/ml C48/80 (D). Data are expressed as percentage of total cellular content. Dots represent mean values of N = 3 (A and C) or N = 4 (B and D). Vertical lines represent SE. NGF, nerve growth factor; C48/80, compound 48/80. *P < 0.05, ***P < 0.01, and ****P < 0.0001 vs. basal group (Student’s t-test).
peritoneal lavage. Pretreatment with K252a reduced slightly but not significantly RMCP-6 release in IM animals compared with vehicle (Fig. 2). No differences were found between Sham- and IM-treated groups.

GI transit following IM. Postoperative ileus-like symptoms following IM were observed in our rat model as characterized by delayed intestinal transit ($P < 0.05$, Fig. 3A) and delayed gastric emptying ($P < 0.05$, Fig. 3B) vs. Sham-vehicle. K252a did not alter either basal intestinal transit or gastric emptying in Sham animals and did not prevent the IM-induced delay of GI transit and gastric emptying (Fig. 3, A and B). Two-way analysis revealed that IM is the major factor driving GI motility (76 and 59.19%, $P = 0.0001$).

Intestinal wall inflammation following IM and pretreatment with K252a. MPO activity was increased in the ileal wall after IM although was not considered significant ($P > 0.05$ vs. Sham-vehicle) (Fig. 4A). Two-way analyses revealed that at least 50% of observed variance is due to IM.

IM also increased the expression of IL-6 gene in the ileal wall compared with Sham group ($P < 0.05$). Treatment with K252a significantly reduced IL-6 upregulation in IM rats ($P < 0.05$ vs. IM-vehicle, Fig. 4B).

Mast cell distribution and density in intestinal wall following IM and K252a pretreatment. Only one to two CTMCs mainly located in the serosa were observed per slide. Furthermore, CTMC density was not altered by IM or K252a treatment. MMC density was also unaltered after IM (Sham-vehicle 7.84 ± 0.78 vs. IM-vehicle 6.6 ± 0.76 MMCs per ×40 field, $P > 0.05$) or K252a pretreatment (Sham-K252a 7.06 ± 1.06 and IM-K252a 8.50 ± 1.44 $P > 0.05$).

RMCP-6 and RMCP-2 gene expression in the ileal wall remained unchanged across the experimental groups.

NGF and TrkA gene expression. Intestinal manipulation or K252a treatment did not affect the expression of NGF within the wall of the ileum. However, K252a treatment reduced the expression of TrkA in both Sham- and IM-treated animals compared with IM-vehicle treated rats ($P < 0.05$, Fig. 5B).

**DISCUSSION**

In this study we demonstrate the ability of the tropomyosin-related kinase receptor antagonist K252a to inhibit NGF-evoked degranulation of PMCs in vitro. In addition, we demonstrate that K252a is able to block some of the functional consequences of IM, such as upregulation of intestinal IL-6 gene expression and mast cell degranulation, but it does not affect the motor inhibition observed in the POI model.

Previous studies demonstrated that NGF stimulates degranulation of PMCs (13, 20) possibly via the NGF high-affinity receptor TrkA (4, 9, 22, 25, 29). Our study confirms this by showing that stimulation of isolated PMCs with NGF evokes a degranulatory response. In addition, K252a prevented the NGF-evoked degranulation, without affecting the degranulation evoked by the MC degranulator C48/80 (13). C48/80 does not interact with membrane receptors but directly activates G protein αi subunit protein (1, 19). The fact that K252a did not prevent β-hexosaminidase release evoked by C48/80, could be explained by the different mechanism of action of both drugs and suggests that K252a blocking activity is specific to NGF-induced MC degranulation.

We next evaluated the MC stabilizing properties of K252a in vivo, in the rat model of POI on the intestinal inflammation and transit. In line with previous studies, IM in our model resulted in a rapid degranulation of PMCs, as judged by the peritoneal increase of the CTMC-specific product RMCP-6 (3, 7, 23, 26). Intestinal manipulation also resulted in a delay of the GI transit and produced inflammation of manipulated intestinal segments as previously reported (10, 11).

K252a pretreatment attenuated the release of RMCP-6 in vivo following IM, thus further demonstrating that K252a has MC stabilizing properties. Jardí et al. (9) demonstrated that MC express TrkA receptor. Taken together, these observations...
suggest that TrkA modulates MC activity and furthermore, that K252a could have a role for MC-mediated disorders.

Similarly, we also observed that K252a decreases IL-6 expression after IM. However, K252a did not prevent neutrophil activation after IM, as judged by tissue MPO activity. These findings suggest that, in our model, K252a is involved specifically in the inactivation of IL-6 secreted from intestinal resident cells including MCs or macrophages (8, 31), cell types with TrKA receptors, rather than newly recruited neutrophils. Therefore, TrKA receptors seem not to interfere with other mechanisms leading to migration of circulating neutrophils as observed in other models (18, 30). In fact, only mononuclear cells have been identified as the most prominent cellular source for IL-6 in the intestine after IM (12).

Our study also demonstrates that K252a effectively blocks NGF receptor since TrkA mRNA levels were decreased in treated rats. NGF-TrkA interactions promote overexpression of TrkA on cell surface and this may explain our finding in rats receiving K252a (6). This finding further corroborates that K252a inhibits MC activation as MCs express TrkA (9). In fact, we reported attenuation of RMCP-6 release into the peritoneal cavity after K252a pretreatment.

However, GI motility was not improved by K252a. This is in contrast to a previous study in a rat model of food allergy reporting modulation of colonic motility in animals treated with K252a on a daily basis for 4 wk (9). So we cannot confirm or discard an effect of K252a. We suggest that the lack of effect of K252a on motility in our study could be related to the fact that

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**Fig. 4.** Evaluation of inflammation in ileum specimens collected 24 h after induction of the model and following pretreatment with K252a or vehicle. MPO cellular infiltrate assessed as MPO activity (A) and gene expression of proinflammatory IL-6 (B). Data are expressed as means ± SE of N = 3–5. Dots represent individual values and vertical lines SE. A: P > 0.05 is the overall P value of the statistical test. B: *P < 0.05, **P < 0.01 (2-way ANOVA with Sidak’s multiple-comparison test). MPO, myeloperoxidase; IL-6, interleukin-6.

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**Fig. 5.** Gene expression of rat mast cell proteases RMCP-6 (A, left) and RMCP-2 (A, right) and nerve growth factor (NGF, B, left) and its receptor TrkA (B, right) in ileum 24 h after induction of the model and following pretreatment with K252a or vehicle. Data are expressed as means ± SE of N = 4–5. Dots represent individual values and vertical lines SE. *P < 0.05 (2-way ANOVA with Sidak’s multiple-comparison test).
K252a was given only 20 min prior to IM, which is possibly not enough time to completely prevent IM-induced hypomotility. In a similar way we cannot discard that K252a also acts on other cells expressing the TrkA receptor (5, 12). However, the fact that K252a specifically stabilizes mast cells allows us to conclude that blocking of TrkA receptors can be a target for MC stabilization and therefore be useful in those diseases where MCs are involved.

In conclusion, K252a attenuated PMC degranulation and upregulation of IL-6 induced after IM in a POI model in the rat, thus showing 1) a potential use for treatment of MC-mediated disorders and 2) an anti-inflammatory effect. Therefore, we suggest that K252a and TrkA receptor should be further explored in intestinal inflammatory disorders with concomitant activation of MCs.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.B., J.R., and P.V. conception and design of research; S.B. and J.R. performed experiments; S.B., J.R., and P.V. interpreted results of experiments; S.B., J.R., and P.V. prepared figures; S.B., J.R., and P.V. edited and revised manuscript; S.B., J.R., and P.V. approved final version of manuscript.

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