British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01296.x
www.brjpharmacol.org

RESEARCH PAPER

Central activation of the cholinergic anti-inflammatory pathway reduces surgical inflammation in experimental post-operative ileus

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Keywords
post-operative ileus; intestinal muscle inflammation; cholinergic anti-inflammatory pathway; semapimod; intracerebroventricular; dorsal motor nucleus; paraventricular nucleus; macrophages; mice

Received
7 February 2010
Revised
8 January 2011
Accepted
23 January 2011

BACKGROUND AND PURPOSE

Electrical stimulation of the vagus nerve reduces intestinal inflammation following mechanical handling, thereby shortening post-operative ileus in mice. Previous studies in a sepsis model showed that this cholinergic anti-inflammatory pathway can be activated pharmacologically by central administration of semapimod, an inhibitor of p38 mitogen-activated protein kinase. We therefore evaluated the effect of intracerebroventricular (i.c.v.) semapimod on intestinal inflammation and post-operative ileus in mice.

EXPERIMENTAL APPROACH

Mice underwent a laparotomy or intestinal manipulation 1 h after i.c.v. pre-treatment with semapimod (1 $\mu g \cdot kg^{-1}$) or saline. Drugs were administered through a cannula placed in the left lateral ventricle 1 week prior to experimentation. Twenty-four hours after surgery, gastric emptying was measured using scintigraphy, and the degree of intestinal inflammation was assessed. Finally, activation of brain regions was assessed using quantitative immunohistochemistry for c-fos.

KEY RESULTS

Intestinal manipulation induced inflammation of the manipulated intestine and significantly delayed gastric emptying, 24 h after surgery in saline-treated animals. Semapimod significantly reduced this inflammation and improved gastric emptying. Vagotomy enhanced the inflammatory response induced by intestinal manipulation and abolished the anti-inflammatory effect of semapimod. Semapimod but not saline induced a significant increase in c-fos expression in the paraventricular nucleus, the nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve.

CONCLUSIONS AND IMPLICATIONS

Our findings show that i.c.v. semapimod reduces manipulation-induced intestinal inflammation and prevented post-operative ileus. This anti-inflammatory effect depends on central activation of the vagus nerve.

Abbreviations

DMV, dorsal motor nucleus of the vagus nerve; i.c.v., intracerebroventricular; IL, interleukin; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus; TNF, tumour necrosis factor

Introduction

The vagus nerve plays a crucial role in the control of gastrointestinal (GI) function, including secretion, visceral perception and motility. In addition, there is strong evidence indicating that the vagus nerve modulates the innate immune system, as electrical stimulation of the vagus reduced levels of tumour necrosis factor (TNF)α and prevented arterial hypotension after endotoxin injection (Borovikova *et al.*, 2000b). Similarly, we have demonstrated that vagus nerve stimulation reduced the inflammatory response to mechanical manipulation of the intestine during surgery, thereby preventing surgery-induced delayed gastric emptying (De jonge *et al.*, 2005). This anti-inflammatory effect was mediated by acetylcholine (ACh) interacting with nicotinic (nACh) receptors (nomenclature follows Alexander *et al.*, 2009) located on macrophages, leading to a reduction in macrophage activation and cytokine production (Wang *et al.*, 2003). This so-called cholinergic anti-inflammatory pathway may represent an additional regulatory system controlling the inflammatory response to a wide range of threats to the organism. Inflammation is sensed by the brain via afferent nerve fibres or through cytokine signalling in the general circulation, and both routes are integrated in the nucleus of the tractus solitarius (NTS) (Tracey, 2007). After processing of this information, the motor neurons of the vagus nerve are believed to be activated, and an anti-inflammatory signal is sent back to the inflamed area (Tracey, 2007). The presence of such a feedback loop or reflex and its anatomical connections are still hypothetical and need to be demonstrated. Nevertheless, this may represent an interesting mechanism to control inflammation in a number of disorders. In contrast to anti-inflammatory cytokines and the hormonal control by corticosteroids [through the hypothalamic–pituitary–adrenocortical (HPA) axis], this control via the autonomic nervous system might provide an integrated response that is rapid and target-specific. Clearly, it may also provide new therapeutic means to control or dampen inflammation, not only in case of sepsis or ileus, but also in other inflammatory diseases like rheumatoid arthritis and inflammatory bowel diseases.

Semapimod, a tetravalent guanyl hydrazone, also known as CNI-1493, prevents macrophage activation via inhibition of mitogen-activated protein kinase (MAPK) signalling (Lowenberg *et al.*, 2005). Recently, Wehner *et al.* (2009) found that single systemic doses of semapimod prevented post-operative ileus via inhibition of macrophage activation. Conversely, while studying the effect of semapimod in cerebral ischaemia, Meistrell *et al.* (1997) found that central application of this drug could reduce systemic inflammation. Further studies revealed that semapimod, when infused intracerebroventricularly (i.c.v.), is up to 100 000 times more effective than after intravenous administration (Bernik *et al.*, 2002b). In addition, electrophysiological studies have shown enhanced activity of the vagus nerve after infusion of semapimod (Borovikova *et al.*, 2000a). These findings strongly suggest that in addition to systemic MAPK inhibition, semapimod also represents a pharmacological and centrally acting activator of the cholinergic anti-inflammatory pathway.

Animal studies on the pathogenesis of post-operative ileus have shown that gentle small bowel manipulation during abdominal surgery results in a distinct inflammation of the muscularis propria (Kalff *et al.*, 1999; de Jonge *et al.*, 2003). This local innate inflammatory response activates an adrenergic inhibitory neural reflex leading to generalized hypomotility or ileus (de Jonge *et al.*, 2003). Reduction of the inflammatory response by pre-treatment with intercellular adhesion molecule (ICAM)-1 inhibitory antibodies or antisense oligonucleotides normalizes gastric emptying (de Jonge *et al.*, 2003; The *et al.*, 2005), further illustrating the crucial role of this inflammatory process in the pathogenesis of post-operative ileus. We have shown earlier that both electrical stimulation of the vagus nerve (de Jonge *et al.*, 2005) and systemic administration of selective nACh agonists (The *et al.*, 2007) had an anti-inflammatory effect on surgery-induced intestinal inflammation, suggesting that activation of the cholinergic anti-inflammatory pathway indeed may represent an interesting approach to treat intestinal inflammation.

In the present study, we evaluated whether pharmacological activation of the vagus nerve by central application of semapimod also leads to reduced inflammation and prevention of ileus. In addition, we performed immunohistochemical analysis for c-fos in the brain stem to assess the involvement of the motor nucleus of the vagus nerve.

Methods

Animals

All animal care and experimental procedures were according to the guidelines of the Ethical Animal Research Committee of the University of Amsterdam and with their approval. Female Balb/C mice (Harlan Nederland, Horst, The Netherlands), age 12 to 15 weeks, were kept under environmentally controlled conditions (light on from 8:00 a.m. till 8:00 p.m., in ambient temperatures 20–22°C (55% humidity), with water and rodent non-purified diet provided *ad libitum*.

Study protocols

First, the efficacy of semapimod given i.c.v. was evaluated in our mouse model of post-operative ileus (de Jonge *et al.*, 2003). An i.c.v. cannula was placed in the left lateral ventricle of the brain 7 days prior to surgery, as described below. Sixty minutes before the surgical procedure, animals were treated with semapimod (1 $\mu g \cdot kg^{-1}$ i.c.v.) or its vehicle (saline) in a volume of 5 μL administered in 10 min, using an infusion pump (22 multiple syringe pump, Harvard Apparatus, Holliston, MA). Twenty-four hours after surgery, gastric emptying of a semi-liquid non-caloric test meal was determined using a scintigraphic imaging technique (Bennink *et al.*, 2003). After completion, mice were killed by cervical dislocation, and ileal segments (4–6 cm proximal to the caecum) were quickly excised for the assessment of intestinal inflammation.

In a different set of experiments, a sub-diaphragmatic bilateral vagotomy was performed 30 min prior to infusion of semapimod or vehicle to determine vagus nerve involvement. In this set of experiments, the inflammatory response, but no gastric emptying, was assessed, as the latter is impaired after vagotomy.

Finally, to identify the brain nuclei involved in the central activation of the cholinergic anti-inflammatory pathway,

c-fos expression was studied after i.c.v. treatment with semapimod or saline. A swivel equipped infusion pump was used to administer the drugs, allowing the animals to move freely in their usual environment. Swivel pumps were connected at 8 a.m. in all animals, and infusion was started only after 4 h to minimize stress-induced brain activity. Three hours after i.c.v. administration of saline or semapimod, mice were anaesthetized (see below for details) and were transcardially perfused (1.6 mL·min^{-1}) with 8 mL of a 0.9% NaCl solution, followed by 50 mL of 4% paraformaldehyde in phosphate buffer (0.1 mol·L^{-1}; pH 7.4). After perfusion, the brain, brainstem and proximal spinal cord were carefully removed, post-fixed overnight in the same fixative at 4°C and cryoprotected until further analysis in 30% sucrose solution containing 0.05% sodium azide at 4°C.

The anaesthetic procedure used in all the current study protocols was as follows; intraperitoneal (i.p.) injection of 10 mL·kg^{-1} of an anaesthetic solution containing 0.078 mg·mL^{-1} fentanyl citrate, 2.5 mg·mL^{-1} fluanisone (Hypnorm; Janssen, Beerse, Belgium) and 1.25 mg·mL^{-1} midazolam (Dormicum; Roche, Mijdrecht, The Netherlands).

Placement of the i.c.v. cannula

In anesthetized animals, a cannula (23 G needle) was stereotaxically implanted into the left lateral cerebral ventricle using the following coordinates from Bregma: 0.46 mm posterior, 1.0 mm lateral and 2.2 mm ventral. Dental cement was used to secure the cannula to three screws inserted into the skull.

Surgical procedure

Anesthetized mice underwent a laparotomy alone or a laparotomy followed by small intestinal manipulation as described previously (de Jonge *et al.*, 2003). In short, a midline incision was made, and the peritoneal cavity was opened along the linea alba under sterile conditions. The small intestine was carefully exteriorized from the distal duodenum until the caecum and gently manipulated for 5 min using sterile moist cotton applicators. Contact or stretch of stomach or colon was strictly avoided. After repositioning of the intestinal loops, the abdomen was closed using a two-layer continuous suture (Mersilene Softsilk 6-0, Ethicon, Somerville, NJ, USA). Mice recovered from surgery in a temperature-controlled cage set at 32°C with free access to water but not to food. Twenty-four hours after surgery, gastric emptying was measured. Thereafter, mice were anaesthetized and killed by cervical dislocation. The small intestine was removed, flushed in ice-cold phosphate-buffered saline (PBS) and snap frozen in liquid nitrogen or fixed in ethanol for further analysis.

Sub-diaphragmatic vagotomy

In anaesthetized mice, a midline incision was made, and a retractor was placed. Under microscopic view, both the left and right vagal nerve trunks were cut, distal from the diaphragm but proximal to the division of the hepatic branch. During this procedure, the i.p. organs were protected and kept moist using sterile gauze soaked in saline. Any palpation or manipulation of the small intestine was carefully avoided. The abdomen was closed using a two-layer continuous suture (Mersilene Softsilk 6-0). Animals were kept in a temperature-controlled cage at 32°C until drug infusion (30 min later) and surgery (60 min after drug infusion). Microscopic inspection and post-mortem evaluation of the stomach distention were utilized to determine a successful vagotomy procedure.

Measurement of gastric emptying

As previously described, gastric emptying rate was determined after gavage of a semi-liquid, non-caloric test meal (0.1 mL of 3% methylcellulose solution containing 10 MBq of ^{99m}Tc-Albures) (Bennink *et al.*, 2003; The *et al.*, 2005). Mice were scanned using a gamma camera set at 140 keV (Bennink *et al.*, 2003). The entire abdominal region was scanned for 30 s, immediately and 80 min after gavage. During the scanning period, mice were conscious and manually restrained. The static images obtained were analyzed using Hermes computer software (Hermes, Stockholm, Sweden). Gastric retention was calculated by determining the percentage of activity present in the gastric region of interest compared with the total abdominal region of interest (The *et al.*, 2005).

Assessment of intestinal muscle inflammation

Postmortem, the mesentery was removed from the intestine, which was cut open along its mesenteric border. Fecal content was washed out with ice-cold PBS and the tissue fixed in 100% ethanol for 10 min. Fixed preparations were kept in 70% ethanol at 4°C until further analysis. Before final analysis, segments were stretched 1.5 times to their original size and pinned down on a glass dish filled with 70% ethanol after which the mucosa was carefully removed. Tissues were stained for myeloperoxidase (MPO) as described below.

Staining for MPO

Fixed preparations were rehydrated by incubation in 50% ethanol and PBS, pH 7.4 for 5 min. To identify MPO-positive cells, intestinal preparations were incubated for 10 min with 3-amino-9-ethyl carbazole (Sigma, St. Louis, MO) as a substrate, dissolved in sodium acetate buffer (pH 5.0) to which 0.01% H_2O_2 was added (de Jonge *et al.*, 2003). For quantification, the number of MPO-positive cells in five randomly chosen 1 mm^2 fields was counted. Tissue sections were coded so that the observer was unaware of the surgical and pharmacological treatment of the specimens.

Immunohistochemistry and quantification of c-fos expression in the CNS

C-fos immunohistochemistry was performed according to Bonaz *et al.* (1994), with modifications. After fixation, the brain was embedded in Tissue-Tek (Sakura Finetek Inc., Torrance, CA), and 40 µm transverse sections were cryostat-cut. Free-floating sections were washed with Tris-buffered saline (TBS; pH 7.4) three times and incubated overnight at 4°C with the primary polyclonal sheep antibody (catalogue number OA-11-824A, batch number: c-Fos 294 K) (0.3 µg·mL^{-1}; Sigma Genosys, St. Louis, MO) in 0.25% gelatin and 0.5% Triton X-100 in TBS. Next, sections were washed in TBS three times and incubated with biotinylated rabbit anti-sheep antiserum (BA-6000; Vector Laboratories, Burlingame, CA) for 1.5 h at room temperature. After washing in TBS 3 times, sections were processed for avidin– biotin–peroxidase

(Vectorstain ABC kit, PK-4000; Vector Laboratories), and peroxidase was visualized by using diaminobenzidine (D5637; Sigma Aldrich, St. Louis, MO) in 0.02% nickel sulphate in TBS as the chromogen.

A counterstaining with Cresyl Violet (histological staining to delineate neuronal populations) was performed on the 40 µm section after c-fos staining. Sections of 40 µm collected from Bregma –6.96 mm until –8.12 mm exhibit both the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus nerve (DMV), as reported from the *Mouse Brain Atlas* (Franklin and Paxinos, 2007). We assessed c-fos expression on sections from bregma –7.20 mm until –7.76 mm (in saline/semapimod groups) as these sections represent the level in which sensory vagal afferents of the GI tract terminate in the NTS (intestine in the subnuclei commissuralis and medialis and the stomach in the medialis and gelatinosus subnuclei) and motor efferent fibres of the vagus nerve that target the GI tract (lateral DMV) (Altschuler *et al.*, 1991; Fogel *et al.*, 1996; Travagli *et al.*, 2006). Therefore, we selected non-adjacent sections (from bregma –7.20 mm until 7.76 mm, separated by at least 80 µm) for c-fos positive cells counting. Our data represent a mean of the relative density of c-fos positive cells counts on six to eight sections.

The identification of the paraventricular nucleus (PVN) of the hypothalamus (bregma –0.46 mm until –1.22 mm) was performed with the help of the Cresyl Violet counterstaining and the *Mouse Brain Atlas*. The counting of c-fos-positive cells on non-adjacent sections was performed in the PVN nuclei without regard to the parvocellular and magnocellular subdivisions.

Data analysis

Prism 4 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS Statistics 16 (SPSS Inc., Chicago, IL, USA) software for Mac OS were used to perform statistical analysis and create graphs. The data were non-parametrically distributed. If more than two sets of data were compared, a Kruskal–Wallis test was performed to assess whether the cohort of data was statistically different. When variance of medians was statistically significant, the Mann–Whitney test was used to identify the statistical differences within the cohort. When two treatment groups were compared, the Mann–Whitney test was used. $P < 0.05$ was considered statistically significant and results are shown as means ± s.e.mean.

Results

Semapimod administered i.c.v. ameliorates post-operative ileus and diminishes manipulation-induced intestinal muscle inflammation

Manipulation of the small intestine during abdominal surgery initiated a significant increase in gastric retention 24 h after the procedure when compared with mice undergoing laparotomy alone ($P < 0.001$) (Figure 1). The gastric stasis illustrating the extent of post-operative ileus was accompanied by a marked influx of MPO-positive inflammatory cells into the manipulated intestinal segment (Figure 2B). This local leukocyte recruitment was not observed after a laparotomy alone (Figure 2A) and was statistically significant ($P = 0.001$; Figure 3). Treatment with semapimod 1 µg·kg^{-1} i.c.v. ameliorated the manipulation-induced delay in gastric emptying ($P = 0.02$; Figure 1). In line with this observation, the number of MPO-positive cells in the intestinal muscle layer also diminished significantly in the semapimod-treated group ($P = 0.003$; Figures 2D and 3). In contrast, semapimod did not alter gastric retention ($P = 0.4$; Figure 1) nor the extent of intestinal muscle infiltration with MPO-positive cells ($P = 0.3$; Figures 2C and 3) compared with saline-treated mice that underwent a laparotomy alone.

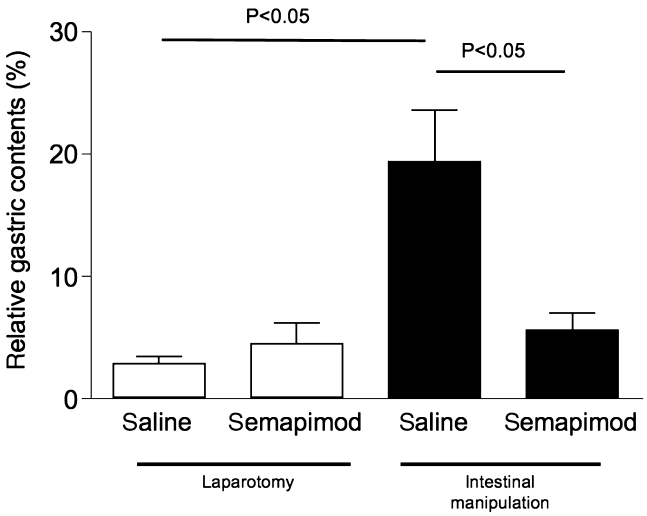

Figure 1

Effect of saline (vehicle) or semapimod i.c.v. pre-treatment on gastric retention 24 h after laparotomy alone or laparotomy followed by gentle intestinal manipulation. Gastric retention was determined 80 min after gavage of a semi-liquid test meal. Data are expressed as mean ± s.e.mean; $n = 8$.

The anti-inflammatory effect of semapimod is mediated through the vagus nerve

In order to assess the involvement of the vagus nerve, experiments were repeated with mice subjected to a sub-diaphragmatic vagotomy prior to start of the initial study protocol. As gastric motility is strongly affected by vagotomy, gastric emptying was not assessed in these animals. Vagotomy by itself did not induce any significant leukocyte recruitment to the intestinal wall (Figure 3 compare 'saline' with 'VGX saline', open bars). Intestinal manipulation, however, resulted in a significant increase of MPO-positive cells infiltrating the muscularis propria 24 h after surgery when compared with laparotomy alone ($P = 0.004$; Figure 3). This influx of inflammatory cells was even more pronounced when compared with intestinal manipulation in mice not subjected to vagotomy ($P = 0.04$; Figures 2B, F and 3). Most importantly, the anti-inflammatory effect of semapimod pre-treatment was absent in mice subjected to sub-diaphragmatic vagotomy ($P = 0.4$; Figures 2F, H and 3). These results are in line with previous reports (Borovikova *et al.*, 2000a; Pavlov *et al.*, 2006) suggesting that the anti-inflammatory effect achieved with semapimod administered i.c.v. is mediated through vagus nerve signalling.

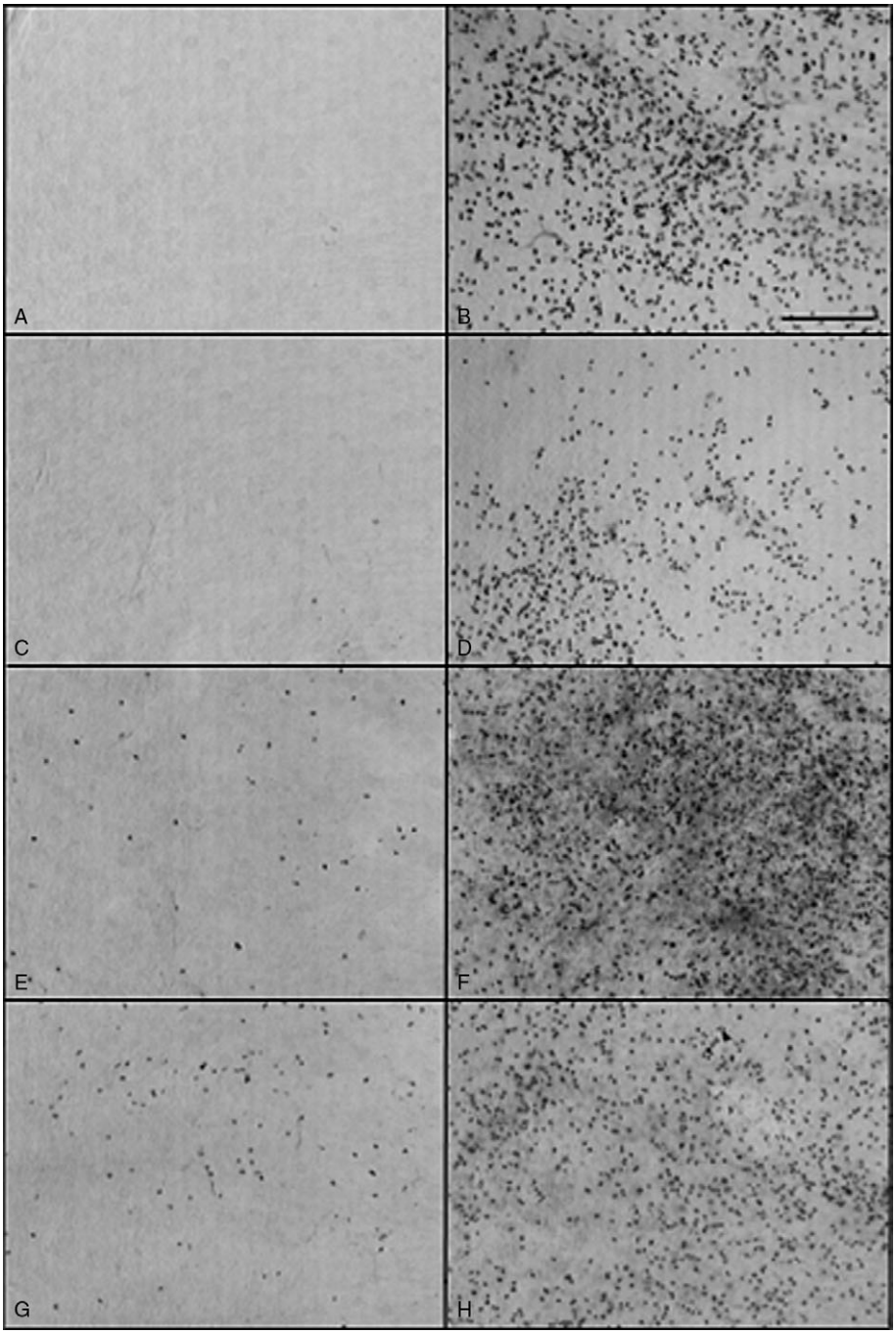

Figure 2

MPO staining of muscle whole mounts from mice that underwent a laparotomy alone (A), a laparotomy with intestinal manipulation (B) after pretreatment with saline (vehicle) i.c.v. or laparotomy alone (C) and intestinal manipulation (D) after pretreatment with semapimod i.c.v. In a second series of experiments, pretreatment and surgical intervention were repeated after sub-diaphragmatic vagotomy; laparotomy alone (E) and saline intestinal manipulation (F) after saline i.c.v. or laparotomy alone (G) and intestinal manipulation (H) after pretreatment with semapimod i.c.v. Scale bar represents 25 μm.

Semapimod induced c-fos expression in brain stem nuclei

To obtain more insight in the central mechanism through which semapimod activates the cholinergic anti-inflammatory pathway, immunohistochemical analysis of the brain for c-fos was performed. Based on a previous report demonstrating enhanced activity of vagal efferent nerve fibres upon semapimod administration (Borovikova *et al.*, 2000a), we first assessed c-fos expression in the dorsal motor nucleus of the vagus nerve (DMV) and the nucleus of the

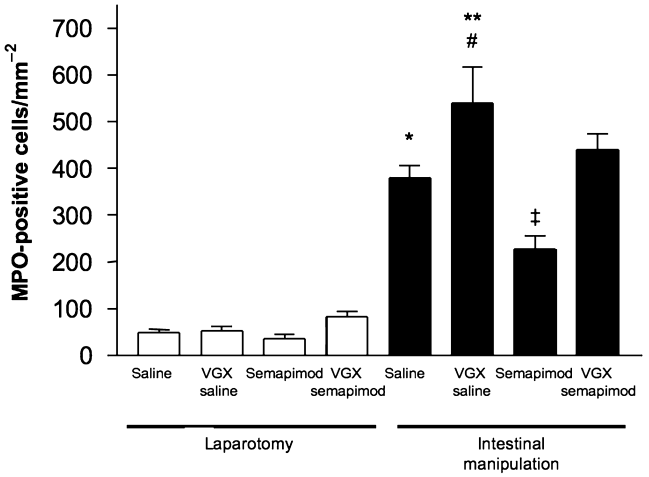

Figure 3

Effect of saline (vehicle) or semapimod i.c.v. pre-treatment on manipulation-induced inflammatory cell recruitment to the muscularis propria. Quantitative analysis of the number of MPO-positive cells 24 h after laparotomy alone or laparotomy followed by gentle intestinal manipulation. Mice that underwent a sub-diaphragmatic vagotomy prior to treatment are marked by VGX. $P < 0.05$ for * (saline vs. saline + intestinal manipulation), # (saline + intestinal manipulation vs. saline + vagotomy + intestinal manipulation), ‡ (saline + intestinal manipulation vs. semapimod + intestinal manipulation), ** (saline + vagotomy vs. saline + vagotomy + intestinal manipulation). Data are expressed as mean ± s.e.mean $n = 8$.

solitary tract (NTS) (Figure 4A and B). Quantification of the absolute amount of c-fos labelling is unlikely to be accurate; therefore, comparison of relative density of labelling is sufficient. Quantitative c-fos analysis 3 h after infusion of semapimod showed an increased number of c-fos-positive neurons in both the NTS ($P = 0.02$) and the DMV ($P = 0.002$) when compared with those after saline infusions (Figure 5B–C). Next we also evaluated c-fos activity in the PVN as it projects on the DMV. Here, c-fos expression 3 h after i.c.v. injection of semapimod was significantly increased compared with saline infusion ($P = 0.03$; Figures 4C, D and 5A).

Discussion

Manipulation-induced inflammation of the intestine, a process orchestrated by cells involved in the innate immune system, such as mast cells and macrophages (Mikkelsen, 1995; Kalff *et al.*, 2000; 1998; 2003; de Jonge *et al.*, 2004; 2003; Wehner *et al.*, 2005; 2007), is now generally believed to play an important role in the pathophysiology of prolonged post-operative ileus. Recently, we showed that vagus nerve stimulation reduces this inflammation and thereby shortens post-operative ileus. Semapimod, a tetravalent guanylhydrazone also known as CNI-1493, is known to be a p38 MAPK inhibitor. It has also been shown to be a stimulant, acting centrally, of this so-called cholinergic anti-inflammatory pathway (Borovikova *et al.*, 2000a; Bernik *et al.*, 2002b). In the present study, we demonstrated that i.c.v. semapimod reduced the inflammatory cell influx in response to intestinal manipulation and improved post-operative gastric emptying. This anti-inflammatory effect was abolished by vagotomy, and i.c.v. semapimod induced increased c-fos expression in the PVN and DMV. Our findings are in line with these earlier reports suggesting that central administration of semapimod activates the cholinergic anti-inflammatory pathway leading to a reduction of the manipulation-induced intestinal inflammatory cell infiltration and restoration of gastric emptying (Borovikova *et al.*, 2000a; Bernik *et al.*, 2002b).

Macrophages, present as a network between the circular and longitudinal muscle layers of the intestine (Mikkelsen, 1995), have been shown to play an important role in the pathogenesis of sustained post-operative ileus in rodents (Kalff *et al.*, 1998; Wehner *et al.*, 2007). These phagocytes lie in close proximity to the myenteric plexus and carry nACh receptors enabling neuro-immune interaction (de Jonge *et al.*, 2005). Recently, we demonstrated that the manipulation-induced inflammation can be diminished by electrical stimulation of the vagus nerve in our experimental mouse model for post-operative ileus (de Jonge *et al.*, 2005) A similar anti-inflammatory effect of vagus nerve stimulation has been demonstrated in sepsis, ischaemia–reperfusion, inflammatory bowel disease and pancreatitis (Borovikova *et al.*, 2000b; Bernik *et al.*, 2002a; Ghia *et al.*, 2006; Van Westerloo *et al.*, 2006) and is currently referred to as the cholinergic anti-inflammatory pathway. Stimulation of the efferent vagus nerve results in reduction in the release of pro-inflammatory cytokines, that is TNFα, interleukin (IL)-1β and IL6, which is associated with improved outcome in experimental septic shock (Borovikova *et al.*, 2000b). The peripheral mechanism of this cholinergic anti-inflammatory response is mediated through nACh receptors expressed on macrophages (Wang *et al.*, 2003; van der Zanden *et al.*, 2009). Activation of these receptors results in JAK2/STAT3 signalling (de Jonge *et al.*, 2005), inhibition of NF-κB signal transduction (Wang *et al.*, 2004) and enhanced phagocytosis (van der Zanden *et al.*, 2009). Indeed, systemic application of selective α7 nACh receptor agonists mimics the effect of vagus nerve stimulation in experimental models for inflammatory bowel disease, pancreatitis and post-operative ileus (Ghia *et al.*, 2006; van Westerloo *et al.*, 2006; The *et al.*, 2007).

Wehner *et al.* (2009) recently studied the role of MAPK signalling in surgery-induced intestinal inflammation and post-operative ileus. These authors found that phosphorylation of p38 MAPK was increased 15 to 60 min after surgical manipulation. This process was most extensive in intestinal macrophages. Inhibition of p38 MAPK phosphorylation by i.v. or i.p. treatment with semapimod reduced the inflammatory response and prevented post-operative ileus in a dose of 5 mg·kg^{-1}. In this setting, semapimod not only decreased pro-inflammatory mediator synthesis but also nitric oxide (NO) activity, an important inhibitory neurotransmitter in the GI tract. In addition to this peripheral mechanism of action, others have found semapimod to be a central activator of the cholinergic anti-inflammatory pathway (Borovikova *et al.*, 2000a; Bernik *et al.*, 2002b). In the present study, we found that i.c.v. semapimod indeed suppressed inflammation in our mouse model for post-operative ileus, reflected by the decreased number of MPO-positive cells present in the intestinal muscle layer. Application of 1 ug·kg^{-1} semapimod

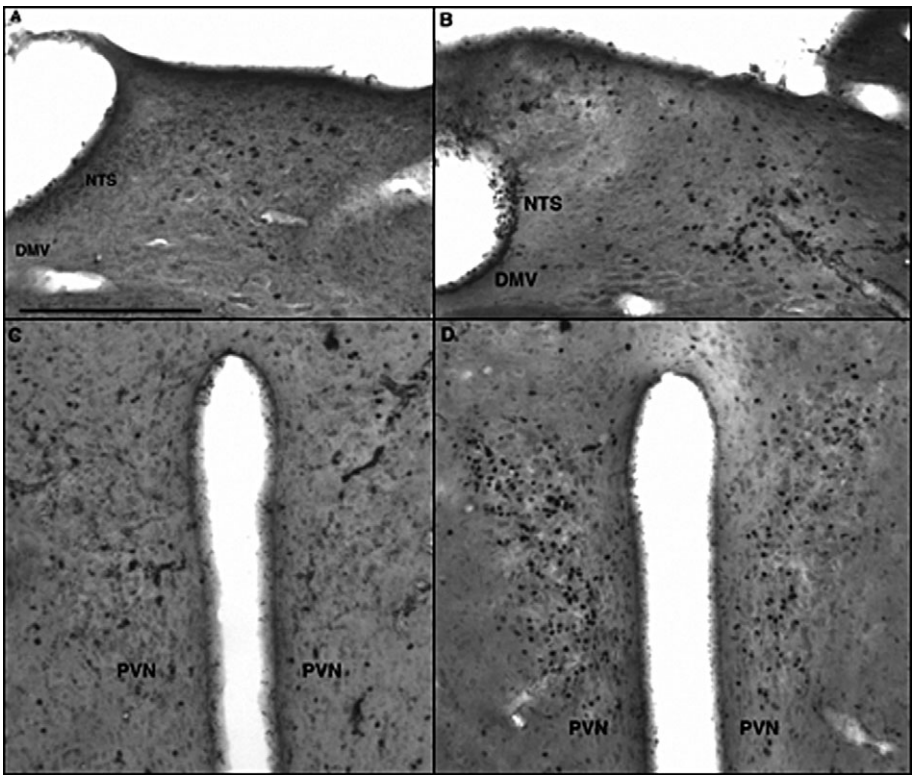

Figure 4

Immunohistochemistry for c-fos in the DMV and NTS 3 h after treatment with saline (A) or semapimod i.c.v. (B) and PVN 3 h after treatment with saline (C) or semapimod i.c.v. (D) Scale bar represents 200 μm.

i.c.v. diminished manipulation-induced inflammatory cell influx, subsequently improving gastric emptying. It is important to emphasize that the dose used in our experiments was 100 times below the dose shown to be ineffective in reducing inflammation when administered systemically. When delivered peripherally, semapimod only showed significant anti-inflammatory properties at doses above 300 $\mu g \cdot kg^{-1}$ (Bernik *et al.*, 2002b). The anti-inflammatory effect achieved with i.c.v. semapimod was abolished by sub-diaphragmatic vagotomy, suggesting a centrally mediated anti-inflammatory pathway dependent on vagal nerve integrity. Whether vagotomy also abolished the beneficial effect on gastric emptying could not be studied as vagotomy results in almost complete retention of gastric content. This is in line with previous observations by us (The *et al.*, 2007) and others (Ghia *et al.*, 2006). Nevertheless, our current data strongly suggest that, in addition to the previously established peripheral inhibition of p38 MAPK (Wehner *et al.*, 2009), central activation of the vagus nerve contributes to the anti-inflammatory properties of semapimod.

Pavlov *et al.* (2006) have demonstrated high affinity of semapimod for M_1 muscarinic receptors Moreover, they showed activation of the cholinergic anti-inflammatory pathway by central administration of muscarinic agonists such as muscarine and the M_1 receptor selective agonist MCN-A-343 with reduction of TNFα release in an endotoxemia model (Pavlov *et al.*, 2006). However, the exact brain areas involved remain to be clarified. We observed an increase in the number of c-fos-positive neurons in the DMV after semapimod but not after saline. Accepting that semapimod interacts with central M_1 receptors (Pavlov *et al.*, 2006), direct activation of the DMV is rather unlikely as this brain nucleus lacks M_1 receptors (Hoover *et al.*, 1985). In line with this finding, we demonstrated c-fos activation in the PVN after i.c.v. administration of semapimod. This finding and the knowledge that the PVN is interconnected with the DMV (Rogers *et al.*, 1980) suggest that activation of the cholinergic anti-inflammatory pathway by semapimod might be indirect, via interaction with M_1 muscarinic receptors in the PVN. More studies however are required to confirm this hypothesis. In addition to increased c-fos expression in the DMV, we also observed enhanced c-fos expression in the NTS following i.c.v. administration of semapimod. This is in line with electrophysiological findings by Zhang *et al.* (1999). These authors indeed demonstrated that, although NTS neurons are predominantly inhibited by electrical stimulation of PVN neurons, a minority of NTS neurons were activated. In contrast, we did not observe an increase in c-fos expression in non-parasympathetic regions of the CNS (i.e. visual cortex and hippocampus; data not shown).

Interestingly, we also demonstrated an increase in the inflammatory cell influx induced by intestinal manipulation in vagotomized animals. Similar findings have been reported in other models of inflammation (Borovikova *et al.*, 2000a) and of sepsis (Borovikova *et al.*, 2000b). For example, vagotomy increased the mortality rate in animals subjected to

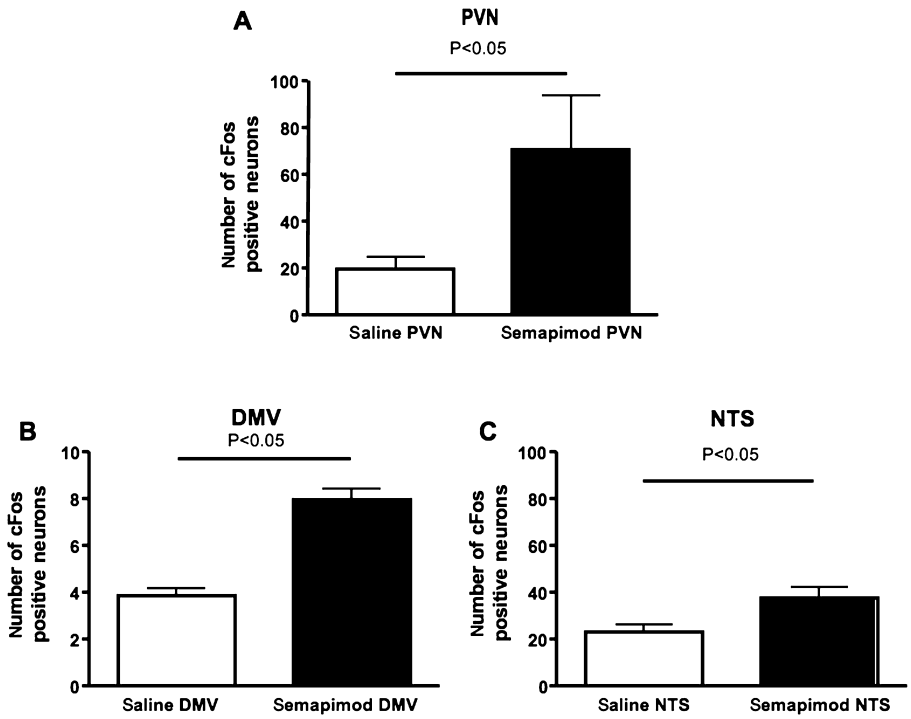

Figure 5

Expression of c-fos in the PVN (A), DMV (B) and NTS (C), 3 h after i.c.v. saline or semapimod treatment. Data are expressed as mean ± s.e.mean; $n = 6$ for all groups.

haemorrhagic shock, associated with an increase in TNFα levels (Guarini *et al.*, 2004). Similarly, the degree of colitis induced by dextran sulphate sodium (Ghia *et al.*, 2006) and pancreatitis (van Westerloo *et al.*, 2006) was significantly augmented after vagotomy. Together with our findings, these data would suggest endogenous activation of the cholinergic anti-inflammatory pathway by the ongoing peripheral inflammatory response and would fit with the hypothesis that the vagus nerve exerts an important role in modulating the innate immune system. Interestingly, we have recently been able to provide the first neuro-anatomical evidence for such a reflex (Cailotto *et al.*, 2010).

Although our data support the existence of an endogenous anti-inflammatory pathway, it needs to be stressed that this hypothesis still remains to be proven. Although we showed enhanced neuronal activity in the PVN, DMV and NTS in response to i.c.v. semapimod infusion possibly via competitive binding to M_1 muscarinic receptors (Pavlov *et al.*, 2006), this finding only indicates that semapimod evokes a stimulatory neural response. Whether this results in excitation of the vagal anti-inflammatory pathway cannot be concluded from the present results. Finally, semapimod was initially developed as a macrophage-selective L-arginine inhibitor (Bianchi *et al.*, 1995). Later studies revealed that semapimod exerts its anti-inflammatory effect via p38 MAPK inhibition and not only by affecting NO synthesis in macrophages (Cohen *et al.*, 1997). More recent work by Tracey's group identified semapimod as a potential pharmacological activator of the cholinergic anti-inflammatory pathway (Meistrell *et al.*, 1997; Borovikova *et al.*, 2000a; Bernik *et al.*, 2002a). These studies suggest that semapimod is not selective or specific.

In conclusion, we showed that in addition to its peripheral inhibitory effect on p38 MAPK (Wehner *et al.*, 2009), semapimod administered i.c.v. reduced inflammatory cell recruitment into the intestinal muscle layer after surgical handling. Our current data strongly suggest that semapimod exerted its effect through central activation of the cholinergic anti-inflammatory pathway. Moreover, we have provided indirect evidence that semapimod activated the DMV via activation of the PVN. These findings further demonstrate the anti-inflammatory properties of the cholinergic anti-inflammatory pathway. Further understanding of its working mechanism and exploration of its clinical application are warranted and are the subject of ongoing investigations.

Acknowledgements

FOT and RvdW were supported by the Technology Foundation STW, applied science division of NWO and the technology program of the ministry of Economic Affairs (NWO-STW, grant no. AKG.5727). WJdJ was supported by a VIDI grant from the Netherlands Organisation for Scientific Research (NWO). GEB was supported by a VICI grant from the Netherlands Organisation for Scientific Research (NWO) and by a grant (Odysseus program, G.0905.07) of the Flemish 'Fonds Wetenschappelijk Onderzoek' (FWO).

Conflict of interest

The authors state no conflict of interest.

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