Chapter 5

Endogenous vagal activation dampens intestinal inflammation independently of splenic innervation in postoperative ileus

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Abstract

Postoperative ileus is encountered by patients undergoing open abdominal surgery and is characterized by intestinal inflammation associated with impaired gastrointestinal motility. We recently showed that inflammation of the gut muscularis triggered activation of the vagal efferent pathway mainly targeting the inflamed zone. In the present study we investigate further the modulatory role of endogenous activation of the vagal motor pathway on the innate immune response. Intestinal or splenic denervation was performed two weeks prior to intestinal manipulation (IM) or laparotomy (L). Twenty-four hours post-surgery, the gastrointestinal transit, immune cell influx, and pro-inflammatory cytokine levels were measured in the gut muscularis. Manipulation of the small intestine led to a delay in intestinal transit, an influx of leukocytes and increased pro-inflammatory cytokine expression. Surgical lesion of the vagal branch that selectively innervates the small intestine did not further delay the intestinal transit but significantly enhanced the expression levels of the pro-inflammatory cytokines IL-1β and IL-6 in the gut muscularis. Splenic denervation did not affect intestinal inflammation or gastrointestinal transit after intestinal manipulation. Our study demonstrates that selective vagotomy, leaving the splenic innervation intact, increases surgery-induced intestinal inflammation. These data suggest that endogenous activation of the vagal efferent pathway by intestinal inflammation directly dampens the local immune response triggered by intestinal manipulation independently of the spleen.
Introduction

Postoperative Ileus (POI) is experienced by almost every patient undergoing open abdominal surgery. The pathophysiology of POI is characterized by a transient impairment of the gastro-intestinal tract leading to nausea, pain and discomfort for the patient. It is now well documented that the underlying mechanism relies on the recruitment of leukocytes as well as the production of pro-inflammatory cytokines (i.e., IL-β, IL-6, TNF-α) and induction of enzymes (i.e., Cox-2) in the gut muscularis after handling of the intestine [1,2].

During the last decade the vagus nerve was discovered as an essential player in the regulation of the immune response. Indeed, in diverse models of inflammation, increased vagal activity by electric stimulation of the vagus nerve was reported to dampen inflammation by suppressing the production of pro-inflammatory cytokines. The spleen was recently identified to play an essential role in mediating this anti-inflammatory effect of the vagus nerve in both endotoxemia [3,4], colitis [5,6] and local inflammation [7]. This phenomenon is thought to rely on the existence of synaptic connections between the vagus nerve and the sympathetic splenic nerve in celiac ganglia [8]. Activation of the vagus nerve leads to the release of noradrenaline by the splenic nerve which activates the production of acetylcholine (ACh) by splenic T cells. Binding of ACh to receptors present on macrophages was shown to inhibit their secretion of pro-inflammatory cytokines such as TNF-α, leading to dampening of the inflammatory response. Evidence of the existence of synaptic contacts between the vagus nerve and sympathetic post-ganglionic neurons in the celiac ganglion on which this model relies is however missing. Recent studies have on the contrary described the absence of such connections [9-11].

Several studies also demonstrated the anti-inflammatory property of the vagus nerve in POI. Indeed, vagus nerve stimulation (VNS) applied prior to the handling of the intestine down-regulates the secretion of pro-inflammatory cytokines by resident macrophages, inhibits the influx of immune cells to the gut muscularis and thereby prevents the delay in the gastrointestinal transit [12-14]. Besides, we provided substantial functional evidence that VNS directly suppresses pro-inflammatory cytokine secretion by macrophages in the gut muscularis independently of the spleen [14]. So far, most studies performed to unravel the cholinergic anti-inflammatory pathway regulating postoperative ileus
used electrical activation of the vagus nerve prior to intestinal manipulation and the subsequent intestinal inflammation. Data on the existence of an intrinsic manipulation-induced vagal reflex modulating the intestinal inflammation underlying postoperative ileus are however scarce. We recently demonstrated that intestinal inflammation triggered the activation of vagal motor neurons innervating the small intestine as well as the spleen [15]. The exact contribution of the vagal innervation targeting the intestine and splenic innervation in the modulation of the manipulation-induced inflammatory response underlying postoperative ileus has not yet been investigated.

In this study, we hypothesized that endogenous activation of vagal efferent fibers innervating the small intestine induced by intestinal manipulation, similar to vagus nerve stimulation, exerts a direct suppressive effect on pro-inflammatory cytokine release by immune cells during POI. We anticipate that this vagal anti-inflammatory effect is not mediated through the splenic nerve.

**Material and methods**

**Mice**

Ten to 12 week-old female Balb/c were purchased from Harlan Nederland (Horst, The Netherlands) and housed in a Specified Pathogen Free facility with a 12/12 light/dark cycle under constant conditions of temperature (20 +/- 2º C) and humidity (55%) and *ad libitum* food and water. Denervation procedures were performed under a mixture of fentanylcitrate/fluanisone (Hypnorm, Janssen, Belgium) and midazolam (Dormicum, Roche, The Netherlands) (5mg/mL). The anesthetic mixture was in a ratio of 1:1:2 of Hypnorm, Dormicum and water respectively and each mouse received 0.1mL/10g of that mixture by intraperitoneal injection. For the intestinal manipulation procedure, a mixture of ketamine (Ketalar 100 mg/kg) and xylazine (Rompun 10 mg/kg), known to have a short-lasting effect [16], was injected intraperitoneally. All efforts were made to minimize the suffering of the animals. All experiments were performed in accordance with the guidelines of the Laboratory Animal Use of the Netherlands and approved by the Ethical Animal Research Committee of the Academic Medical Center of Amsterdam.

**Surgical procedures and sample collection**

Selective vagal denervation of the intestine (Intx) and splenic denervation (Splx) were
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performed as previously described [15]. Vagal intestinal denervation was achieved by cutting the right celiac branch of the vagus nerve supplying the jejunum, ileum and cecum. Splenic denervation was achieved by cutting noradrenergic fibers running along blood vessels supplying the spleen and by cutting nerve fibers present in the conjunctive tissue located at each tip of the spleen. Mice were monitored and weighed daily following the denervation procedure. Both sham-operated, Intx and Splx mice regained their initial weight and produced normal stool within 5 days after the surgical procedure. Two weeks after sham operation or denervation, mice underwent Laparotomy (L) or Laparotomy followed by Intestinal Manipulation (IM), as previously described [15]. After opening the abdominal cavity by a midline incision, the small intestine was carefully externalized from the peritoneal cavity and placed on a moist gauze pad. The small bowel was manipulated by compression with two moist cotton swabs: the two cotton swabs were placed on each side of the small intestine and manipulation was performed so that the luminal content of the small intestine was moved aborally. The entire small bowel was manipulated twice from the distal duodenum to the cecum. No contact or manipulation of the stomach or colon was applied. Sacrifice was performed 24h after surgery by transcardiac perfusion with PBS during anesthesia with pentobarbital (0.1mL of a 50mg/mL solution).

**Tyrosine Hydroxylase staining**

The completion of splenic nerve lesion was assessed by a Tyrosine Hydroxylase (TH) immunohistochemical staining on spleen sections. Eight μm sections of frozen spleens were air-dried, fixed in ice cold acetone for 2 min and air-dried again. Sections were blocked with 0.1% Triton (Sigma, St Louis, MO) 2% Normal Goat Serum (Dako Cytomation, Glostrup, Denmark) in TBS for 30 min. After a 2-hour incubation at RT with an anti-mouse TH antibody (Sigma, St Louis, MO), sections were incubated with a Brightvision Poly-AP-Anti Rabbit antibody (Immunologic, Duiven, The Netherlands) for 60 min at RT. The staining was revealed by incubating the sections with an alkaline-phosphatase solution AP kit III (Vector Laboratories, Burlingame, The United States) in Tris HCl with 5 mM Levamisole (Sigma, St Louis, MO) for 35 min. Sections were air-dried and mounted in gelatin-glycerol (Sigma, St Louis, MO). The absence of positive signal determined a complete splenic denervation. The success rate of the splenic denervation was 95%. One mouse was excluded from the analysis as its splenic denervation was not complete.
Measurement of the gastrointestinal transit

The gastrointestinal transit was measured using the non-absorbable tracer 70 kDa fluorescein isothiocyanate-labeled dextran (FD70) as previously described [14,17]. Briefly, 1.5 hour before sacrifice, mice were fed with 10 μL of a 6.25 mg/mL solution of FD70 in distilled water. The entire gastrointestinal tract (from stomach to colon) was divided into 15 segments (stomach, 10 segments of equal length for the intestine, cecum, and 3 segments of equal length for the colon). Each segment was flushed with PBS and FD70 concentration was assessed by fluorimetry in the supernatant of each segment. The distribution of FD70 was determined by calculation of the Geometric Center (GC) with GC=Σ(% of total fluorescent signal per segment x segment number)/100).

RNA isolation, RT-PCR and QPCR

The mucosa of segments 5, 6 and 7 (ileum) of the small intestine was stripped from the muscularis with 2 fine pincets. Intestinal muscularis of these 3 segments was snap-frozen. Total mRNAs of the frozen intestinal muscularis were extracted after homogenization of the samples in TriPure isolation reagent according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN). cDNA synthesis was performed using the Revertaid first strand cDNA synthesis kit (Fermentas, Germany) and Real-time PCR was performed using a SYBR green master mix (Roche Applied Science, Indianapolis, IN) on a Lightcycler480 (Roche Applied Science, Indianapolis, IN). Primer sets were synthesized by Invitrogen (Bleiswijk, The Netherlands) and are described in Table 1. Raw data of the genes of interest were analyzed using the LinRegPCR program (AMC, The Netherlands) [18] and normalized with reference genes chosen after analysis with the Genorm software.

Cytokine analysis by ELISA

Segments 2, 3 and 4 (jejunum/ileum) of the small intestine were snap frozen and stored until further use. Small intestinal segments were homogenized in Greenberger Lysis Buffer. Analysis of murine IL-1β, IL-6 and TNF-α was performed using the commercially available ELISA kits according to the manufacturer’s instructions (R&D systems, Abingdon, UK). Total protein content of the samples was measured using the BCA colorimetric assay (Thermoscientific, Breda, The Netherlands). The concentration of
the protein of interest was normalized on the total protein content of each sample.

**Myeloperoxidase quantification by Immunohistochemistry**

Segment 8 (ileum) of the small intestine was stored in 70% ethanol until further use. The mucosa was removed from the muscularis with 2 fine pincets and stained for Myeloperoxidase (MPO) as a marker of leukocytic infiltration. Briefly, the whole mount intestinal muscularis segment were stained with a 3-amino-9-ethyl carbazole (Sigma, St Louis, MO), 0.01% $H_2O_2$ in Sodium Acetate buffer (pH=5) for 20 min, as previously described [15]. Sections were analyzed using a plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives) connected to a color-camera (JVC KY-F55 3CCD). Random counting of MPO positive cells was performed for each section, as previously described [15].

**Statistical analysis**

Statistical analysis was performed using the SPSS 19.0 software (SPSS Inc, Chicago, IL). The data are expressed as mean±SEM. Normal distribution was assessed using the Kolmogorov-Smirnov test. Square-root normalization was applied to non-normal data sets. A 2-way ANOVA was performed to determine the interaction between denervation (Sham vs Intx/Splx) and treatment (L vs IM). When significance was observed with the 2-way ANOVA, an unpaired Student t-test was performed to evaluate the significance between Sham vs Intx/Splx or L vs IM.

<table>
<thead>
<tr>
<th>Forward primer 5’-3</th>
<th>Reverse primer 5’-3</th>
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<tr>
<td>HRPT</td>
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<td>Cox-2</td>
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<tr>
<td>IL-10</td>
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<td>TGF-β</td>
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<tr>
<td>CD45</td>
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<tr>
<td>F4/80</td>
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**Table 1. Primer sequences used for QPCR analysis**
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Results

Modulatory role of vagal motor efferent fibers in response to intestinal inflammation

Intestinal inflammation induced by handling of the small intestine delayed the gastrointestinal transit, as shown by the decrease in calculated geometric center (GC) (Fig. 1A) (11±1 vs 4±1 for Sham L vs Sham IM). We also reported a strong influx of immune cells to the gut muscularis, i.e., increased expression level of the general hematopoietic marker CD45 (L vs IM: 1±0 vs 5±1; p<0.001) and the macrophage marker F4/80 (L vs IM: 1±0 vs 3±0; p<0.001) and increased numbers of MPO+ cells (neutrophils/monocytes) (Fig. 1B and C). Ablation of the vagal input to the small intestine did not affect intestinal transit in control mice (Laparotomy) and did not further delay the gastrointestinal transit in the inflamed intestine. In line, the immune cell influx was not affected by the denervation.

However, a significant effect of vagal denervation was observed on the expression levels of pro-inflammatory cytokines in the gut muscularis. Indeed, Intx IM mice displayed a significant increase in the mRNA (Sham IM vs Intx IM: 8±1 vs 29±9; p=0.03) and protein level of IL-1β (Sham IM vs Intx IM: 8±1 vs 29±9; p=0.04). A significant increase in the mRNA level of IL-6 (Sham IM vs Intx IM: 6±2 vs 21±7; p=0.039) as well as a trend towards increased IL-6 protein level (Sham IM vs Intx IM: 13±4 vs 20±7; p=0.08) was also observed in Intx mice compared to Sham-operated animals 24h after IM. Of note, a non-significant trend was observed in the mRNA levels of TNF-α (Sham IM vs Intx IM: 8±2 vs 18±7; p=0.11) and Cox-2 (Sham IM vs Intx IM: 7±2 vs 10±3; p=0.34). As described previously, intestinal manipulation led to an increase in the expression of the anti-inflammatory cytokines IL-10 and TGFβ [17,19]. Vagal intestinal denervation however did not affect the expression level of these anti-inflammatory cytokines (Fig. 2A and B).

Splenic innervation does not participate in the vagal regulatory effect on the intestinal immune response

We previously demonstrated that the spleen is not involved in the anti-inflammatory effect of VNS during POI [14]. To determine whether the endogenous vagal anti-inflammatory effect triggered in response to manipulation of the intestine was also independent of splenic innervation, we investigated the effect of selective splenic denervation on IM-induced inflammation and delay in gastrointestinal transit.
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**Figure 1.** Intestinal vagal denervation does not influence the impairment of the gastrointestinal transit or the influx of immune cells to the small intestine. Lesion of the vagal fibers targeting the intestine was performed prior to Laparotomy (L)/Intestinal Manipulation (IM). (A) Intestinal manipulation (IM) triggers a delay in the gastrointestinal transit 24h post-surgery but intestinal denervation (Intx) does not affect the severity of this delay. Cm=cecum (B) IM triggers enhanced expression of the hematopoietic marker CD45 and the macrophage marker F4/80 in the gut muscularis 24h post-surgery. Intx doesn’t affect the expression level of these markers. (C) IM triggers an enhanced number of myeloperoxidase positive (MPO⁺) cells in the gut muscularis 24h after surgery. Intx does not affect the number of MPO⁺ cells in the gut muscularis. Scale bar represents 50µm. Data are expressed as mean±SEM (n=6-7 animals per group). * p<0.05; ** p<0.01; *** p<0.001

Completion of splenic denervation was assessed by immunohistochemical staining for tyrosine hydroxylase, an enzyme present in noradrenergic fibers. The absence of positive signal determined a successful splenic denervation (Fig. 3A). The IM induced-delay in gastrointestinal transit was similar in sham-operated and splenic denervated mice (Fig. 3B). Similarly, no effect of the splenic denervation was observed in the number of immune cells infiltrating the gut muscularis (Fig. 3C), and the mRNA and protein levels of pro- (i.e., IL-1β, IL-6, TNF-α and Cox-2) or anti-inflammatory cytokine levels (i.e., IL-10 and TGFβ) (Fig. 4A and B) confirming that the spleen does not participate in the vagal anti-inflammatory pathway in POI.
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Discussion

In the present study, we demonstrate that selective vagal denervation of the intestine aggravates pro-inflammatory cytokine production in the gut muscularis in response to intestinal manipulation but does not affect influx of immune cells or delay in the intestinal transit. This vagal immunomodulatory effect targets directly the gut muscularis and does not involve the splenic innervation.

We recently provided anatomical evidence of endogenous activation of a vagal reflex in response to intestinal inflammation [15]. Activation of vagal neurons was observed in both the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMV) 24h after manipulation of the intestine demonstrating that the vagus nerve senses and responds to IM-induced inflammation. Here, we demonstrate that vagal innervation of the small intestine is essential to dampen the pro-inflammatory cytokines IL-1β and IL-6, known to increase early on after surgery. Interestingly the production of IL-1β and IL-6 in the gut wall is not restricted to the sole immune cells as enteric neurons are also able to produce these pro-inflammatory cytokines [20,21]. However, we recently demonstrated that VNS failed to dampen the inflammation in POI in wild-type irradiated mice reconstituted with α7nAChR−/− bone marrow.

This strongly demonstrates that the anti-inflammatory effect of the vagus nerve on the production of IL-1β and IL-6 relies on the presence of the α7nAChR cholinergic receptors on innate immune cells (i.e., macrophages and neutrophils) rather than enteric neurons [14]. Notably, especially IL-1β has recently been identified to play an essential role in the development of POI [21]. Given the crucial role of IL-1β in POI, a tight control of its production is of extreme importance. Our data demonstrating an increase in this cytokine following selective vagal denervation of the intestine indicate that IM-induced inflammation activates the vagal anti-inflammatory pathway reducing IL-1β production and contributing to restrict the inflammatory response.

Influx of mainly neutrophils and monocytes is considered to mediate the reduction in gastrointestinal motility in the postoperative period, mainly by release of nitric oxide and prostaglandins [1,2]. Previously, we showed that VNS significantly reduces this influx and shortens POI. Conversely, one might anticipate that selective vagal denervation of the intestine will increase cellular influx, an effect that we previously observed when cervical vagotomy was performed prior to manipulation of the intestine [12].
Figure 2. Lack of vagal intestinal innervation enhances the expression level of pro-inflammatory cytokines in the gut muscularis after IM. (A) Increased mRNA levels of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α are observed 24h after Intestinal manipulation (IM). Vagal intestinal denervation (Intx) increases the expression level of IL-6 and IL-1β after IM in comparison with sham-operated animals. (B) Cytokine levels as measured by ELISA 24h after IM. Intx leads to a significant increase in IL-1β and a trend towards increased IL-6 in the small intestine 24h after IM. Data are expressed as mean±SEM (n=12-14 animals per group). * p<0.05; *** p<0.001
present study, we fail to observe increased cellular influx to the gut muscularis in mice lacking vagal input to the intestine. Influx of cells to the gut muscularis represents a very early step in the pathophysiology of postoperative ileus whereas endogenous activation of the motor part of the vagus nerve occurs once the inflammation is settled [15]. This may explain why vagal denervation of the intestine does not alter the influx of cells after intestinal manipulation. Moreover, we previously showed that mild manipulation of the intestine leads to less production of pro-inflammatory cytokines in the intestine as compared to a severe manipulation of the intestine [22]. In this same study however, the influx of cells to the gut muscularis was as high in mice undergoing mild intestinal manipulation as in mice undergoing severe intestinal manipulation. This seems to indicate that the influx of cells after intestinal manipulation represents an on/off system that reaches its maximum intensity independently of the severity of the manipulation or the inflammation and may explain this absence of aggravation of cellular influx in intestinal-denervated mice. Moreover, the increase in the levels of pro-inflammatory cytokines observed after intestinal vagal denervation in the absence of a greater influx of cells implies that the vagus nerve exerts an anti-inflammatory role targeting immune cells residing in the gut muscularis and/or on infiltrating immune cells once they have reached the gut muscularis.

VNS was previously shown to prevent the delay in the gastrointestinal transit demonstrating the effectiveness of vagal signaling in ameliorating POI [13,14]. Although we observed increased inflammation 24h after IM in intestinal-denervated mice compared to sham-operated mice, we failed to report a further delay in the gastrointestinal transit. Importantly, VNS is applied pre-emptively to intestinal handling thereby preventing the release of pro-inflammatory cytokines by resident macrophages and consequently inhibiting the underlying cause leading to intestinal paralysis. On the other hand, endogenous activation of the vagal efferent pathway is only triggered once the intestinal inflammation is already settled and inflammatory signals such as pro-inflammatory cytokines and enzymes are released and detected by sensory vagal afferents. This endogenous pathway most likely contributes to resolution rather than prevention of the inflammatory response. This discrepancy might explain why, even though the endogenous vagal anti-inflammatory effect is able to dampen the production of pro-inflammatory cytokines by innate immune cells, it is not sufficient to prevent the impairment of the gastrointestinal motility. Of note, the delay in gastrointestinal transit observed after intestinal manipulation in our hands is very severe. It is therefore likely
Figure 3. The vagal anti-inflammatory effect is independent of splenic innervation. Splenic denervation (Splx) was performed prior to Laparotomy (L)/Intestinal Manipulation (IM) (A) Immunohistochemical staining targeting Tyrosine Hydroxylase (TH) in sham animals (a) revealed the presence of noradrenergic fibers alongside blood vessels, trabeculae and in the white and red pulp of the spleen. The absence of TH+ fibers in spleen sections of spleen-denervated (Splx) mice (b) was taken as a proof of completion of splenic denervation. (B) Splx does not affect the severity of the delay in the gastrointestinal transit or (C) the influx of cells to the gut muscularis 24h after IM. Scale bar represents 50µm. Data are expressed as mean±SEM (n=11-12 animals per group). * p<0.05; ** p<0.01; *** p<0.001
that, as for the cellular influx to the muscularis, this delay is already at its maximum and cannot be increased by denervation.

The action mode of the anti-inflammatory effect triggered by VNS was shown to rely on splenic innervation during sepsis and colitis [4,6-8]. In the context of POI however, VNS was shown to modulate the intestinal inflammation independently of the splenic innervation [14]. In these inflammatory models, little is known about the exact neural networks implicated in the inflammation-induced endogenous vagal anti-inflammatory effect. Here we demonstrate for the first time that also under endogenous conditions, splenic innervation is not implicated in the vagal neural network regulating postoperative ileus. On the contrary, we show that the vagal neural circuitry activated by IM-induced inflammation directly exerts its anti-inflammatory effect on the inflamed organ, i.e., the intestine. Furthermore, the approach we chose in the present study allowed us to provide evidence that the cholinergic anti-inflammatory pathway is also endogenously activated by intestinal inflammation. Notably, this results in a reduction of pro-inflammatory cytokines in the muscularis thereby contributing to resolution of the inflammatory response.

In conclusion, our study provides further insights in the cholinergic anti-inflammatory pathway regulating POI, showing that the vagal pathway activated endogenously during POI exerts an anti-inflammatory effect independently of splenic innervation.
Figure 4. The vagal anti-inflammatory effect is independent of splenic innervation. A. Splx mice display identical increased levels of IL-1β, IL-6 and TNF-α as sham-operated mice 24h after IM on mRNA level (A) as well as on a protein level (B). Data are expressed as mean±SEM (n=11-12 animals per group). * p<0.05; ** p<0.01; *** p<0.001
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References


