The vagus nerve as a modulator of intestinal inflammation
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Citation for published version (APA):
van der Zanden, E. P. M. (2011). The vagus nerve as a modulator of intestinal inflammation

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Cholinergic agonists interact with immunomodulatory actions of neuropeptides VIP and SP

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Manuscript in preparation
ABSTRACT

INTRODUCTION Vagus nerve activity ameliorates intestinal inflammation. Its mechanisms may involve attenuation of pro-inflammatory cytokine production by macrophages (MF) and inhibition of inflammatory processes via the release of acetylcholine (ACh). However, vagus nerve stimulation may also modulate the release of neuropeptides with immune-modulatory potential, such as substance P (SP) and vasoactive intestinal polypeptide (VIP). Here, we questioned whether vagus nerve released ACh may relay its anti-inflammatory potential via modulation of release of VIP or SP.

AIMS & METHODS: Electrical vagal nerve stimulation (VNS) was performed using bipolar electrodes, after which intestinal tissue was analyzed for expression of VIP and VIP receptors VPAC1-2, as well as Substance P and its receptor NK-1. In vitro, peritoneal mouse MF were pretreated with Ach/nicotine (30min, 10µM) and/or VIP or SP (30min, 10µM), followed by 3hrs LPS challenge (100ng/mL). Cytokine levels were determined using ELISA, NF-kB activity was measured in RAW MF stably transfected with NF-kB luciferase reporter construct. Calcium measurements were performed using Fluo-4-AM.

RESULTS: VNS modulated expression levels of VIP and SP in ileum and colonic tissue. Expression of VIP and its receptors VPAC1 and VPAC2 was induced by VNS, whereas the other hand, VNS resulted in a gradual decline in SP expression. In vitro, VIP inhibited LPS-induced TNF production in RAW MF down to 52.4 +/- 4.2% of LPS alone. When ACh and VIP (10 μM) were co-administered, LPS induced TNF production was decreased almost down to unstimulated levels (25±4.2% of LPS alone). On the other hand, SP enhanced NF-kb transcriptional activity and TNF production in RAW MF. ACh reduced TNF production and NF-kB activation in MF significantly, whereas co-application of nicotine and SP attenuated LPS induced NF-kB activation and TNF production even further, down to 57.2±5.1% and 52.4±3.0% respectively compared to substance P treatment alone. Nicotine pretreatment showed similar results. Intracellular calcium was not affected using either treatment.

CONCLUSION: Cholinergic agonists reduce TLR4 activation on peritoneal macrophages a mechanism that involves nAChRs, and interference with VIP and SP pathways. These data suggest that the vagus nerve anti-inflammatory effect may be amplified via modulation of neuropeptide expression.
INTRODUCTION

Electrical stimulation of vagal efferent fibers inhibits inflammatory processes in various experimental animal models of inflammatory disease, such as sepsis\textsuperscript{1}, rheumatoid arthritis\textsuperscript{2}, post-operative ileus\textsuperscript{3} and DSS-colitis\textsuperscript{4}. \textit{In vitro} data reveal that activation of nicotinic acetylcholine receptors (nAChR) on macrophages can attenuate pro-inflammatory cytokine release\textsuperscript{1} and enhance phagocytosis\textsuperscript{5}. These findings might explain the anti-inflammatory effect of activation of the vagus nerve. However, it remains to be elucidated if acetylcholine (ACh) released from vagus nerve termini actually reaches the immune cells, and if so, in what quantities. Given the short half-life of ACh, cholinergic modulation of immune cell activation most likely requires close contact and there is currently no strong evidence that parasympathetic neurons indeed innervate macrophages. Therefore, it is likely that, next to the 'classical' direct anti-inflammatory effect of acetylcholine on tissue macrophages, vagus nerve stimulation affects immune cells via post-ganglionic mechanisms involving alternative neurotransmitters, such as neuropeptides or catecholamines.

In septic models, the protective effect of vagus nerve stimulation has been ascribed to modulation of splenic release of catecholamines\textsuperscript{6}. Alternatively, in the gastrointestinal tract, the vagus nerve mainly synapses with neurons of the enteric nervous sytem (ENS) and vagus nerve activation leads to the release of several neuropeptides at the nerve endings in the intestine. Neuropeptides are neuronal signaling molecules, that diffuse into surrounding tissues and bind to their corresponding receptors, affecting nearby muscle, epithelium, endothelium, and immune cells. Besides the known functions that neuropeptides exhibit in the gastrointestinal tract (GI tract), such as secretion of salivary, gastric fluids, intestinal fluids and electrolytes, neuropeptides are increasingly appreciated as modulators of the immune response.

Substantial evidence shows that substance P and vasoactive intestinal peptide (SP and VIP), two neuropeptides that are abundantly expressed in the gut, have important neuroimmunomodulatory properties in the intestine. Increased SP expression is observed in both tissue and nerve fibers in the colons of patients with UC\textsuperscript{7} and substance P receptor antagonists attenuate disease activity in a DSS-colitis rat model\textsuperscript{8}. \textit{In vitro} data reveal that substance P enhances NF-kB transactivation and chemokine and cytokine responses in murine macrophages\textsuperscript{9}. In contrast, vasoactive intestinal peptide (VIP), displays potent anti-inflammatory properties, including inhibition of leukocyte migration and stimulation of IgA production by B lymphocytes\textsuperscript{10}. Moreover, VIP administration reduces clinical symptoms and cytokine profiles in mice models of experimental colitis\textsuperscript{11}. Hence, substance P and VIP can modulate the intestinal immune response, however, the exact mechanism via which these neuropeptides exert their immunomodulatory effects is unknown. It is a complex system, in which secretion and action of a single neuropeptide can be influenced by other neuropeptides, neurotransmitters, cytokines, hormones and drugs.
The present study shows that VNS modulates expression levels of VIP and SP in ileum and colonic tissue. In vitro, substance P enhances NF-kB transactivation and TNF production, whereas VIP reduces inflammation in peritoneal macrophages. Cholinergic agonists nicotine and acetylcholine prevent the pro-inflammatory effects of SP, while the anti-inflammatory effects of VIP are enhanced. Taken together, our data suggest that acetylcholine, released upon vagus nerve stimulation, can affect immunomodulatory actions of neuropeptides VIP and SP.

MATERIALS AND METHODS

General reagents. (-)-Nicotine, Acetylcholine, LPS, VIP and substance P were from Sigma-Aldrich (Zwijndrecht, the Netherlands). ELISA kits for TNF were from R&D Systems (Abingdon, UK).

Mice. For electrical vagal nerve stimulation, 12- to 15-week-old female BALB/c mice (Harlan Nederland) were kept in environmentally controlled conditions (light on from 08:00 to 20:00; water and rodent nonpurified diet ad libitum; temperature, 20–22 °C; 55% humidity). All animal experiments were approved by the local animal experimental committees.

Electrical vagal nerve stimulation. Mice were anesthetized by intraperitoneal injection of a mixture of fentanyl citrate and fluanisone (Hypnorm; Janssen) and midazolam (Dormicum; Roche). The vagus nerve was stimulated as described12. The right cervical vagal branch was prepared free from the carotid artery and was ligated with 6-0 silk suture. The part distal from the ligation was attached to a bipolar electrode and 5 V of stimulation with a frequency of 5 Hz and a duration of 2 ms was applied for 15 min. In sham-operated mice, the cervical skin was opened and was left for 15 min covered by moist gauze. Groups of three mice were electrically stimulated and ileum and colonic tissue were collected at 4, 8 and 24 h after stimulation.

NF-κB activity assay. Peritoneal mouse RAW264.7 macrophages (ATCC, Middlesex, UK) were stably transfectioned with a NF-kB luciferase reporter construct (Clontech, Mountain View, CA) in which a PDNA3.1(+) derived neomycin resistance TK cassette was inserted. Transfection was performed using electroporation. In short, 2*10^6 RAW264.7 cells were scraped and resuspended in 100µL Nucleofector V reagent (Amaxa Biosystems Inc.) with 2 µg of highly purified NF-kB-luciferase construct, mixed carefully and transferred to a 0.2-cm Amaxa electroporation cuvette. The cuvette was placed in the Amaxa nucleofection device and nucleofected according to manufacturer’s instructions using program D-32. Immediately after transfection, cells were cultured in RPMI 1640/10% FCS o/n. Transfected cells were selected in 102
the culture medium supplemented with neomycin (1000µg/ml) for 14 days. Resistant cells were subcloned and clones were cultured up to 20 passages with RPMI containing 1000ug/ml neomycine. Clones stably expressing the NF-kB reporter construct were used.

Cell culture and stimulation. Murine RAW264.7 cells (ATCC, Middelsex, UK) and RAW cells stably expressing the NF-kB luciferase reporter construct were grown in RPMI-1640 medium with antibiotics and L-glutamine (Gibco, Breda, The Netherlands), supplemented with 1% heat-inactivated fetal bovine serum at 37°C in a humidified incubator with 5% CO₂. For experiments, cells were transferred to 48-well suspension plates (Greiner-Bio, Alphen aan de Rijn, The Netherlands) at 2.5 x10^5 cells/well. After o/n incubation, the medium was removed and replaced with RPMI containing 1% serum. Cells were pretreated with vehicle, nicotine or acetylcholine 30 minutes prior to SP or VIP treatment. VIP or SP were applied for another 30min, cells were subsequently stimulated with 100ng/ml LPS for 3 hours. TNF levels were analyzed using the TNF sandwich ELISA kit. For NF-kB luciferase measurement, the medium was removed after 3 hrs. of LPS stimulation; the cells were washed three times with ice-cold PBS and lysed with Passive Lysis Buffer supplied in the LuciferaseTM Reporter Assay Kit (Promega Corporation, Madison, WI), the lysate was assayed for luciferase activity according to the manufacturer’s instructions.

Ca^{2+} measurement. RAW264.7 cells were loaded with the fluorescent dye 10 μM Fluo-4-AM (Invitrogen, Darmstadt, Germany) and 2.5 mM probenecid (Sigma-Aldrich) in HEPES buffer for 20 min at RT. Cells were washed three times to remove the dye and perfused with HEPES solution (+2.5mM probenecid) for another 20 min. ATP, nicotine, acetylcholine, VIP and SP (all 10µM) were applied with rapid ejections using a picospritzer. The dye was excited with a HC482/35 filter and fluorescence was monitored through a HC529/28 filter. Calcium fluxes were measured until 20 sec after application of ATP, nicotine, ACh, VIP or SP.

Statistical analyses. Data are expressed as mean ± standard error (SEM). Statistical analyses were performed using the non-parametric Mann-Whitney U test. A probability value (P) of less than 0.05 was considered significant.

RESULTS

VNS modulates VIP and SP expression
To assess whether VNS can modulate VIP or SP receptor expression in vivo, we applied VNS for 15 minutes and collected mucosal and muscle tissue from the ileum
and colon 4, 8, or 24 hrs after VNS. Using QPCR, we determined expression levels of VIP, or its main receptors VPAC-1 and -2. VPAC1 is constitutively expressed in both stimulated and unstimulated macrophages, whereas VPAC2 is only expressed in activated macrophages. Moreover, we tested the effect of VNS on the expression of Substance P and its receptor NK-1. Figure 1A shows that, after an initial drop in VIP expression (4 hrs after VNS), VIP expression is enhanced 24 hrs after VNS stimulation in ileum. In colon, VNS significantly enhances VIP expression after 4 hrs. On the other hand, SP expression is gradually reduced by VNS in ileum and colonic tissue. In figure 1B, it is demonstrated that VNS enhances expression of VPAC1 in mucosal colon tissue after 4 hrs. Besides that, VNS does not alter expression levels of VIP receptors VPAC1-2 or SP receptors NK-1 in colonic or ileum tissue at other timepoints. In duodenal and jejunum tissue, no differences in expression levels of VIP, SP and its receptors upon VNS were observed (data not shown).

**ACh, nicotine and VIP reduce NF-kB activity and cytokine release in a cumulative manner**

Next we studied the potential of VIP and ACh/nicotine to modulate MF activity in vitro; To test the immunomodulatory capacity of VIP on MF, RAW macrophages were treated with VIP for 30 minutes before 3 hrs LPS challenge. VIP inhibited LPS-induced TNF production and NF-kB activation in a dose dependent fashion in RAW macrophages, down to resp. 27+/−6.2% and 52.4+/−4.2% of control release (Fig 2A and 2B). ACh or nicotine pretreatment (30 min) attenuated LPS-induced TNF production down to 59±4.5% and 60.6±4.5% confirming previous studies (Fig 2C). Interestingly, when ACh and VIP, or nicotine and VIP, were co-administered, LPS induced TNF production was decreased almost down to unstimulated levels (ACh+VIP: 25±4.2%; nicotine+VIP 23.2±3.1% of LPS) (Fig 2C).

**The pro-inflammatory activity of SP is neutralized by cholinergic agonists**

Subsequently, we examined whether ACh or nicotine could alter immuno-modulatory actions of SP. Cells were treated with different concentrations of SP 30 minutes prior to 3 hours LPS (100ng/ml) challenge. SP enhanced TNF-production and NF-kB activity dose-dependently in macrophages, up to resp. 132±7.4% and 135±6.3% of LPS (Fig. 3A and 3B). To assess whether ACh or nicotine affects SP induced increase in NF-kB transactivation and TNF release, we pretreated cells with ACh or nicotine for 30 minutes prior to substance P (30min) and LPS challenge (3hrs). ACh reduced LPS induced TNF production and NF-kB activation in macrophages down to 63±3.0% and 69±4.0% of LPS treated cells. Nicotine attenuated NF-kB activation and TNF production to the same extent, down to 75,9+/−5.3% and 69.5+/−3.3% (Fig. 3B). However, co-application of SP with ACh attenuated LPS induced TNF production and NF-kB activation even further, down to 53,2±5.1% and 44±3.0% respectively compared
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Figure 1. Modulation of VIP and SP expression upon VNS Electrical VNS was performed using bipolar electrodes for 15 minutes and after 4, 8 or 24hrs, mucosal and muscle tissue of the ileum (left panel) and colon (right panel) were collected. (A) Tissue was analyzed using QPCR for VIP (grey lines; open dots) and SP (black lines; closed squares) expression levels. In Figure 1B, expression levels of VPAC1 (black closed triangles), VPAC-2 (grey rhombus) and NK-1 (open squares) were depicted. All values are corrected for B-actin and expressed as % of sham stimulated tissue. Experiments were performed in triplicate. Asterisks indicate significant differences (P<.05) vs SHAM stimulation.

Figure 1. Modulation of VIP and SP expression upon VNS Electrical VNS was performed using bipolar electrodes for 15 minutes and after 4, 8 or 24hrs, mucosal and muscle tissue of the ileum (left panel) and colon (right panel) were collected. (A) Tissue was analyzed using QPCR for VIP (grey lines; open dots) and SP (black lines; closed squares) expression levels. In Figure 1B, expression levels of VPAC1 (black closed triangles), VPAC-2 (grey rhombus) and NK-1 (open squares) were depicted. All values are corrected for B-actin and expressed as % of sham stimulated tissue. Experiments were performed in triplicate. Asterisks indicate significant differences (P<.05) vs SHAM stimulation.

to substance P treatment alone. In conjunction, when cells were treated with SP and nicotine, LPS-induced TNF production and NF-KB activation were decreased to 57.2±5.1% and 52.4±6.6% as compared to SP alone (Fig. 3C and 3D). These data suggest that cholinergic agonists can neutralize the pro-inflammatory actions of the neuropeptide SP.
Figure 2. VIP and ACh/nicotine reduce inflammatory mediators in a cumulative manner. RAW macrophages were incubated with a dose range of VIP (0-1000nM) for 30min prior to 3hrs LPS stimulation (100ng/ml). NF-kB activity was determined using luminescence and TNF production with ELISA. TNF levels (A) or NF-kB activity (B) were measured after 3hrs. and depicted as % of vehicle treated cells. Data are mean ± s.e.m. of three independent experiments done in triplicate. *, P < 0.05

Figure 3. Co-application of cholinergic agonists with SP neutralizes pro-inflammatory actions of SP RAW cells were incubated with SP (0-1000nM) for 30min, followed by 3hrs LPS stimulation (100ng/ml). (A) TNF levels or (B) NF-kB levels were determined and represented as % of vehicle treated cells. (C and D) Cells were pretreated with 10µM ACh/nicotine for 30min, then stimulated with 10µM VIP, followed by 100ng/ml LPS challenge. TNF levels were measured after 3hrs. Data are mean ± s.e.m. of three independent experiments done in triplicate. *, P < 0.05.
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Calcium measurements
To elucidate the cellular mechanism behind the immuno-modulatory actions of ACh, VIP and SP on macrophages, we analyzed whether modulation of SP and VIP pathways by ACh was reflected by altered cellular calcium flux. However, we found that the effects of ACh, VIP and SP, are independent of calcium signaling pathways, as cells respond potently to adenosine triphosphate (ATP), but calcium fluxes were not affected by exposure to ACh, VIP or SP alone, or in combination.

Figure 4. Exposure to ACh, VIP, or SP, alone or in combination, does not affect calcium fluxes. RAW264.7 cells were loaded with Fluo-4-AM and probenecid in HEPES buffer. ATP, ACh, VIP and substance P (all 10µM), alone or in combination were applied with rapid ejections and calcium fluxes were measured until 20 sec application of either of the substances. Data are representative of four independent experiments.

DISCUSSION
Intestinal immune homeostasis requires strictly controlled regulatory mechanisms. Evidence is mounting that these factors can be modulated by signals emerging from the nervous system. For instance, the parasympathetic and sympathetic pathways can restrain inflammatory responses and affect innate immune cell reactivity. Vagus nerve stimulation ameliorates inflammation via acetylcholine. However, vagus nerve stimulation may also affect immune cells via postganglionic mechanisms involving alternative neurotransmitters, such as neuropeptides. For decades, neuropeptides are recognized as modulators of the immune system that can play a crucial role in the pathogenesis of inflammation7-11. Here, we show that VNS can affect expression of neuropeptides in the gut and in vitro, cholinergic activation affects immuno-modulatory actions of neuropeptides.

In vivo, VNS has shown to ameliorate disease in diverse inflammatory mouse models. However, in vitro, cholinergic agonists reduce LPS-induced TNF production in macrophages only to a moderate extent that can not entirely explain the in vivo effect of VNS. Therefore, we reasoned that neuropeptides, which are abundantly present in the intestine, could play a role in the the anti-inflammatory effects observed after VNS. We showed that VNS modulated expression of VIP and SP in ileum and colonal tissue.
Since the cholinergic anti-inflammatory effects have been extensively studied in macrophages, we performed our *in vitro* experiments using peritoneal mouse macrophages. These experiments clearly display that co-application of VIP and ACh or nicotine reduces inflammation in RAW macrophages in a cumulative manner. However, it is unclear if the VNS-induced modulation of VIP receptors is macrophage mediated. Presumably, other immune cells can be affected as well. For example, it is reported that DC isolated from the PP exhibit increased levels of VPAC1 and VPAC2 mRNA as compared to peripheral DC's, indicating that these PP DC are susceptible to modulation by VIP\textsuperscript{16}. Therefore, our future prospects are to investigate if besides macrophages, other immune cells such as DC, are involved in the anti-inflammatory actions of ACh and VIP.

The mechanism via which vagus nerve released ACh can co-operate with VIP to reduce inflammation is still unclear. Suprisingly, calcium fluxes were unaffected by application of ACh, nicotine, VIP or SP. Nevertheless, we and others previously showed that ACh does induce kinase signaling\textsuperscript{17}, therefore, we would have expected that ACh modulated calcium flux. Presumably, the cell culture system used was not suitable for measurement of gradual changes in calcium flux, or the experiment set-up was not sensitive enough.

Delgado et al demonstrated that the anti-inflammatory effect of VIP on RAW cells is mediated via two intracellular pathways: affecting both NF-kB binding and the composition of the cAMP responsive element binding complex (CREB/c-Jun)\textsuperscript{18}. The working mechanism of ACh ultimately involves modulation of STAT3 pathways\textsuperscript{6} and prevention of NF-kB p65 transcriptional activity\textsuperscript{19}, following nAChR activation. Possibly, VIP and ACh act synergestic in the inhibition of nuclear translocation of NF-kB. Alternatively, the cross-talk between VIP and ACh could be extracellular instead of intracellular. This is supported by the finding that, in isolated parasympathetic neurons of rat ganglia, VIP selectively increased the affinity of nAChR for their agonists, thereby potentiating ACh-evoked whole cell currents in rat cholinergic neurons\textsuperscript{20}. Otherwise, it is possible that ACh modulates VIP and SP receptor expression which renders cells more susceptible to immuno-modulation. Finally, studies in rodents have demonstrated that VIP, SP and ACh can be locally produced by inflammatory cells such as macrophages, lymphocytes, and dendritic cells. Possibly, the nAChR-mediated effects of ACh could be potentiated in presence of locally released VIP.

We show here that VNS modulates VIP and SP expression in the gut, and *in vitro*, cholinergic agonists neutralize the pro-inflammatory SP effects and further enhance anti-inflammatory VIP actions in intestinal macrophages. These data suggest that the anti-inflammatory effects of cholinergic activation *in vivo* may be mediated not only via direct ACh binding to nAChR, but also via modulation of co-released neuropeptides SP and VIP.
REFERENCE LIST


