Lipid-rich enteral nutrition regulates mucosal mast cell activation via the vagal anti-inflammatory reflex

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Lipid-rich enteral nutrition regulates mucosal mast cell activation via the vagal anti-inflammatory reflex. The majority of mast cells resides in the gut wall. Here, they function as an important part of the immunological barrier between the internal milieu and luminal content. Mucosal mast cell degranulation is associated with gut barrier dysfunction, an event that has been implicated in the development of excessive systemic inflammation and distant organ injury in settings of surgery and sepsis. Further support for a pivotal role of intestinal mast cells in the development of intestinal injury and inflammation was derived in intestinal ischemia and reperfusion models in which anthistaminic and mast cell stabilizing drugs were shown to inhibit neutrophil influx, cytokine production, and both local and remote tissue injury. Therefore, regulation of early mucosal mast cell activity is a potential strategy to attenuate the acute immune response and prevent inflammatory complications.

Nutritional activation of a hard-wired vagal anti-inflammatory reflex is a physiological approach to modulate the acute inflammatory response and preserve intestinal integrity. The present study investigates intestinal mast cell responsiveness upon nutritional activation of the vagal anti-inflammatory reflex during acute inflammation. Mucosal mast cell degranulation was induced in C57/B6 mice by administration of Salmonella enterica LPS. Lipid-rich enteral feeding prior to LPS significantly decreased circulating levels of mouse mast cell tryptase compared with isocaloric low-lipid nutrition or fasting. CCK-1R blockage reversed the inhibitory effects of lipid-rich feeding, whereas stimulation of the peripheral CCK-1R mimicked nutritional mast cell inhibition. The effects of lipid-rich nutrition were negated by nAChR blockers chlorisondamine and α-bungarotoxin and vagal intestinal denervation. Accordingly, release of β-hexosaminidase by MC9 mast cells following LPS or IgE-ovalbumin complexes was dose dependently inhibited by acetylcholine and nicotine. Application of GSK1345038A, a specific agonist of the nAChR α7, in bone marrow-derived mast cells from nAChR β2−/− and wild type indicated that cholinergic inhibition of mast cells is mediated by the nAChR α7 and is independent of the nAChR β2. Together, the present study reveals mucosal mast cells as a previously unknown target of the nutritional anti-inflammatory vagal reflex.

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in groups of three to four per cage. Prior to the experiments, mice were drug and test naïve and had ad libitum access to standard rodent diet, chow and water. Mice were randomly assigned to 25 study groups. In three groups of eight animals (fasted or fed with lipid-rich or low-lipid nutrition), the nutritional effects on LPS-induced mast cell activation were investigated. In 16 groups of six animals, the role of the vagal anti-inflammatory reflex was studied by using diverse agonists and antagonists. Involvement of the vagus nerve was studied in four groups of eight animals. Group sizes were based on previously reported effects of enriched nutrition on circulatory mast cell markers and earlier experiments in which agonists and antagonists were applied and selective vagotomy was performed (6, 8, 31). In two groups of six animals (ketotifen and vehicle), the importance of mast cells in the development of LPS-induced inflammation and intestinal barrier loss was studied. The experimental protocols were carried out following institutional guidelines and approved by the Animal Ethics Committees of Maastricht University Medical Centre + and the University of Amsterdam. This study is reported in accordance with the ARRIVE guidelines.

**Experimental design of the animal studies.** Mice received an intraperitoneal dose of LPS (2 mg/kg) from Salmonella enterica serotype Minnesota Re 595 (Sigma, St. Louis, MO), dissolved in sterile saline (200 μg/ml; pH 7.4) (11). Animals displayed sickness behavior shortly following LPS. Animals were either fasted from 4 h prior to LPS administration or received liquid lipid-rich or liquid control low-lipid nutrition at 2 h (0.2 ml) and 45 min (0.2 ml) before LPS via oral gavage. To assess early mucosal mast cell activity, animals were killed at 30 min following LPS. The involvement of mast cells in the response to LPS was investigated by administration of ketotifen (1 mg/kg; Novartis Pharma, Arnhem, the Netherlands) or vehicle (sterile saline) intraperitoneally at 48, 24, and 4 h before LPS injection. These animals were killed at 90 min post-LPS by venacava puncture following anesthesia (pentobarbital; 200 mg/kg ip). Samples were coded and stored at −80°C.

Selective denervation of the intestine was performed at 14 days prior to LPS challenge, as described before (6). Animals were anesthetized by an intraperitoneal injection of a mixture of fentanyl citrate–fluanisone (Hypnorm; Jansen, Beerze, Belgium), midazolam (Dormicum, Roche, Midirecht, the Netherlands), and water in a ratio of 1:1:2, respectively. Fentanyl (0.02 mg/ml/100 g from 10×-diluted stock solution 50 mg/ml) was administered subcutaneously after surgery for analgesia. Following a midline abdominal incision, the right celiac branch of the vagus nerve, which supplies the jejunum, ileum, and cecum, was cut by removal of the fat/connective tissue in which the nerve is embedded (6).

**Nutritional composition.** The lipid-rich nutrition contained 50.4 energy percent (en%) fat, 8.7en% protein, and 40.9en% carbohydrates (30). The fat fraction contained 30% phospholipids. The low-lipid control nutrition contained 16.0en% fat, 8.7en% proteins, and 75.3en% carbohydrates. The protein and carbohydrate composition of the two feedings were identical. The amount of fat in the control nutrition was similar to that present in standard rodent chow and the lipid-rich nutrition was isocaloric and isonitrogenous to the control nutrition. The lipid source was soy lecithin. Midazolam (0.22% sodium pyruvate, nucleoside mix (adenosine, guanosine, cytidine, uridine, thymidine; 7 μg/ml each), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (28). In addition, 1% of MC/9 medium constituted of the supernatant of IL-3 producing transfected Chinese hamster ovary (CHO) cells (a kind gift of Dr. Renaud, Brussels, Belgium). CHO cells were cultured in Glasgow Minimum Essential Medium enriched with 10% FCS, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, nucleoside mix (adenosine, guanosine, cytidine, uridine, thymidine; 7 μg/ml each), 100 IU/ml penicillin and 100 μg/ml streptomycin (25). MC9 cultures were split when 80% confluence was reached. Under these conditions, cell populations duplicated in 10 h.

**Culture and stimulation of MC9 and bone marrow-derived mast cells.** MC9 (kindly provided by Dr. Ito, Kobe, Japan), a murine cell line with a mast cell phenotype, was cultured at 37°C with 5.0% CO2 in RPMI-1640 (Life Technologies, Carlsbad, CA), supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (28). In addition, 1% of MC9 medium constituted of the supernatant of IL-3 producing transfected Chinese hamster ovary (CHO) cells (a kind gift of Dr. Renaud, Brussels, Belgium). CHO cells were cultured in Glasgow Minimum Essential Medium enriched with 10% FCS, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, nucleoside mix (adenosine, guanosine, cytidine, uridine, thymidine; 7 μg/ml each), 100 IU/ml penicillin and 100 μg/ml streptomycin (25). MC9 cultures were split when 80% confluence was reached. Under these conditions, cell populations duplicated in 10 h.

Bone marrow mast cells were cultured from nAChR β2−/− mice (kindly provided by Dr. Maskos, Paris, France) or C57/Bl6 wild-type mice as described earlier (9). In brief, femoral bone marrow cells were maintained in vitro for 4 wk in RPMI 1640 complete medium (Life Technologies) supplemented with 10% FCS, in the presence of stem cell factor (50 ng/ml; Pepro Tech, Rocky Hill, NJ), interleukin-3 (1 ng/ml; Pepro Tech) and 6% bone marrow mast cell supplement (containing 20% MEM nonessential amino acids, 1% l-glutamine, 0.22% sodium pyruvate, 0.005% β-mercaptoethanol). During culture, medium was refreshed once weekly. After this culture period, mast cells represented more than 95% of the total cells as determined by toluidine blue staining on cytospin preparations.

Stimulation of the afferent vagus nerve in fasted animals was assessed by administration of 6 μg/kg pegylated-CCK9 (produced by Dr. Verhaegs, Kortrijk, Belgium) dissolved in sterile saline. Pegylated-CCK9 specifically binds to CCK-1R and does not cross the blood brain barrier (48). To investigate the effects of afferent vagus stimulation via the transient receptor potential vanillloid 1 (TRPV1), the specific agonist SA13353 (12 μg/kg; kindly provided by Dr. Tsuji, Osaka, Japan) or vehicle (saline with 1% Cremophor EL, Sigma) was employed (37). Conforming with the recommendations of the producers, 0.1 mg/kg terbutaline and 10 mg/kg theophylline were administered subcutaneously at 15 min prior to SA13353 to counteract its acute respiratory and cardiovascular effects.

The role of peripherally localized nAChR was studied by administration of chlorisondamine diiodide (125 μg/kg; Tocris Bioscience, Bristol, UK) or vehicle (saline). Next, the nAChR α5 antagonist α5-bungarotoxin (2 μg/kg; Sigma), which does not pass the blood brain barrier, or its vehicle (PBS), was applied. Whereas α5-bungarotoxin is widely used as a blocker of the nAChR α5, affinity has also been reported for other nAChR subunits, including α1, α6, and α10 (52).

**Intestinal permeability.** Gut wall permeability was assessed in 5-cm segments of terminal ileum, of which the distal end was located at 5 cm of the ileocecal valve. Segments were gently flushed and filled with 0.4 ml of Tris buffer (125 mM NaCl, 10 mM fructose, 30 mM Tris; pH 7.5) containing 40 μg/ml of the 44-kDa enzyme horseradish peroxidase (HRP; Sigma). After ligation of both ends, the filled segments were incubated in 1 ml Tris buffer at room temperature for 45 min. Next, the ileal segment was carefully removed and HRP activity in the buffer was measured spectrophotometrically at 450 nm after addition of tetramethylbenzidine as a substrate (8).

**Culture and stimulation of MC9 and bone marrow-derived mast cells.** MC9 (kindly provided by Dr. Ito, Kobe, Japan), a murine cell line with a mast cell phenotype, was cultured at 37°C with 5.0% CO2 in RPMI-1640 (Life Technologies, Carlsbad, CA), supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (28). In addition, 1% of MC9 medium constituted of the supernatant of IL-3 producing transfected Chinese hamster ovary (CHO) cells (a kind gift of Dr. Renaud, Brussels, Belgium). CHO cells were cultured in Glasgow Minimum Essential Medium enriched with 10% FCS, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, nucleoside mix (adenosine, guanosine, cytidine, uridine, thymidine; 7 μg/ml each), 100 IU/ml penicillin and 100 μg/ml streptomycin (25). MC9 cultures were split when 80% confluence was reached. Under these conditions, cell populations duplicated in 10 h.

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Before stimulation, mast cells were washed, resuspended in Tyrode’s buffer (10 mM HEPES, 130 mM saline, 6.2 mM D-glucose, 3.0 mM KCl, 1.4 mM CaCl2, 1.0 mM MgCl2, and 0.1% BSA) and plated in triplicate in 96-well flat bottom microplates (1.5 × 10^5 per well; Corning, Lowell, MA). To study the mast cell response to LPS, MC/9 cells were incubated for 2 h with 100 µg/ml LPS of *Salmonella enterica* (11). IgE-mediated degranulation of MC/9 cells was induced by incubation of the cells for 2 h with murine monoclonal anti-ovalbumin (OVA) IgE (5 µg/ml, kindly provided by Dr. Kiniwa, Taiho Pharmaceutical, Tokushima, Japan) followed by 1 h incubation with 100 µg/ml OVA (Sigma) (39). Bone marrow-derived mast cells were stimulated with *Salmonella enterica* LPS (100 ng/ml), peptidoglycan (10 ng/ml), or compound 48/80 (1 mg/ml) for 3 h. Compound 48/80 is widely used as an inducer of mast cell degranulation (26). Cell cultures were centrifuged and supernatants were collected. Cell pellets were lysed in Tyrode’s buffer containing 1% Triton X-100. Stimulation experiments were performed at least twice. Throughout the experiments, cells were kept at 37°C.

To simulate neuronal activity, MC/9 cells and bone marrow-derived mast cells were preincubated with a serial dilution of the vagal neurotransmitter acetylcholine or the nAChR agonist nicotine (both Sigma, dissolved in Tyrode’s buffer) at 1 h prior to LPS, IgE, peptidoglycan, or compound 48/80. Acetylcholine was applied in presence of 2 mM cholinesterase inhibitor pyridostigmine bromide (Sigma). Bone marrow-derived mast cells were also stimulated with the selective nAChR α3 agonist GSK1345038A (supplied by Glaxo-SmithKline) at the indicated concentrations.

β-Hexosaminidase assay. The enzymatic activities of β-hexosaminidase in MC/9 cell supernatants and lysates were measured using 2 mM 4-nitrophenol-N-acetyl-β-D-glucosaminide (Sigma) dissolved in 0.2 M citrate buffer (pH 4.5) at 37°C for 2 h in a 5% CO2 humidified atmosphere. The reaction was quenched by addition of Tris (pH 9.0). Production of p-nitrophenol was detected by absorbance at 405 nm (2). The activity of bone marrow-derived mast cells was assessed by use of a 4-methylumbelliferyl glucosaminide (4-MUG) substrate solution (3.79 mg 4-MUG/ml DMSO) in 0.1 M citrate buffer (pH 4.5). The reaction was stopped by adding 0.2 M glycine buffer (pH 10.7). Fluorescence was measured by using a multilwell plate reader at an emission wavelength (λ) of 360 nm and excitation wavelength (λ) of 460 nm. The percentage of degranulation is calculated as follows: 

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\frac{(a - b)(t - b)}{\alpha} \times 100, \quad \text{where} \quad \alpha \text{ is the amount released from unstimulated cells (basal release)}
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Flow cytometric analysis. To confirm the mast cell characteristics of MC/9, the mast cell-specific CD200R3-binding rat anti-mouse antibody Ba91 (kindly provided by Dr. Karasuyama, Tokyo, Japan) was used for flow cytometry (27). Cells were washed, resuspended in FACS buffer (PBS with 2% BSA), and incubated for 30 min with Ba91 or appropriate isotype control (Hycult Biotech, Uden, the Netherlands). Next, after being washed twice, cells were incubated for 30 min with a FITC-conjugated goat anti-rat IgG (HP1101, Hycult Biotech). After another washing, cells were resuspended in FACS buffer and analyzed on a FACSsort cytometer (BD, Franklin Lakes, NJ). For each measurement, 10,000 cells were acquired.

Statistical analysis. All animals were included in the analysis. The Kruskal-Wallis test with Dunn’s posttest was applied to perform multiple comparisons between groups (Figs. 1, 2A, 3, 5C, and 6). A two-tailed Mann-Whitney U-test was used for comparisons between two groups (Figs. 2B and 4). All data are displayed as means ± SE. *P* values smaller than or equal to 0.05 were considered statistically significant. Prism 5.02 for Windows (GraphPad Software, San Diego, CA) was used for computations.

**RESULTS**

Lipid-rich enteral nutrition reduces LPS-induced mast cell degranulation in mice. Circulatory MMCP I levels were measured to assess mucosal mast cell activation following in vivo LPS exposure. Whereas MMCP I levels were undetectable in healthy animals (not shown), a strong MMCP I increase was observed at 30 min following LPS (Fig. 1). Lipid-rich nutrition significantly decreased MMCP I levels compared with fasted (*P* < 0.001) and low-lipid treated controls (*P* < 0.05). MMCP I levels in low-lipid treated animals were not statistically different from the fasted group.

Stimulation of peripheral cholecystokinin-1 receptors inhibits mast cell degranulation. To investigate involvement of the CCK-1R-dependent vagal pathway in the observed mast cell inhibition by lipid-rich nutrition, A70104, an antagonist of peripheral CCK-1R, was employed (12). Administration of A70104 prior to LPS abolished the effects of lipid-rich nutrition on MMCP I release (Fig. 2A; *P* < 0.05). No effect of A70104 was seen in fasted animals. In vehicle-treated animals, MMCP I levels were significantly reduced in the lipid-rich fed group compared with fasted controls (*P* < 0.01). No statistical differences were observed between the fasted and lipid-rich fed animals that received A70104. The effect of peripheral CCK-1R signaling on mast cell reactivity was further explored by administration of pegylated-CCK9. Previously, pegylated-CCK9 was shown to inhibit the acute inflammatory response via CCK-1R (31). Here, pegylated-CCK9 significantly reduced MMCP I levels compared with vehicle in fasted animals (Fig. 2B; *P* < 0.05). To investigate whether mast cell inhibition could also be obtained by CCK-R independent stimulation of the afferent vagus nerve, we employed SA13353, a specific agonist of the vanilloid receptor TRPV1 that is abundantly expressed on the afferent nerve (37). A trend toward decreased MMCP I levels following administration of SA13353 was observed; however, no statistical differences were obtained (Fig. 2B).

Nicotinic acetylcholine receptors and the intestinal vagal innervation are crucial in the inhibition of LPS-induced mast cell degranulation by lipid-rich nutrition. Previously, activation of nAChR was shown to be pivotal in the anti-inflamma-
tory effects of vagus nerve signaling (5, 33). First, we investigated the involvement of nAChR in the nutritional inhibition of LPS-induced mast cell degranulation using chlorisondamine, a nonspecific peripheral nAChR antagonist. Chlorisondamine prevented the decrease of MMCP I that was observed in lipid-rich treated mice receiving vehicle (veh) compared with fasted mice receiving vehicle (Fig. 3A; \( P < 0.05 \)). In particular the \( \alpha_7 \)-subunit of the nAChR was reported to mediate cholinergic inhibition of cytokine release in macrophages. Therefore we evaluated the effects of a nAChR \( \alpha_7 \) antagonist, \( \alpha \)-bungarotoxin, on nutritional mast cell inhibition (51). Administration of \( \alpha \)-bungarotoxin abrogated the effects of lipid-rich nutrition (Fig. 3B; \( P < 0.05 \)), implicating involvement of the nAChR \( \alpha_7 \) in the vagal inhibition of mast cell activity by lipid-rich nutrition. Neither chlorisondamine nor \( \alpha \)-bungarotoxin significantly affected MMCP I levels in fasted animals, indicating that these compounds lack an intrinsic mast cell-stimulatory effect.

To confirm the involvement of the vagus nerve in the anti-inflammatory effects of lipid-rich nutrition, selective in-
Cultured MC/9 cells were following LPS and IgE exposure. MMCP I (data not shown). Ketotifen did not significantly alter circulatory levels of fasted animals compared with animals that received vehicle; B and reduced ketotifen before LPS decreased IL-6 concentrations in plasma (Fig. 4A; P < 0.05). In addition, ketotifen reduced ileal permeability to HRP compared with vehicle (Fig. 4B; P < 0.05). Ketotifen did not significantly alter circulatory levels of MMCP I (data not shown).

Acetylcholine and nicotine reduce mast cell degranulation following LPS and IgE exposure. Cultured MC/9 cells were employed to further assess the effects of cholinergic signaling on mast cell activation. The affinity of Ba91, an antibody that specifically binds to the CD200R3 located on murine mast cells, for MC/9 cells confirmed the mast cell characteristics of the cell line (Fig. 5A) (27). First, MC/9 cells were activated by exposure to Salmonella enterica LPS. Both acetylcholine and nicotine dose-dependently decreased LPS-induced mast cell degranulation as assessed by quantification of β-hexosaminidase release (Fig. 5B).

To confirm the inhibitory effects of acetylcholine and nicotine on mast cell activation with a mast cell-specific stimulus, sensitization of MC/9 cells with anti-OVA IgE and subsequent exposure to OVA was applied. Whereas anti-OVA IgE and OVA independently did not affect mast cell activity (data not shown), the combination led to strongly enhanced degranulation, which was set as 100% (Fig. 5C). Cholinergic control of the IgE-induced response was assessed by preincubation with 1 μM of acetylcholine or nicotine. The concentrations of acetylcholine and nicotine were based on the abovementioned experiments using LPS. IgE-mediated mast cell activation was significantly reduced by acetylcholine as well as nicotine (Fig. 5C; P < 0.01 and P < 0.05, respectively).

A selective agonist of the α7-subunit of the nicotinic acetylcholine receptor inhibits mast cell degranulation. MC/9 cells possess a mast cell phenotype; however, the nAChR subtypes that are expressed by these cells have not been determined so far. The involvement of the α7- and β2-subunits was investigated in bone marrow-derived mast cells. Stimulation with peptidoglycan resulted in enhanced β-hexosaminidase release (i.e., 34% of the release following administration of compound 48/80) compared with LPS, confirming Ikeda and Funaba (17). In line with the observations in MC/9 cells, both acetylcholine and nicotine reduced bone marrow-derived mast cell activity following peptidoglycan stimulation (Fig. 6A; P < 0.05 for 0.1 mM nicotine and P < 0.05 for 0.1 mM acetylcholine). A highly specific agonist of the nAChR α7-, GSK1345038A, reduced mast cell degranulation to an extent comparable to nicotine and acetylcholine (Fig. 6A; 0.1 mM GSK: P < 0.05). Next, involvement of the β2-subunit was studied. Similar to wild types, in mast cells derived from β2−/− mice, a dose-dependent reduction of β-hexosaminidase was observed following administration of acetylcholine (Fig. 6B; 10 μM: P < 0.01 and 100 μM: P < 0.001), nicotine (10 μM and 100 μM: both P < 0.001), and GSK1345038A (10 μM: P < 0.05 and 100 μM: P < 0.01).

**DISCUSSION**

The present study identifies stimulation of the hard-wired vagal anti-inflammatory reflex by enteral lipid-rich nutrition as a strong inhibitor of mucosal mast cell reactivity. Since mast cells are recognized as early and pivotal regulators of the innate immune response, these findings support nutritional intervention in patients prone to develop an excessive inflammatory response.

In the present study, exposure of mice to the general inflammatory trigger of LPS resulted in increased levels of MMCP I, a protein secreted by mucosal mast cells in the gastrointestinal tract (3). The detection of MMCP I as soon as 30 min following LPS may represent a direct mast cell-stimulatory effect of LPS, since murine mast cells express TLR4, the LPS signaling receptor (34). The in vivo findings were reflected by...

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**Fig. 4.** Mast cells contribute to cytokine release and intestinal compromise following LPS exposure. Administration of histaminic receptor antagonist ketotifen before LPS decreased IL-6 concentrations in plasma (A) and reduced leakage of horseradish peroxidase (HRP) through the intestinal wall (B) in fasted animals compared with animals that received vehicle; n = 6 for all groups.

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enhanced release of protease β-hexosaminidase in MC/9 cultures following exposure to LPS from the same origin. Interestingly, various authors report enhanced cytokine production by mast cells following LPS stimulation, but no increase of degranulation (38). Since different mast cell responses are reported following distinct agonists to the same mast cell receptor, arguably the discrepancy between our work and a part of the literature may be explained by the ligand selected (35).

The notion that different mast cell populations display different responses to stimuli is supported by our observation that administration of TLR2 agonist peptidoglycan in bone marrow-derived mast cells resulted in enhanced β-hexosaminidase release, whereas the response to LPS was limited (29, 38).

Following recognition of pathogens or endogenous danger signals, mucosal mast cells are among the first immune cells to

**Fig. 5.** Cholinergic agents inhibit LPS and IgE-induced mast cell degranulation. A: flow cytometry analysis shows affinity of MC/9 cells for mast cell-specific murine CD200R3 antibody Ba91, confirming the mast cell characteristics of the cell line. B: exposure to LPS resulted in release of 17.6 ± 0.5% of total cellular β-hexosaminidase. The released amount of β-hexosaminidase was set as 100%. Acetylcholine and nicotine dose dependently inhibited MC/9 mast cell degranulation after 2 h of stimulation with LPS. C: exposure to anti-ovalbumin IgE followed by OVA resulted in increased MC/9 degranulation. Administration of 1 μM of either acetylcholine (ach) or nicotine (nic) significantly reduced MC/9 degranulation. All experiments were performed at least twice.

![Graph showing cholinergic agents inhibit LPS and IgE-induced mast cell degranulation](image)

**Fig. 6.** α7-Subunits but not β2-subunits of the nicotinic acetylcholine receptor are involved in cholinergic mast cell inhibition. A: stimulation with peptidoglycan in bone marrow-derived mast cells from C57/Bl6 mice resulted in release of 30.8 ± 1.4% of total cellular β-hexosaminidase. The released amount of β-hexosaminidase was set as 100%. Acetylcholine, nicotine, and nAChR α7 agonist GSK1345038A (GSK) dose dependently reduced β-hexosaminidase release. B: in bone marrow-derived mast cells from β2−/− mice, 30.1 ± 1.5% of total cellular β-hexosaminidase was released by peptidoglycan. The degranulation was significantly decreased by nicotine, acetylcholine, and GSK1345038A. All experiments were performed at least twice. *P < 0.05 vs. control, #P < 0.01 vs. control, †P < 0.001 vs. control.
become activated in the intestine during the innate immune response (1, 13). Via instant release of biologically active chemokines, cytokines, vasoactive amines, and proteases, mucosal mast cell activity plays a pivotal role in the recruitment of neutrophils, increase of vascular and mucosal permeability, and the development of local intestinal damage (1, 15, 40). Evidence has been provided that these local intestinal events contribute to the development of systemic inflammatory complications (7, 10, 23). Therefore, mast cell regulation is currently appreciated and tested as a potential therapeutic strategy in several intestinal diseases (20, 22, 24). In the present study, the contributory role of mast cells in the development of inflammation and loss of gut wall integrity is confirmed by the observation that administration of histamine-1 receptor antagonist ketotifen prior to LPS resulted in decreased IL-6 concentrations and improvement of gut barrier function. Whereas ketotifen has also been reported to exert mast cell stabilizing properties, next to histamine receptor-1 antagonism and weak inhibition of leukotrienes and phosphodiesterases (16), it did not affect circulatory MMCP I concentrations, which is in accordance with Kloker et al. (24).

Here, lipid-rich nutrition is identified as a strong inhibitor of mucosal mast cell degranulation. Peripheral CCK-1R are demonstrated to be crucial in the nutritional modulation of mast cells. These data are supported by an earlier report that demonstrated a critical role for CCK-1R in the anti-inflammatory effects of lipid-rich nutrition following hemorrhagic shock (31). The observation that CCK-1R antagonists did not affect MMCP I levels in fasted animals confirmed that the observed CCK-1R-mediated inhibition of mast cell responsiveness depends on nutritional intake. In line with our findings, Vergara et al. (50) reported mast cell inhibition by infusion of high doses of CCK. Since CCK release in the proximal intestine is enhanced by dietary lipids and proteins, in the present study lipid-rich and isocaloric low-lipid feedings were applied that proved to be effective in previous studies (8, 30). Enrichment of the feeding with lipids resulted in strongest mast cell inhibition, thus stressing the importance of carefully designed nutritional compositions to obtain optimal anti-inflammatory effects in the intestine.

CCK released in the gut wall can activate the central nervous system via the afferent vagus nerve but also via the humoral route (4). In the present model we employed pegylated-CCK9, a well-characterized activator of the afferent vagus nerve that solely acts peripherally (48). Pegylated-CCK9 reduced MMCP I levels, thus mimicking the nutritional inhibition of mast cells and confirming the importance of the neural route in this context. It should be noted that the vagus nerve consists of several types of neurons that respond to different stimuli (49). To investigate whether afferent vagus stimulation attenuates mast cell responsiveness also following a nonpeptide trigger, next the vanilloid receptor TRPV1 was stimulated. The TRPV1 is abundantly expressed on afferent nerve endings in the gastrointestinal tract with diverse functions including the transmission of nociception (47). Although TRPV1 agonist SA13353 was previously reported to reduce inflammation in models of endotoxemia and arthritis (37), in the present study SA13353 did not significantly reduce MMCP I levels. This observation suggests that stimulation of the afferent vagus nerve needs to be specific, e.g., via nutrition and CCK-1R, to inhibit mast cell activity. In theory, our data do not exclude the possibility that CCK binds to mast cells directly. Indeed, evidence has been provided that mouse fetal skin-derived mast cells possess CCK-receptors; however, to our knowledge these observations have not been replicated in intestinal mast cells (14). In addition, the finding that nAChR antagonists and selective vagotomy abrogate the nutritional effects on mucosal mast cells indicates that direct actions of CCK do not play a major role in vivo.

Nutritional effects on mucosal mast cell reactivity were inhibited by chlorisondamine and α-agonist, pointing at a crucial role for nAChR activation. α-Bungarotoxin is commonly used as a nAChR α7 agonist, although affinity has been reported also for other subunits like α9 and α10 (52). Furthermore, selective intestinal vagal denervation abrogated the mast cell inhibitory effects of lipid-rich nutrition. Cholinergic inhibition of mast cells was further investigated in an in vitro setting. First, inhibition of MC/9 by acetylcholine and nicotine was shown following LPS. Acetylcholine was demonstrated to be a stronger mast cell inhibitor compared with nicotine, which is in accordance with previous macrophages studies (5). Because the range of nAChR subtypes that are expressed by MC/9 cells has not been determined, the role of the nAChR α7- and β2-subunit was investigated in bone marrow-derived mast cells. In these cells, stimulation of the nAChR α7 was shown to inhibit mast cells to a similar extent compared with nicotine and acetylcholine. Interestingly, the β2-subtype, which was previously shown to mediate the cholinergic stimulation of phagocytosis (46), was not involved in the mast cell inhibitory effects of nicotine and acetylcholine. Taken together, this study points toward an important role for nAChR α7 in nutritional mast cell inhibition, although our studies do not exclude other subunits that are expressed on mast cells that are targeted by the vagal nerve, to be involved. Further studies, e.g., using knockout mice, are needed to provide direct evidence on the role of nAChR α7 and other nAChR subunits.

In this study, cholinergic agents were shown to inhibit mast cell activation after a mast cell-specific trigger of anti-OVA IgE followed by OVA. These data are in congruence with studies of Kageyama-Yahara et al. (19) and Mishra et al. (36) that showed cholinergic inhibition of bone marrow-derived mast cells and the rat mast cell/basophil cell line RBL-2H3 and involvement of the nAChR α7. The present data on cholinergic modulation of mast cells fit in the growing body of literature concerning the extensive and complex interactions between intestinal mast cells and neurons (41, 44, 47). The majority of intestinal mast cells is apposed to nerve fibers, thus providing also a microanatomic basis for communications between nerves and mast cells in the intestinal wall (42). The findings of the present study do not exclude an additional direct inhibitory effect of n-3 and n-6 polyunsaturated fatty acids on mast cell activation, as shown previously in vitro (18). However, since CCK-1R and α7 nAChR antagonists blunted the nutritional effects, a direct mast cell inhibitory effect of small quantities of enteral lipids as used in the present study appears not to be of substantial importance.

Taken together, regulation of mast cell activity is a promising approach that could be therapeutic in various settings of intestinal and systemic inflammation. The present study provides evidence that early mucosal mast cell activity during a general inflammatory response is inhibited by nutritional stimulation of the CCK-1R and nAChR-mediated vagal anti-in-
flamatory reflex. These findings, giving novel insight in neuroimmune interactions triggered by enriched enteral nutrition, may contribute to more goal-directed clinical interventions.

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