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

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Male C57/B6 mice, aged 10–12 wk, were purchased from Charles River Laboratories (Maastricht, the Netherlands). Mice were housed under controlled conditions of temperature and humidity.
in groups of three to four per cage. Prior to the experiments, mice were drug and test naive and had ad libitum access to study groups’ food, water, and chow. 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 and earlier experiments in which agonists and antagonists were groups of eight animals. Group sizes were based on previously

Stimulation of the afferent vagus nerve in fasted animals was assessed by administration of 6 μg/kg pegylated-CCK9 (produced by Dr. Verbaes, Kortrijk, Belgium) dissolved in sterile saline. Pegylated-CCK9 specifically binds CCK-1R and does not cross the blood brain barrier (48). To investigate the effects of afferent vagus stimulation via the transient receptor potential vanilloid 1 (TRPV1), the specific agonist SA13353 (12 mg/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 α7 antagonist α7-bungarotoxin (2 μg/kg; Sigma), which does not pass the blood barrier or, or its vehicle (PBS), was applied. Whereas α7-bungarotoxin is widely used as a blocker of the nAChR α7, 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 MC/9 and bone marrow-derived mast cells.** MC/9 (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 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). MC/9 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 C57Bl6 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.
Before stimulation, mast cells were washed, resuspended in Tyrode's buffer (10 mM HEPES, 130 mM saline, 6.2 mM d-glucosamine, 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 \times 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 a concentration of 2 mM cholinesterase inhibitor pyridostigmine bromide (Sigma). Bone marrow-derived mast cells were also stimulated with the selective nAChR α7 agonist GSK1345038A (supplied by GlaxoSmithKline) 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 DMDSO) 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 multichannel 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: \((|a - b|/(a + b)) \times 100\), where \(a\) is the amount of β-hexosaminidase released from stimulated cells that are pretreated with cholinergic agents, \(b\) is the amount released from unstimulated cells (basal release by cells incubated with Tyrode's buffer only), and \(t\) is β-hexosaminidase released by stimulated cells without cholinergic agents.

**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 FACSort 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 vagus 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-inflammato-
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 animals (Fig. 3A; \( P < 0.05 \)) compared with fasted controls (\( P < 0.05 \)).

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-vivo vagotomy was performed.
testinal vagotomy was performed 2 wk prior to LPS challenge. As expected, no gastric distention was observed after selective vagal denervation of the intestine, indicating that gastric motility was not affected by the surgical procedure and that selective denervation of the intestinal region was achieved (6). Whereas lipid-rich nutrition reduced MMCP 1 levels in sham-operated animals (Fig. 3C; P < 0.05), no alterations in MMCP 1 levels were observed between fed and fasted animals that underwent intestinal denervation.

The antihistaminic drug ketotifen reduces cytokine release and preserves intestinal integrity following LPS. Earlier studies implicated a prominent role for mast cells in the development of the general inflammatory response and intestinal barrier dysfunction (1, 21). To assess whether mucosal mast cells are involved in cytokine release and loss of gut wall integrity following LPS exposure, fasted animals were pretreated with ketotifen. Ketotifen, which possesses histamine-1 receptor antagonizing as well as mast cell stabilizing properties, significantly decreased plasma levels of proinflammatory cytokine IL-6 following LPS compared with animals receiving vehicle (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 1 (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 1, a protein secreted by mucosal mast cells in the gastrointestinal tract (3). The detection of MMCP 1 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...
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.

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 C57Bl/6 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.
Nutritional effects on mucosal mast cell reactivity were inhibited by chlorisondamine and α-agonists, 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 polysaturated fatty acids on mast cell activation, as shown previously in vitro (18). However, since CCK-1R and αα2nAChR 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-
flammary 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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DISCLOSURES

M. Skynner works for GlaxoSmithKline, the company that produces GSK1345038A.

AUTHOR CONTRIBUTIONS


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