Nutritional stimulation of cholecystokinin receptors inhibits inflammation via the vagus nerve

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The immune system in vertebrates senses exogenous and endogenous danger signals by way of complex cellular and humoral processes, and responds with an inflammatory reaction to combat putative attacks. A strong protective immunity is imperative to prevent invasion of pathogens; however, equivalent responses to commensal flora and dietary components in the intestine have to be avoided. The autonomic nervous system plays an important role in sensing luminal contents in the gut by way of hard-wired connections and chemical messengers, such as cholecystokinin (CCK). Here, we report that ingestion of dietary fat stimulates CCK receptors, and leads to attenuation of the inflammatory response by way of the efferent vagus nerve and nicotinic receptors. Vagotomy and administration of antagonists for CCK and nicotinic receptors significantly blunted the inhibitory effect of high-fat enteral nutrition on hemorrhagic shock-induced tumor necrosis factor-α and interleukin-6 release ($P < 0.05$). Furthermore, the protective effect of high-fat enteral nutrition on inflammation-induced intestinal permeability was abrogated by vagotomy and administration of antagonists for CCK and nicotinic receptors. These data reveal a novel neuroimmunologic pathway, controlled by nutrition, that may help to explain the intestinal hyporesponsiveness to dietary antigens, and shed new light on the functionality of nutrition.

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The immune system in vertebrates senses exogenous and endogenous danger signals by way of complex cellular and humoral processes, and responds with an inflammatory reaction to combat putative attacks (1). Although inflammation is essential to protect the host from invasion of potentially harmful pathogens, an overwhelming inflammatory response that leads to tissue damage, increased vascular permeability, and organ injury has to be avoided (2, 3). In the gastrointestinal tract, hyperactivation of the immune system to commensal bacteria and dietary antigens is inhibited continuously to maintain homeostasis, and to allow absorption and utilization of nutrients (4). Recently, we showed that dietary fat strongly reduced the systemic inflammatory response after hemorrhagic shock; this indicated a direct interaction between specific food components and the systemic immune response (5, 6).

Ingestion of food triggers a cascade of responses, such as initiation of gut contractility and regulation of food intake, by way of hard-wired connections and chemical messengers (e.g., cholecystokinin [CCK] and PYY_{3-36}) (7–10). Besides regulation of metabolism, the parasympathetic nervous system recently was identified to inhibit macrophage activation by way of the vagus nerve through binding of acetylcholine to α-7 nicotinic receptors located on macrophages (11, 12). Central or peripheral stimulation of this so-called "cholinergic antiinflammatory pathway" reduced plasma TNF-α in endotoxic shock, and blunted NF-κB activation after hemorrhagic shock by way of efferent vagal nerve fibers (13–15). We reasoned that high-fat enteral nutrition, sensed in the gastrointestinal tract, activates the parasympathetic nervous system, and leads to inhibition of the inflammatory response by way of efferent vagal fibers.

RESULTS AND DISCUSSION

To investigate whether a neural based anti-inflammatory pathway is involved in the effect

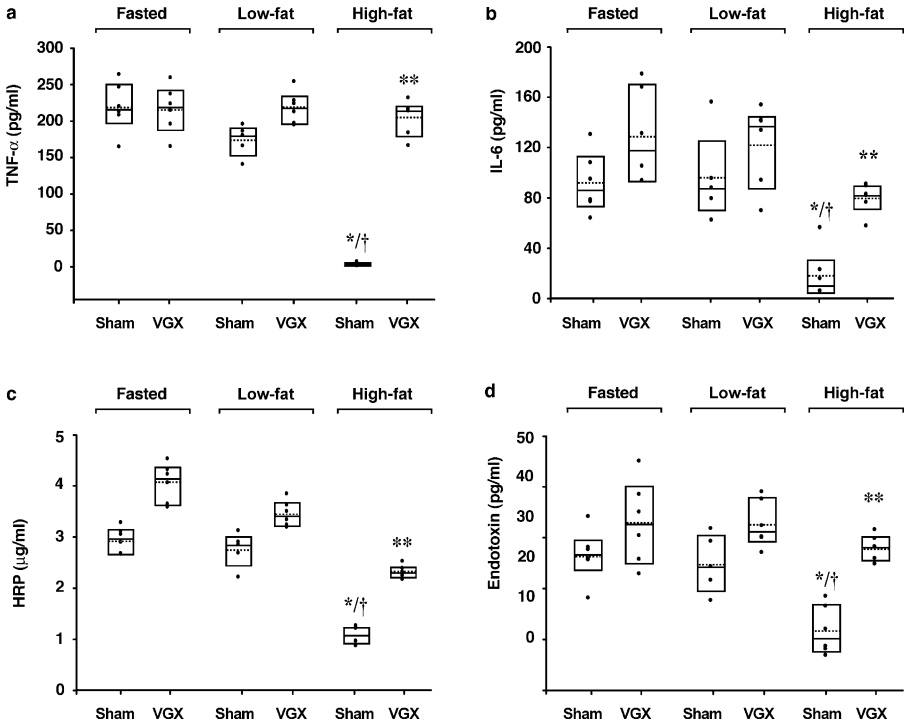

Figure 1. Vagotomy blunts the inhibitory effect of high-fat enteral nutrition on the inflammatory response and preserves gut barrier function. Rats ($n = 6$ per group) were fasted or fed low-fat or high-fat enteral nutrition before (Sham) vagotomy (VGX) and hemorrhagic shock (Hem. Shock). Inhibition of TNF-α (a), IL-6 (b), leakage of HRP in ileum (c) and endotoxin (d) after hemorrhagic shock by high-fat nutrition is reversed by vagotomy. Data are solid dots, mean (dashed line), median (solid line), 25th and 75th percentiles. *$P < 0.01$ versus fasted Sham + Hem. Shock; **$P < 0.01$ versus high-fat treated Sham + Hem. Shock; †$P < 0.05$ versus low-fat treated Sham + Hem. Shock.

of high-fat enteral nutrition, Sprague-Dawley rats were subjected to (sham) vagotomy, 45 min before induction of hemorrhagic shock as described in Materials and methods. Animals were fasted or fed enterally with high-fat or low-fat nutrition 18 h, 2 h, and 45 min before hemorrhagic shock was induced. Inflammatory mediators and gut barrier function were assessed 90 min after shock.

Typically, hemorrhagic shock results in systemic release of proinflammatory cytokines, such as TNF-α and IL-6 (16). In line with our earlier observations, high-fat enteral nutrition (containing 52% [energy %] fat) strongly reduced hemorrhagic shock-induced TNF-α and IL-6 in rats that were subjected to sham vagotomy, compared with low-fat and fasted controls (containing 17% fat) (Fig. 1, a and b). These data show that the percentage of fat in the enteral diet is a determinant of protection, because the inflammatory response was affected only mildly in the low-fat control group. Vagotomy abrogated the high-fat–induced reduction in TNF-α (205 ± 11 pg/ml vs. 5 ± 1 pg/ml [sham]; $P < 0.01$) and IL-6 levels (80 ± 5 pg/ml vs. 19 ± 9 pg/ml [sham]; $P < 0.01$) after hemorrhagic shock compared with rats that underwent a sham vagotomy.

Changes in intestinal barrier function were evaluated by determination of bacterial translocation to distant organs, leakage of horseradish peroxidase (HRP) in isolated ileum-segments, and plasma endotoxin levels as described in Materials and methods.

In line with previous reports (16, 17), the inflammatory response in control shock-rats was paralleled by bacterial translocation to distant organs (Table I), an increased permeability for HRP, and detectable endotoxin levels (Fig. 1, c and d). Impairment of gut barrier function after hemorrhagic shock likely is caused by proinflammatory cytokines, because application of cytokines (e.g., TNF-α) to intestinal cells increased intestinal permeability; decreased inflammatory cytokines prevented loss of intestinal barrier function (18–20). In accordance with high-fat enteral nutrition–induced inhibition of the inflammatory response, circulating endotoxin levels, permeability of ileum segments for HRP, and bacterial translocation to distant organs were reduced compared with low-fat–treated and fasted sham vagotomized rats. Vagotomy reversed this protection of high-fat nutrition and led to elevated plasma endotoxin levels (from 12 ± 2 pg/ml to 28 ± 1 pg/ml, $P < 0.01$), increased leakage of HRP in ileum segments (from 1.1 ± 0.7 μg/ml to 2.3 ± 0.5 μg/ml, $P < 0.01$) and increased bacterial translocation (from 16 CFU/g tissue to 328 CFU/g, $P < 0.01$), (Fig. 1, c and d; Table I). Based on these findings we concluded that a parasympathetic neural control mechanism underlies the protective effect of enteral nutrition containing a high percentage of fat.

Nutrition activates the autonomic nervous system in several ways, based on the nature of its composition. Dietary fat typically results in release of CCK, a potent neuro-endocrine signal-

Table I. Total bacterial translocation to distant organs after hemorrhagic shock

Groups	High fat	Fasted
	CFU/g tissue	*CFU/g tissue*
Sham-VGX	16 (0–65)[a]	412 (206–517)
VGX	328 (183–1459)	542 (164–849)
Sham-VGX + CCK-ra	267 (158–837)[b]	
Sham-VGX + vehicle (CCK-ra)	57 (23–217)	
Sham-VGX + chlorisondamine	226 (34–1410)[b]	172 (56–488)
Sham-VGX + vehicle (Chlor.)	22 (5–71)	

All rats were subjected to hemorrhagic shock. Mesenteric lymph nodes, spleen, and liver were cultured at sacrifice (90 min). High fat–treated or fasted rats were subjected to (sham) vagotomy (VGX) and treated with CCK-receptor antagonists (CCK-ra), chlorisondamine, or vehicle where indicated. Total bacterial translocation is expressed as CFU/g tissue. Results are median (range); $n = 6$/group.
[a]$P < 0.05$ compared with VGX.
[b]$P < 0.05$ compared with vehicle treated.

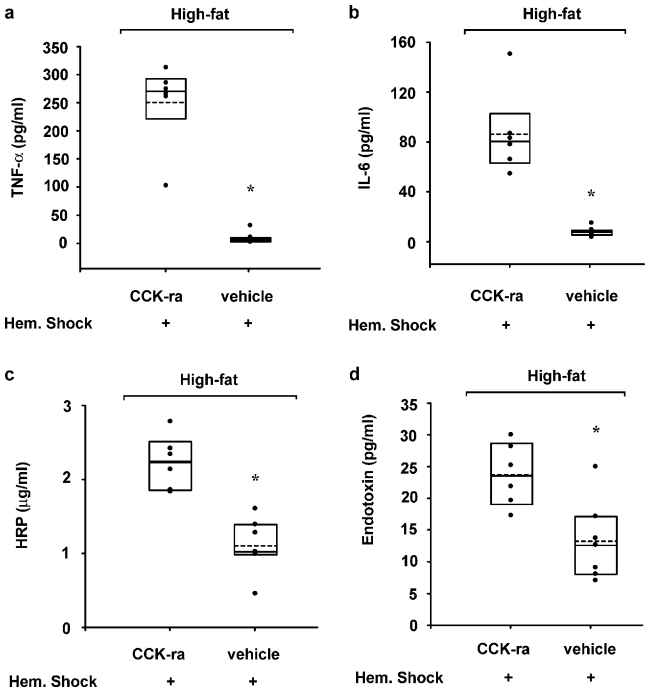

Figure 2. CCK-receptor antagonists increase the inflammatory response and deteriorate gut barrier function in high-fat treated rats subjected to sham vagotomy and hemorrhagic shock. High-fat treated rats ($n=6$ per group) were intravenously injected with CCK-receptor A+B antagonist (CCK-ra) or vehicle before hemorrhagic shock. TNF-α (a), IL-6 (b), leakage of HRP in ileum (c) and endotoxin (d) were increased after hemorrhagic shock in high-fat treated rats injected with CCK-receptor A+B antagonists in high-fat treated rats vagotomized before hemorrhagic shock. Data are solid dots, mean (dashed line), median (solid line), 25th and 75th percentiles. *$P < 0.01$ versus vehicle treated group.

ing molecule that activates nerve cells by way of CCK-A and CCK-B receptors (for review see reference 21). To investigate the role of CCK in the protective effect of high-fat enteral nutrition on the host response to hemorrhagic shock, rats underwent a sham vagotomy and received CCK-A (500 μg/kg) and CCK-B (500 μg/kg) receptor antagonists (22, 23) or vehicle (90% saline, 5% Tween, 5% DMSO) intravenously 25 min before hemorrhagic shock. CCK-receptor blockade potentially alters lipid digestion. However, levels of circulating triglycerides in rats treated with CCK-receptor antagonist (187 ± 8 mg/dL) were similar compared with rats treated with vehicle (200 ± 16 mg/dL), indicating that lipid absorption in the acute phase is unaffected.

Administration of CCK-A and CCK-B receptor antagonists enhanced plasma TNF-α (251 ± 30 pg/ml) and IL-6 levels (87 ± 14 pg/ml) following hemorrhagic shock, compared with high-fat treated rats administered vehicle (10 ± 4 pg/ml, $P < 0.01$ and 9 ± 1 pg/ml, $P < 0.01$, respectively) (Fig. 2, a and b). Furthermore, plasma endotoxin was elevated (24 ± 2 pg/ml vs. 13 ± 2 pg/ml [vehicle], $P = 0.01$), permeability for HRP was increased (2.2 ± 0.1 μg/ml vs. 1.1 ± 0.1 μg/ml [vehicle], $P < 0.01$) (Fig. 2, c and d), while more bacteria translocated to distant organs (total 267 CFU/gram [158–837] vs. total 57 CFU/g [23–217] [vehicle], $P < 0.01$), (Table I) in animals injected with CCK-receptor antagonists, compared with vehicle treated controls. These findings cannot be attributed to injection of CCK-receptor antagonists, since stimulation of peritoneal macrophages from rats with both receptor antagonists did not trigger TNF-α release (<10 pg/ml, below detection limit). Furthermore, injection of CCK-receptor antagonists in rats not subjected to hemorrhagic shock did not elict TNF-α release (13 ± 5 pg/ml) and did not cause bacterial translocation (total: 0 CFU/gram (0–7) or increased leakage of HRP (0.6 ± 0.1 μg/ml) in ileum segments, which is not different from healthy control rats. These data show that high-fat enteral nutrition inhibits the proinflammatory response and prevents loss of intestinal barrier integrity by way of activation of CCK receptors.

Stimulation of the parasympathetic nervous system may result in activation of the hypothalamic-pituitary-adrenal (HPA) axis (24). Alternatively, vagal efferent fibers can be stimulated causing inhibition of the inflammatory response by way of nicotinic receptors. To assess activation of the HPA axis, corticosterone levels were measured in plasma. High-fat enteral nutrition enhanced circulating corticosterone after hemorrhagic shock in sham-vagotomized shock animals (19 ± 4 ng/ml) compared with fasted rats (6 ± 4 ng/ml, $P = 0.046$). However, both vagotomy (9 ± 4 ng/ml, $P = 0.09$ compared with sham vagotomized controls [19 ± 4 ng/ml]) and administration of CCK-receptor antagonists (24 ± 1 ng/ml, $P = 0.39$ compared with vehicle treated rats 22 ± 2 ng/ml) did not significantly affect corticosterone levels in high-fat treated rats. These data are in line with earlier reports showing that vagotomy does not affect corticosterone levels (25). It may well be that the increase in circulating corticosterone is an epiphenomenon caused by stress of oral gavage (26).

Next, we studied whether stimulation of CCK-receptors by high-fat enteral nutrition inhibits the inflammatory re-

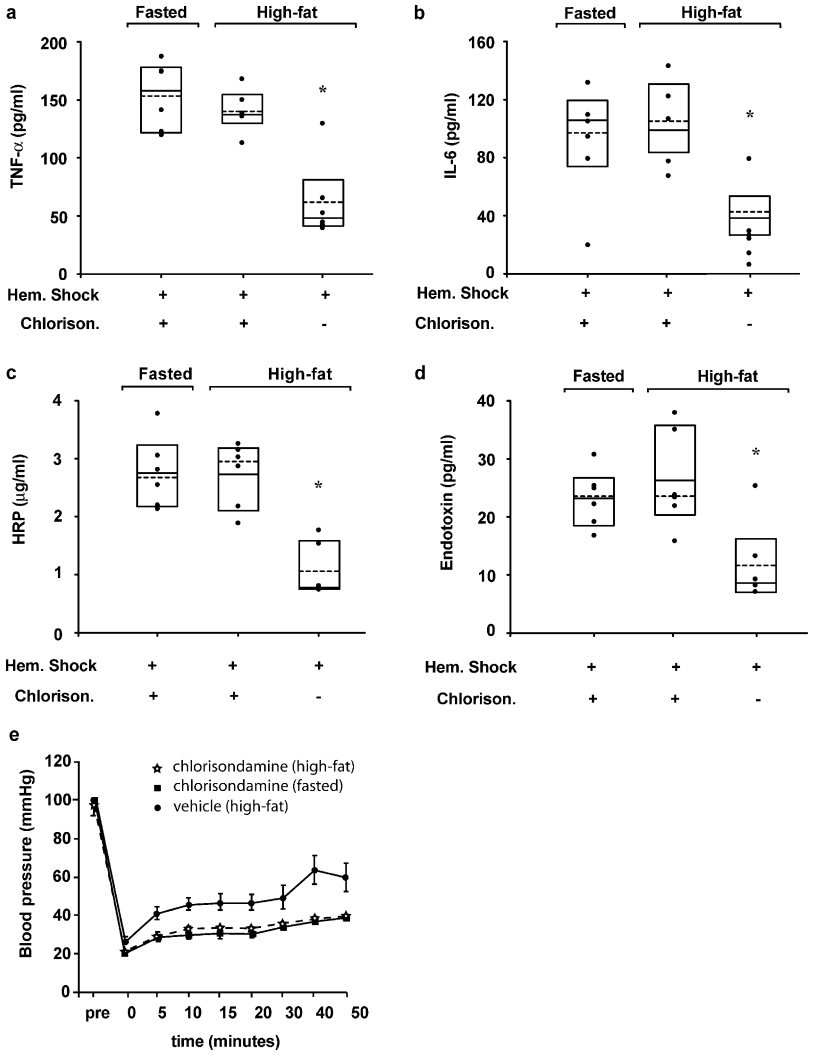

Figure 3. Inhibition of nicotinic receptors increases inflammation and deteriorates gut barrier function in high-fat treated rats subjected to sham vagotomy and hemorrhagic shock. High-fat treated rats ($n = 6$ per group) were injected intravenously with chlorisondamine (Chlorison.) or vehicle before hemorrhagic shock. TNF-α (a), IL-6 (b), leakage of HRP in ileum (c) and endotoxin (d) were increased after hemorrhagic shock in rats injected with chlorisondamine. There were no significant differences between fasted and high-fat treated rats treated with chlorisondamine. Data are solid dots, mean (dashed line), median (solid line), 25th and 75th percentiles. *$P < 0.01$ versus vehicle treated group. Treatment with chlorisondamine did not affect mean arterial pressure (MAP) before and just after hemorrhagic shock (e), although there was a significant difference in MAP between chlorisondamine and vehicle treated rats from 10 to 50 min during the observation period ($P < 0.05$).

sponse by way of the anti-inflammatory efferent vagal pathway, by inhibition of peripheral nicotinic receptors using chlorisondamine diiodide. Chlorisondamine diiodide or vehicle (saline) were administered 25 min before induction of hemorrhagic shock in a dose (0.125 mg/kg) that blocks only peripheral nicotinic receptors (15, 27). A control (fasted) hemorrhagic shock group that received chlorisondamine was included, because inhibition of nicotinic receptors can cause vasodilatation and hypotension (27). Administration of chlorisondamine at this dose did not cause additional hypotension or changes in heart rate before and just after induction of shock (Fig. 3 e). Mean arterial pressure was significantly lower during the 50 min observation period compared with vehicle treated controls, however, this did not affect the shock-induced inflammatory response and loss of gut barrier integrity. Chlorisondamine abrogated the inhibitory effect of high-fat enteral nutrition on circulating TNF-α (140 ± 7 pg/ml vs. 63 ± 14 pg/ml [vehicle], $P < 0.01$) and IL-6 (99 ± 12 pg/ml vs. 30 ± 10 pg/ml [vehicle], $P < 0.05$), (Fig. 3, a and b). TNF-α and IL-6 levels in these high-fat rats treated with chlorisondamine were comparable with those of chlorisondamine-treated fasted rats. Inhibition of nicotinic receptors in high-fat treated rats by way of administration of chlorisondamine led to increased bacterial translocation to distant organs (total 226 CFU/g, $P < 0.05$ vs. total 22 CFU/g [vehicle]), (Table I), increased permeability for HRP in ileum

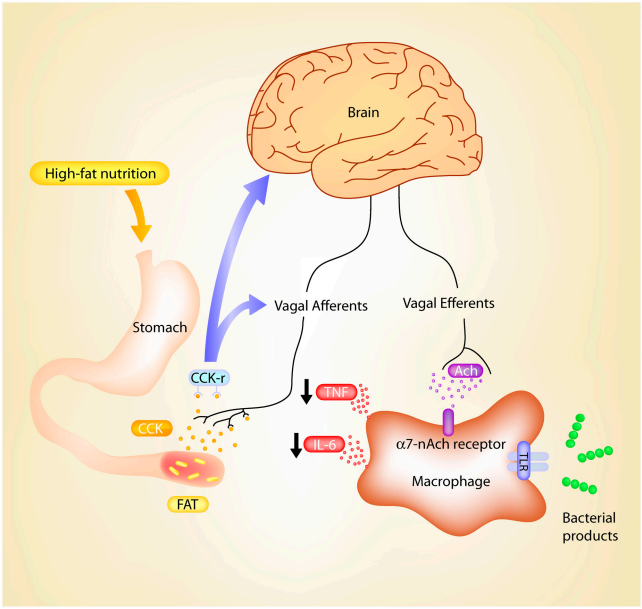

Figure 4. Dietary fat inhibits the inflammatory response by way of stimulation of CCK-receptors leading to activation of nicotinic receptors by vagal efferents. Ingestion of high amounts of fat induces release of cholecystokinin (CCK) that binds to CCK-A and CCK-B receptors (CCK-r) located centrally or on peripheral vagal afferents. Activation of CCK-receptors triggers vagal efferents leading to an increase of acetylcholine (Ach), the principal parasympathetic neurotransmitter. Release of inflammatory cytokines such as TNF-α and IL-6 after activation of Toll-like receptors by bacterial products is inhibited by way of binding of acetylcholine to α-7 nicotinic (α7-nAch) receptors.

segments (2.7 ± 0.2 μg/ml, $P < 0.01$ vs. 1.1 ± 0.2 μg/ml [vehicle]) and elevated plasma endotoxin levels (26 ± 3 pg/ml, $P < 0.05$ vs. 12 ± 3 pg/ml [vehicle]), compared with high-fat treated rats administered vehicle (Fig. 3, c and d). These findings indicate that high-fat enteral nutrition inhibits inflammation by stimulation of nicotinic receptors by way of efferent vagal fibers.

The present study shows that high-fat enteral nutrition stimulates CCK-receptors centrally or peripherally by way of the afferent vagus nerve leading to inhibition of the inflammatory response by way of vagal efferents and nicotinic receptors (Fig. 4). Previously, we showed that the beneficial effects of high-fat enteral nutrition on inflammation and intestinal barrier integrity are specific for the amount of lipids in the enteral nutrition, not related to caloric intake and cannot be attributed to formation of endotoxin neutralizing triacylglycerol-rich lipoproteins (5, 6, 16). The finding that high-fat enteral nutrition inhibits inflammation by way of the vagus nerve provides a functional new mechanism for the interaction between nutrition and the immune response and has widespread implications. It was previously unrecognized that nutrition-induced neuro-endocrine signals such as CCK modulate the immune response by way of the efferent vagus nerve. From a teleological point of view it is functional that a state of immune-hyporesponsiveness is created during feeding. In this way an unwanted response to temporally present high amounts of dietary antigens, biological toxins and destructive endogenous lysozymes in the gut lumen is prevented, gut barrier function is preserved and homeostasis maintained.

We propose that this neural feedback-loop activated by enteral nutrition is an important player in the thus far largely unexplained state of hyporesponsiveness of the immune system in the intestinal tract to dietary antigens and bacterial toxins.

Based on our findings, high-fat enteral nutrition is potentially therapeutic in various inflammatory disorders such as sepsis and inflammatory bowel disease (IBD) characterized by an inflammatory response in which TNF-α is prominent and intestinal barrier function is impaired. In light of this, a fasted state could be a risk factor for developing a potentially lethal inflammatory response after trauma or injury.

MATERIALS AND METHODS

Reagents. CCK-A (Devazepide) and CCK-B (L365, 260) receptor antagonists were gifts from ML Laboratories PLC and dissolved in 90% NaCl, 5% Tween 20, 5% dimethyl sulfoxide (DMSO) to a final concentration of 500 μg/ml. Chlorisondamine diiodide, a nicotinic receptor antagonist was purchased from Tocris Cookson Ltd. and dissolved in saline to a final concentration of 0.125 mg/ml.

Animals. Healthy male Sprague-Dawley rats, weighing 280–420 g (average 360 g) were purchased from Charles River Laboratories, and were housed under controlled conditions of temperature and humidity. Before the start of the experiments, rats were fed ad libitum with standard rodent chow and had free access to water. The experimental protocol was performed according to the guidelines of the Animal Care Committee of the University of Maastricht and approved by the committee.

Experimental design and procedures. A nonlethal hemorrhagic shock model was used as previously described (6, 16). In short, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p); the femoral artery was dissected and cannulated with polyethylene tubing (PE-10) containing heparinized saline (10 IU/ml). Mean Arterial Pressure (MAP) and heart rate (HR) were continuously recorded during a 50-min observation period. At the time of shock (t=0), 2.1 ml blood per 100 g of body weight was taken at a rate of 1 ml/minute (representing 30–40% of the total blood volume). At 45 min before induction of hemorrhagic shock, rats were either subjected to vagotomy or sham vagotomy. In vagotomized animals a ventral cervical incision was made and both vagal trunks exposed. The vagus nerve was ligated at both ends using 4–0 silk suture and divided. In sham-operated animals both vagal trunks were exposed, but the vagus nerve was not ligated and divided.

Before the experiments, rats were fasted ($n = 18$) or fed with low-fat or high-fat enteral nutrition by way of oral gavage ($n = 36$). The high-fat liquid enteral diet contained 6.9% (energy %) proteins, 40.9% carbohydrates, and 52.2% fat; the low-fat nutrition contained 6.9% proteins, 75.4% carbohydrates and 16.7% fat. The amount of fat in the low-fat diet was isocalorical to that present in standard rodent chow and the high-fat liquid enteral diet was isocaloric and isonitrogenous to the low-fat diet. Proteins were derived from lean milk, and the carbohydrate source was a mixture of sucrose and cornstarch. The lipid source was vegetable oil with a fatty acid composition of 8.1% saturated fatty acids; 58.9% monounsaturated fatty acids, of which oleic acid was the main source (57.4%); 28.2% consisted of polyunsaturated fatty acids, of which linoleic acid was the main source (23%); the amount of n-3 and n-6 fatty acids in the high-fat nutrition was <5% of the total fat content. The types of carbohydrates and fat used in both diets were identical. As described before (16), 3 ml was given 18 h before hemorrhagic shock and 0.75

ml at 2 h and 45 min before hemorrhagic shock by way of oral gavage. Fasted and high-fat–treated rats underwent vagotomy or sham vagotomy. To investigate the role of CCK, animals that were fed with high-fat nutrition subjected to sham vagotomy were injected i.v. with CCK-A (500 μg/kg) and CCK-B (500 μg/kg) receptor antagonists ($n = 6$) or vehicle (90% NaCl, 5% Tween 20, 5% DMSO, $n = 6$) 25 min before induction of shock. Potential proinflammatory properties of both CCK-receptor antagonists were investigated by stimulation of peritoneal macrophages isolated from rats ($n = 3$) and injection of CCK-A and CCK-B receptor antagonists in rats not subjected to hemorrhagic shock ($n = 3$). To determine whether the observed effects were specific for stimulation of the cholinergic antiinflammatory pathway, peripheral nicotinic receptors were blocked by intravenous administration of chlorisondamine at 25 min before induction of shock ($n = 6$) in high-fat treated rats, subjected to sham vagotomy. To control for the decrease in MAP (from 100 mm Hg to 65 mm Hg) associated with administration of chlorisondamine, fasted, sham vagotomized rats treated with chlorisondamine were included as controls ($n = 6$). At 90 min after hemorrhagic shock, blood was taken and segments of small bowel were harvested for determination of gut permeability. Plasma was separated by centrifugation, frozen immediately and stored (−20°C) until analysis.

Cytokine analysis. TNF-α, IFN-γ, IL-6 and IL-10 concentrations in arterial blood were determined using a standard ELISA for rat TNF-α and rat IFN-γ (both provided by Hbt, Uden, the Netherlands), rat IL-6 (BD Biosciences) and rat IL-10 (Biosource).

Intestinal permeability. Intestinal permeability for macromolecules was assessed by measuring translocation of the 44-kD enzyme horseradish peroxidase (HRP; Sigma-Aldrich) by the everted gut sac method as described (16).

Microbiological methods. Bacterial translocation to distant organs was assessed as described (6, 16). In short, mesenteric lymph nodes (MLN), the mid-section of the spleen and a liver-segment (IV) were collected aseptically in preweighed thioglycolate broth tubes (Becton Dickinson [BBL] Microbiology Europe) in all rats. Tissue-fragments were homogenized and the entire suspension was transferred to agar plates (Columbia III blood agar base supplemented with 5% vol/vol sheep blood (BBL) (duplicate plates) and Chocolate PolyviteX agar (BioMérieux). After 48 h of incubation, colonies were counted, determined using conventional techniques, adjusted to tissue weight, and expressed as number of CFUs per gram of tissue.

Statistical analyses. Bacterial translocation data are represented as median and range; all other data are represented as mean ± SEM. A Mann-Whitney U test was used for between-group comparisons. Differences were considered statistically significant at $P < 0.05$.

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Note added in proof. de Jonge and colleagues recently showed that stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2–STAT3 signaling pathway (de Jonge, W.J., E.P. van der Zanden, F.O. The, M.F. Bijlsma, D.J. van Westerloo, R.J. Bennink, H.R. Berthoud, S. Uematsu, S. Akira, R.M. van den Wijngaard, and G.E. Boeckxstaens. 2005. *Nat. Immunol.* 6:844–851).

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