The nutritional anti-inflammatory reflex; from rodents to man

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Tim Lubbers

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Chapter 1

General introduction

1. Introduction

1.1 The immune system

1.1.1 The inflammatory response

Inflammation is a normal response to a disturbed homeostasis caused by infection and injury. The immune system is activated to neutralize invading pathogens, remove injured tissue and promote wound healing [1]. This complex mechanism of cellular and humoral components consists of the innate and adaptive immune system [2]. The innate immune system forms the fist line of defense by continuously monitoring the condition of the body and discriminating endogenous cells and components from pathogen associated molecular patterns (PAMPs) or signals of tissue and cell damage, the so-called danger associated molecular patterns (DAMPs) [3]. The cells of the innate immune system rely on pattern recognition receptors (PRR) to rapidly act towards a wide array of potential threats [4], including PAMPs and DAMP's (Figure 1)[4-6]. The best characterized PPRs are toll-like receptors (TLRs), i.e.membrane bound receptors of which the majority is localized on the cell-surface. Activation of TLRs results in production and secretion of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, and interferons via activation of the transcription factor nuclear factor (NF)-κB and interferon regulatory factors [7,8]. In contrast to TLRs, NOD-like receptors (NLR) are PPRs confined to the cytosol that detect intracellular motifs, which enables them to detect PAMPs and DAMPs within the cytosol that cannot be recognized by membrane-bound receptors [9]. NLRs trigger immune responses via activation of NF-κB and formation of inflammasomes, which drive proteolytic processing of cytokine precursors, including pro-IL-1β and pro-IL-18 [6,10]. The NLR-mediated pro-inflammatory response is not confined to pathogen recognition, but can also be initiated by programmed cell death [4]. Next to activation of innate immune responses, TLRs and NLRs are also involved in the crosstalk between the innate and adaptive immune system [4,9].

The innate immune system provides instant protection against infection in a generic manner. After identification of a microbial substance, the innate immune system is able to immediately destroy the pathogen. Upon phagocytosis by antigen presenting cells, the processed pathogen is presented to the adaptive immune system [4]. The adaptive immune system generates a highly targeted response to remove threats from the body and forms the specific line of defense [2].

The adaptive part of the immune system distinguishes itself from its innate counterpart by the ability to develop specific responses to antigens and the generation of immunological memory [4,11]. Together with or following rapidly after a pro-inflammatory stimulus, a regulatory anti-inflammatory response is initiated to

preserve the subtle immunologic balance [12]. This complex response is characterized by 1) upregulation of anti-inflammatory components including TLR inhibitory protein and IL-1 receptor associated kinase (IRAK)-M, 2) release of anti-inflammatory cytokines such as interleukin 10 and transforming growth factor (TGF)-β, 3) apoptosis of immune cells, 4) activation of the autonomic nervous system and 5) release of anticoagulatory proteins [13-15].

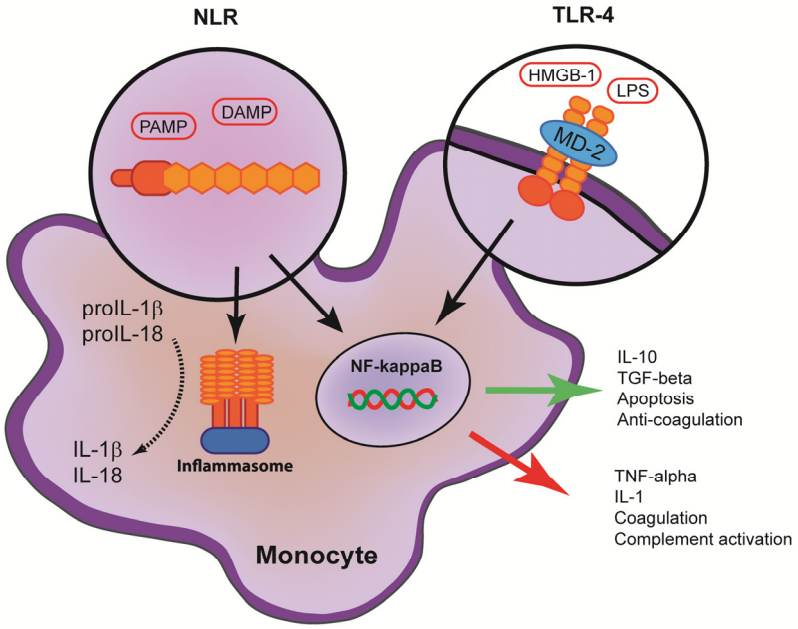

Figure 1: Initiation of the pro- and anti-inflammatory responses during inflammation. Pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), and danger associated molecular patterns (DAMPs), including high-mobility group protein B1 (HMGB-1), activate Toll-like receptors (TLRs) located on the plasma membrane and NOD-like receptors (NLR) within the cytosol. Activation of these pattern recognition molecules triggers various intracellular processes, including NF-kappaB activation and inflammasome formation leading to pro- and anti-inflammatory processes.

1.1.2 Dysregulated inflammatory response

Inflammation is essential to prevent microbial invasion and to promote tissue repair [16]. In several situations, including major surgery, trauma and sepsis, the inflammatory response can become excessive and harmful, leading to tissue damage, multi-organ failure and eventually death [16,17]. To date, the exact

pathophysiology underlying such dysregulation of the inflammatory response remains largely unknown.

The initial hyperinflammatory response, caused by sepsis or major injury, is associated with an excessive release of pro-inflammatory mediators and can progress into a state of immunosuppression, which increases susceptibility to secondary infection [18]. Although various studies describe that the pro- and anti-inflammatory response occur simultaneously, the early net result remains a hyperinflammatory response (Figure 2)[18].

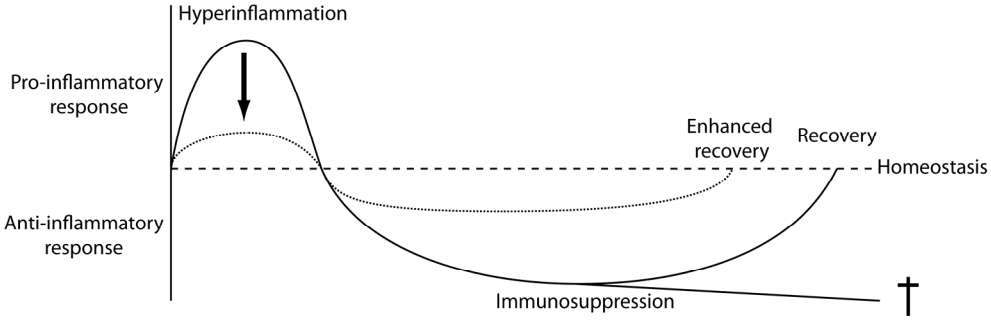

Figure 2: Course of the immunological response following major surgery, trauma and sepsis. Although the pro- and anti-inflammatory responses are activated almost simultaneously, a hyperinflammatory phase predominates the early phase. This hyperinflammatory state is followed by a phase of immunosuppresion. In this phase, the individual either recovers to a state of immunological homeostasis or succumbs to opportunistic infections (†). Interventions aimed at limiting the initial inflammatory response (downward arrow) could prevent major alterations in immunological homeostasis and enhance recovery (dotted line).

Nowadays most patients survive this hyperinflammatory phase as supportive treatments have greatly advanced in the last decades. However, large numbers of patients succumb to opportunistic infections during the subsequent state of immune paralysis [19-21]. The fact that many microbes, which are not particularly virulent in healthy patients, are responsible for secondary infection underlines such an immunosuppressed state in surgical and critically ill patients in the intensive care unit [22,23]. Attenuation of the early hyperinflammatory response has been considered essential to improve sepsis survival [24].

1.2 Immune modulating interventions

1.2.1 Anti-inflammatory treatments

Developing new modalities to treat the inflammatory response during sepsis and following trauma and major surgery has been particularly frustrating. The difficulty of successfully implementing novel treatments has partly been due to lack of understanding the complex immunological interplay that leads to the initial systemic inflammatory response and subsequent compensatory anti-inflammatory response. Most experimental approaches have primarily focused on inhibition of the initial pro-inflammatory response, whilst neglecting the successive immunosuppressive state [18]. The concept that an initial hyperinflammatory response induces significant cell damage and organ failure through uncontrolled cytokine release has evolved from rodent studies, which demonstrated that inhibition of single cytokines improved survival [25]. These findings were supported by human studies, indicating that excessive release of TNF-α is associated with the development of systemic inflammatory response syndrome, organ damage and mortality in sepsis [26]. In addition, circulating levels of TNF-α and IL-6 are correlated with the severity of sepsis in patients [27]. Although these data indicate that excess release of specific pro-inflammatory cytokines is damaging, implementation of anti-inflammatory treatments directed against such cytokines failed to improve survival in patients or even exacerbated their condition [26,28-30]. Moreover, patients effectively treated with cytokine inhibitors for inflammatory bowel disease were reported to be more vulnerable to infections and displayed an increased overall mortality [31,32]. These findings point out that selectively blocking the action of single inflammatory components is potentially harmful as it disrupts elementary immune responses.

Another drawback of clinical studies in which the blockage of single pro-inflammatory components has been tested, is the inadequate timing of these interventions. Most clinical trails using anti-TNF-α or anti-IL-1 were performed in patients with ongoing sepsis [26,28-30]. Excessive activation of the innate immune system during sepsis has been shown to result in a subsequent state of immunosuppression and increased susceptibility to secondary infection [33,34]. In this latter phase, anti-inflammatory treatments will not reduce the inflammatory response but even aggravate immune paralysis. Therefore, timing of immune-modulating treatments is critical. Administration of anti-inflammatory treatments in the early course inflammation should reduce the hyperinflammatory response and potentially limit the subsequent immunosuppressed state (see also Figure 2). On the other hand, interventions executed in the latter immunosuppressed state should be aimed at restoring immune competence.

1.2.2 Restoring immune competence

An immunosuppressed state is characterized by the inability of immune cells to produce pro-inflammatory cytokines [15]. Apoptosis of immune cells also plays a key role in the pathophysiology of immunosuppression, since apoptotic immune cells release significant amounts of anti-inflammatory mediators [25]. In rodent models of sepsis, prevention of apoptosis of lymphocytes and intestinal epithelial cells has been shown to increase survival [35,36]. However, until now apoptosis inhibitors have not been tested in humans due to the risk of uncontrolled cell growth and limited selectivity [13]. Moreover, inhibition of apoptosis will result in accumulation of neutrophils in inflamed tissues and could thus contribute to organ failure since apoptosis is an important mechanism to remove activated neutrophils [13].

Another strategy to enhance immune function during the suppressed state is administration of immune stimulating cytokines. Indeed, administration of interferon-γ or granulocyte-macrophage colony stimulating factor has been shown to restore monocytic function in septic patients in *ex vivo* experiments [37,38]. Although promising results have been reported in experimental models, the impact of this strategy in a clinical setting has not been investigated yet [39].

In conclusion, the development of novel treatment modalities designed to prevent or control a dysregulated inflammatory response should focus on: 1) interventions that broadly affect the immune response without nullifying single inflammatory components and thereby disrupting the complex immunological interplay, 2) timing of the immunomodulating intervention since an anti-inflammatory treatment will improve outcome in the hyperinflammatory state, whilst being detrimental in the immunosuppressed state.

1.2.3 Neuro-immune interactions

In the past two decades, the autonomic nervous system has been identified as a potent modulator of the immune response. Systemic inflammatory mediators released by activated immune cells, including IL-1β and TNF-α and microbial substances, such as LPS, have been shown to activate afferent vagal fibers [40-42]. Receptors for IL-1 as well as TLR4 that recognizes LPS have been identified on the afferent vagus [42,43]. Activation of afferent fibers by these components results in increased neuronal activation, local cytokine mRNA production in the brain and induction of sickness behavior including fever, anorexia, inactivity and pain [44-47]. In this way, the immune system gathers inflammatory information generated in the periphery and serves as extra sensory organ informing the brain about noxious stimuli [48]. The nervous system in turn controls the inflammatory response through humoral and hard-wired mechanisms.

The physiological, behavioral and hormonal responses triggered by this neuro-immune feedback mechanism serve a protective goal via three mechanisms: 1)

induction of fever 2) activation of the hypothalamic-pituitary-adrenal (HPA) axis and 3) stimulation of the cholinergic anti-inflammatory pathway [45,49,50].

Fever raises the core body temperature to a point where bacterial and virusal replication is interrupted, whereas white blood cell numbers rapidly increase and destructive enzymes function optimally. Since every degree of fever requires a 10-15% increase in energy, it is considered that most behavioral changes of the sickness response are directed at saving energy [45].

Cytokine activation of afferent vagal fibers activates a humoral anti-inflammatory pathway via the HPA-axis [51]. Afferent vagal activity is relayed to the hypothalamus, leading to an increased release of adrenocorticotropic hormone (ACTH) from the pituitary gland. Circulating ACTH increases the production and release of corticosteroids from the adrenal gland, which attenuate inflammation via binding to intracellular receptors [52,53]. Additionally, glucocorticoids exert their anti-inflammatory effect by various mechanisms including binding to glucocorticoid responsive elements on glucocorticoid responsive genes, resulting in upregulation of anti-inflammatory gene transcription and reducing pro-inflammatory gene transcription through NF-κB downregulation [54,55].

Nearly a decade ago, a hard-wired mechanism that inhibits inflammation through parasympathetic outflow was described [56]. Electrical stimulation of the vagus nerve limited TNF-α synthesis in the liver, reduced plasma TNF-α levels and prevented development of shock in endotoxemic mice. In this so-called cholinergic anti-inflammatory pathway, acetylcholine is released from activated efferent vagal fibers and binds to the alpha 7 subunit of the nicotinic acetylcholine receptor on inflammatory cells. The anti-inflammatory action is triggered by activation of the signal transducer and activator of transcription 3 (STAT3), which is phosphorylated by the non-receptor kinase, Janus kinase 2 (JAK2) [57]. In addition, activation of the cholinergic pathway was recently shown to enhance the phagocytic potential of macrophages via stimulation of the alpha4/beta2 subunit [58].

Since the discovery of the cholinergic anti-inflammatory pathway, an increasing number of reports documented that stimulation of this hard-wired mechanism at the level of the brain,[59] directly on the vagus nerve [57,60] or in the periphery [6,61], attenuates local and systemic inflammation and improves survival in experimental settings. Although the immuno-modulatory potential of the cholinergic anti-inflammatory pathway appears promising, the feasibility of this pathway to treat inflammatory conditions in the clinical situation remains to be investigated. Electric vagal or pharmacologic stimulation of nicotinic receptors might lead to possible side-effects by generalized and persistent activation of non-associated cells and tissues [62]. Moreover, administration of a selective alpha7 nicotinic receptor agonist has recently been shown to worsen disease in experimental colitis [63].

1.3 The link between enteral nutrients and immune regulation

1.3.1 Sensing of nutrients

Enteral nutrients are sensed in the gastrointestinal (GI)-tract via the gut-brain axis. A well known task of the gut-brain axis is regulation of food intake and energy homeostasis [64]. The short-term regulation comprises initiation and termination of food intake via release of gut peptides and neural mechanisms. Long-term signals regulating energy stores and general health are humoral in nature, such as insulin and the adipokine leptin [65].

First, food passes through the orosensory system, which recognizes the nutrient content of mixed food via taste and smell [64]. These perceptions prepare the GI-tract for efficient processing of the ingested nutrients through cephalic reflexes [66]., Together with the gastric hormone ghrelin, the orosensory signals are the only signals that promote ingestion of nutrients. The other sensory systems, which are discussed subsequently, provide negative feedback signals [64].

Nutrients are sensed within the GI-tract by mechano- and chemosensors on the gut-brain axis. Intraganglionic laminar vagal afferent endings (IGLEs) are the most prominent mechanosensors in the intestine. The volume of ingested food is detected by these IGLEs that are located between the circular and laminar muscle layers. These selective vagal nerve endings respond to passive stretch and active contraction of muscle layers to terminate meal ingestion [67]. The intramuscular arrays (IMAs) are located in the longitudinal and circular muscle layers of the stomach. These receptors are analogue to stretch receptors, which fire at the same rate after prolonged stretch even when the muscle tension decreases [68].

The central nervous system is informed about the absorptive state via taste receptors, release of gut peptides and circulating nutrients (Figure 3)[64,69]. Recently, it was discovered that not only the oral cavity, but also the GI-tract expresses taste receptors. Specialized enteroendocrine cells are able to “taste” sweet, bitter and umami[70]. These taste receptors are located on the apical side of specialized enterocytes. How these receptors communicate with the brain remains focus of future studies [64,70]. Release of gut peptides by enteroendocrine cells is induced by the absorption of the macronutrients carbohydrate, protein and fat. The neuroenteric peptides can signal the brain via the circulation and/or the afferent vagus to inhibit feeding (Figure 3) [64,69]. Ghrelin, Cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and peptide YY are the main gut peptides that control food intake and regulate energy homeostasis. Of these peptides, CCK was the first to be discovered [71]. CCK is produced within the enteric system, predominantly by enterocrine-I cells in the proximal small intestine, and in the central nervous system [72,73]. Ingestion of lipid and also protein results in CCK release[74]. CCK interacts with two types of receptors, namely the CCK-1 and CCK-2 receptor [75]. The CCK-1

receptor is predominantly located in the periphery, while the CCK-2 receptor is mainly located within the central nervous system. However, this distribution is not absolute [69]. CCK has a plethora of digestive functions, including induction of pancreatic secretion and upper-intestinal satiation [73,76]. CCK has been shown to induce short-term satiety via a CCK-1 receptor-mediated vagal afferent pathway [77-79].

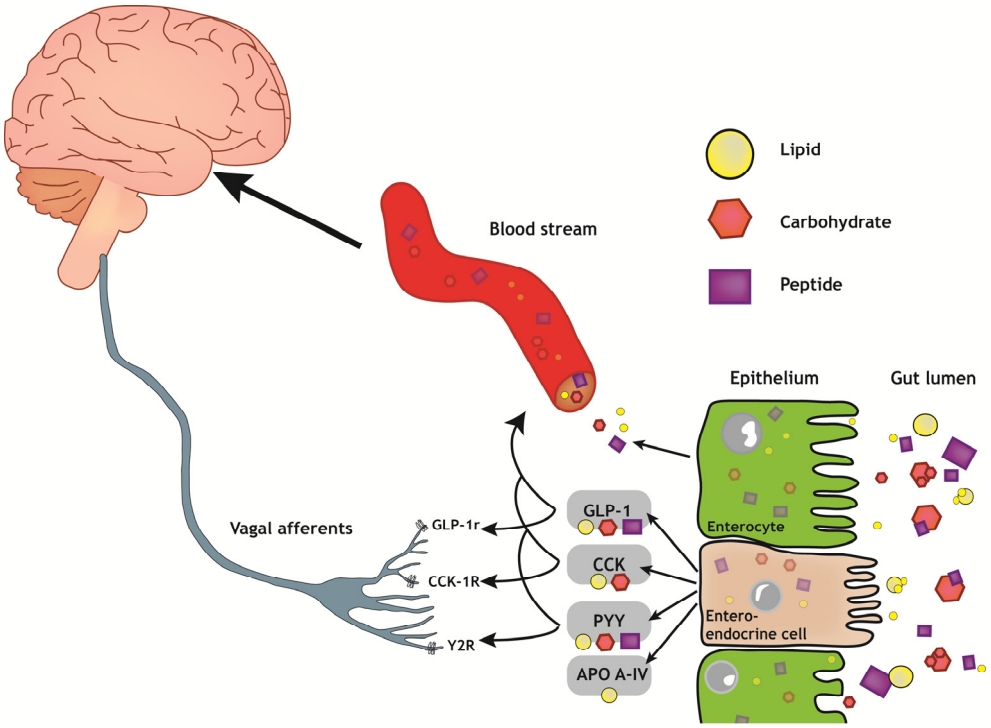

Figure 3: Simplified schematic representation of the pathways that result in the sensing of intraluminal nutrients. Ingested food is digested in the gastrointestinal tract into the macronutrients lipid, carbohydrate and protein. These macronutrients are absorbed by enterocytes or enteroendocrine cells of the small intestine. After absorption, the macronutrients or gastrointestinal hormones, such as glucagon-like peptide 1 (GLP-1), cholecystokinin (CCK), peptide YY (PYY) and apoprotein A-IV (APO A-IV) are secreted into lymphatic or blood vessels and transported to the brain via the circulation. Nutrients and gastrointestinal hormones enter the brain at all levels. Hormones and transmitters in the lamina propria can also rapidly interact with the brain through their relevant receptors on mucosal endings of vagal afferents.

Recent studies have demonstrated that CCK also modulates metabolic processes via the same vagal pathway. Indeed, luminal lipids and CCK can activate a vagovagal reflex that inhibits glucose production in the liver [80,81]. CCK has been termed the "gatekeeper of the vagus nerve" since CCK-1 receptor antagonists abolish the effects of enteral nutrients on plasma levels of ghrelin, PYY and GLP-1 [82-84]. Both peptides are involved in the ileal brake, a mechanism where the presence of nutrients in the distal intestine mediates lower-intestinal satiation by inhibiting proximal GI-motility and gastric emptying [73].GLP-1 and PYY are produced by enterocrine-L cells in the distal intestine upon direct contact with nutrients, especially lipid and carbohydrate, or indirectly upon stimulation of duodenally activated pathways [82,83,85]. Although it is generally accepted that vagus nerve plays a prominent role in GLP-1 induced satiety, intravenous administration of the peptide has been demonstrated to reduce meal size via direct actions on the brain [86,87]. The debate about a vagal or circulatory pathway via which PYY induces satiety is still ongoing [73,88].

The brain itself can detect changes in circulating levels of nutrients with the hypothalamus being the central link in nutrient detection. The arcuate, ventro-medial and lateral nuclei of the hypothalamus possess transporters, enzymes and ion channels that enable them to sense and process nutrients [89]. The nutritional status is then redirected to the periphery via hard-wired pathways to lower food intake and hepatic glucose production [90,91]. Taken together, the versatile gut-brain axis provides instant bidirectional communication to regulate GI and metabolic functions to maintain homeostasis in response to food intake.

1.3.2 The nutritional anti-inflammatory pathway

Besides electric and pharmacologic stimulation of the cholinergic anti-inflammatory pathway, physiologic activation of this potent neuro-immune axis would be a promising intervention to treat inflammatory conditions in the clinical setting. Recently, our group demonstrated in a rodent model of hemorrhagic shock that enteral administration of lipid-enriched nutrition attenuates systemic inflammation and preserves gut-barrier function, resulting in reduced endotoxemia and bacterial translocation [92,93]. Furthermore, lipid-enriched nutrition reduced systemic inflammation and liver damage in a setting of hemorrhagic shock aggravated by pre-exposure to bacterial DNA [94,95]. Further mechanistic studies revealed that the anti-inflammatory potential of lipid-enriched nutrition is executed via the autonomic nervous system [96]. The luminal presence of lipid-enriched nutrition activates the autonomic nervous system via stimulation of CCK receptors. In turn, efferent vagal fibers release acetylcholine that inhibits inflammation by binding to nicotinic receptors on inflammatory cells as described in 2.1.1 [96]. These findings suggest that enteral administration of lipid-enriched nutrition is a

physiologic means to activate the cholinergic anti-inflammatory pathway. Although much is known about the motor arc of this mechanism, the sensory arm that activates the nutritional anti-inflammatory pathway remains subject to further investigation.

1.3.3 Applicability of the nutritional anti-inflammatory reflex

Energy homeostasis has become a major point of interest in hospital care. It is a well known but still underappreciated problem that large numbers of patients admitted to the hospital suffer from malnutrition [97,98]. On top of that, patients are often deprived of enteral nutrition for prolonged periods of time due to standard hospital practice. In surgical patients for instance, preoperative fasting was introduced in the 19th century to minimize the risk of aspiration [99,100]. Additionally, in the postoperative period enteral nutrients are only re-administered based on clinical signs of bowel movement, thereby contributing to the state of nutritional deprivation. Malnutrition and prolonged fasting are proven risk factors for surgical complications and increase hospital stay and costs [101].

During the last decades, the value of pre-operative fasting has been questioned and the catabolic state of surgical and critically ill patients gained interest [99,100]. These novel insights have led to more liberal nutritional regimes, such as a reduced period of pre-operative fasting, pre-operative carbohydrate loading and early administration of enteral nutrition in surgical and critically ill patients [100]. The exact mechanisms behind the beneficial effects of enteral nutrition are not well known. Ingestion of food provides nutrients to the intestine, thereby preserving intestinal health, and activates specific neural and non-neural pathways to maintain digestive, metabolic and immunologic homeostasis [80,96,102,103]. Moreover, it is assumed that adequate nutritional support prevents immunodeficiency induced by caloric deficits [104,105]. In addition to caloric support, prolonged ingestion of nutrition specifically enriched with intrinsic anti-inflammatory compounds, such as long-chain n-3 polyunsaturated fatty acids and glutamine, has been shown to result in immune-modulating effects by influencing specific metabolic processes, including eicosanoid production, glutathione synthesis and generation of heat shock proteins [106,107].

Previous work from our group demonstrated that enteral administration of lipid-enriched nutrition in rodents attenuates systemic inflammation and preserves intestinal health, two major players in the pathophysiology of inflammatory complications, via a hard-wired pathway [96]. Enteral administration of lipid-enriched nutrition fits within the paradigm of optimal nutritional support for hospitalized patients. Moreover, lipid-enriched enteral nutrition directly modulates the inflammatory response at a general level without nullifying single inflammatory components. Physiologic activation of this potent endogenous anti-inflammatory

pathway with lipid-enriched nutrition opens an interesting therapeutic window for use of enteral nutrients as supportive and/or therapeutic modality in the clinical setting.

1.4 Aim of this thesis

As described in this chapter, a strict regulation of the inflammatory response is critical to ensure host survival. Predominantly in surgical, trauma and critically-ill patients, the immune response has the tendency to become dysregulated, resulting in high morbidity and mortality. A well-timed and effective nutritional stimulation of the vagal anti-inflammatory pathway could control the immune response in these cases.

The experiments described in this thesis were performed to provide enhanced insight in the mechanisms underlying activation of the nutritional anti-inflammatory pathway, to explore the therapeutic window of the nutritional intervention and to extend these promising results observed in rodents to man.

First, involvement of the afferent vagus nerve in the anti-inflammatory effects of lipid-enriched nutrition was studied (**chapter 2.1**). This study demonstrated that the nutritional pathway is locally activated in the intestine via CCK-mediated stimulation of afferent vagal fibers. Based on these observations, the intestinal processes that lead to local activation of afferent vagal fibers by lipid-enriched nutrition were investigated in more detail, identifying chylomicron formation and activation of GLP-1 receptors as important components in this nutritional pathway (**chapter 2.2**).

Next, the therapeutic window of lipid-enriched nutrition was explored. Trauma patients form a large population in the surgical clinic. These patients in particular suffer from inflammatory complications [16,108]. To explore a role for lipid-enriched enteral nutrition in the treatment of trauma patients, its anti-inflammatory potential was investigated in a rodent post-shock model to mimic a setting where inflammation and intestinal damage are already present (**chapter 3.1**). These experiments demonstrated that post-injury administration of lipid-enriched nutrition lowers inflammation and reduces intestinal damage.

Postoperative ileus is a major surgical complication following abdominal and even extra-abdominal surgery, leading to increased morbidity and mortality. Recent experimental evidence has shown that intestinal inflammation plays a key role in the pathogenesis of postoperative ileus [57,109]. In the current thesis, the beneficial effects of lipid-enriched nutrition on local intestinal damage and gastrointestinal transit were analyzed in a rat model of postoperative ileus (**chapter 3.2**). These

data revealed that enteral administration of lipid-enriched nutrition attenuates intestinal inflammation and promotes intestinal transit.

Taken together, **Chapter 2** and **chapter 3** of the current thesis revealed that lipid-enriched nutrition activates an anti-inflammatory vagovagal reflex and demonstrated protective effects in several inflammatory rodent models. Moreover, the intervention was also effective in a setting where inflammation and tissue damage are already present.

Given these promising effects in rodents, the last part of this thesis focused on the anti-inflammatory potential of enteral lipid-enriched nutrition in humans using an endotoxemia model. To this end, the anti-inflammatory effects of lipid-enriched nutrition were first studied in a murine model of endotoxemia (**chapter 4.1**). This study revealed that lipid-enriched nutrition reduces organ-specific and systemic inflammation and attenuates intestinal epithelial cell damage during endotoxemia. At the same time, several nutritional compositions were analyzed in healthy subjects to indentify a suitable nutritional intervention to be used in the human study (**chapter 4.2**). Based on the experiments performed in this chapter, continuous duodenal administration of a custom-made lipid- and protein-enriched nutrition was chosen as intervention to be used in a human proof-of-principle study. Lastly, we investigated the immuno-modulatory effects of this lipid- and protein-enriched nutrition in a randomized controlled double-blind intervention study in man (**Chapter 4.3**). The results of this *in vivo* study demonstrated that continuous postpyloric administration of a lipid- and protein-enriched nutrition attenuated systemic inflammation and lowered enterocyte damage in man exposed to endotoxemia.

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Chapter 2.1

Cholecystokinin/cholecystokinin-1 receptor mediated peripheral activation of the afferent vagus by enteral nutrients attenuates inflammation in rats

Tim Lubbers
Jacco J. de Haan
Misha D.P. Luyer
Isabelle Verbaeys
M'hamed Hadfoune
Cornelis H.C. Dejong
Wim A. Buurman
Jan Willem M. Greve.

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Abstract

Background: Enteral nutrition activates humoral and neural pathways to regulate food intake and sustain energy balance. Recently, we demonstrated that enteral nutrition and in particular lipid-rich nutrition modulates inflammation and prevents organ damage.

Objective: The current study investigates activation of the nutritional anti-inflammatory pathway by lipid-rich nutrition.

Methods: Male rats were fasted or fed lipid-rich nutrition prior to hemorrhagic shock. Disruption of afferent vagal fibers with capsaicin (deafferentation) was used to investigate involvement of afferent fibers. Peripheral activation of afferent vagal fibers via cholecystokinin (CCK)-mediated activation of CCK-1 receptors was investigated using administration of the selectively peripheral acting CCK-1 receptor antagonist, A70104 and PEGylated-CCK9. Tissue and blood were collected 90 minutes after shock to assess systemic inflammation and intestinal integrity.

Results: Deafferentation reversed the inhibitory effect of lipid-rich nutrition on systemic levels of TNF-α and IL-6 and on intestinal leakage of horseradish peroxidase and bacterial translocation. Furthermore, the protective effects of lipid-rich nutrition were negated by A70104, indicating that lipid-rich nutrition triggers peripheral cholecystokinin-1 receptors on vagal afferents to modulate inflammation. These findings were substantiated by the fact that pre-treatment of fasted rats with PEGylated-cholecystokinin9, which acts on peripheral CCK-1 receptors, attenuated systemic inflammation and loss of intestinal integrity.

Conclusion: These data demonstrate that enteral lipid-rich nutrition modulates inflammation and preserves intestinal integrity via cholecystokinin release which activates cholecystokinin-1 receptors located on afferent vagal fibers. Taken together, the current study reveals a novel gut-brain-immune axis and provides new insight into the applicability of enteral nutrition to treat inflammatory conditions.

Introduction

Nutrient sensing is an essential feature of the autonomic nervous system to regulate food intake and maintain energy homeostasis. The gastrointestinal tract communicates with the autonomic nervous system via circulating mediators and hard-wired connections, the so-called gut-brain axis [1]. Many of these physiologic processes, such as satiety and regulation of digestive activity, depend on the vagus nerve [2-4]. In addition to a role in nutrient sensing, the vagus nerve has an important immuno-modulatory function. Peripheral vagal afferents are able to sense circulating cytokines, resulting in a thermogenic and humoral anti-inflammatory response [5-7]. Additionally, stimulation of the vagus nerve modulates inflammation and improves outcome in several inflammatory models via the cholinergic anti-inflammatory pathway [8-10].

Recently, we described that lipid-rich nutrition regulates the inflammatory response via activation of the autonomic nervous system [11, 12]. Subsequently nicotinic receptors on inflammatory cells are activated via the vagus nerve, leading to a reduction of cytokine release and organ damage [12, 13]. Activation of this anti-inflammatory pathway via administration of lipid-rich nutrition is an appealing and physiologic intervention to counteract excessive inflammation and organ damage in several diseases [13-15]. Furthermore, this nutritional anti-inflammatory pathway might contribute to the largely unexplained unresponsiveness of the intestinal immune system to dietary and bacterial antigens.

Here, we report on studies directed at the molecular mechanism responsible for the sensing of luminal nutrients by the autonomic nervous system and the creation of a subsequent immuno-modulatory response. First, the involvement of the afferent vagus was assessed via vagal deafferentation with capsaicin. Next, the role of the cholecystokinin (CCK)-1 receptor and the CCK-2 receptor was investigated using selective receptor antagonists. In addition, activation of the nutritional pathway by peripheral CCK-1 receptors was studied with a selective CCK-1 receptor antagonist, which does not cross the blood-brain barrier, and administration of PEGylated-CCK9 (PEG-CCK9), a CCK-agonist that solely acts on peripheral CCK-1 receptors.

The current study reveals a novel gut-brain axis in which enteral nutrients trigger a potent anti-inflammatory response via CCK-mediated activation of peripheral CCK-1 receptors on afferent vagal fibers. Furthermore, our data point at lipid-rich nutrition as an effective and physiologic intervention to modulate inflammation.

Methods

Animals and experimental groups

Male Sprague-Dawley rats, weighing 300-350 g were purchased from Charles River Laboratories (Maastricht, the Netherlands) and housed under controlled conditions of temperature and humidity. Prior to the experiments, rats were fed standard rodent chow ad libitum and had free access to water. The experimental protocols were approved by the Animal Ethics Committee of the Maastricht University Medical Center.

A non-lethal hemorrhagic shock model was used, as previously described [12, 16]. In short, rats were anesthetized with isoflurane (induction 4%, maintenance 1.5%); the femoral artery was cannulated with polyethylene tubing (PE-10) containing heparinized saline (10 IU/ml). At the time of shock, 2.1 ml blood per 100 g body weight was withdrawn at a rate of 1 ml/minute. In all experiments, rats were fasted overnight (18 h) or fed lipid-rich nutrition by oral gavage prior to hemorrhagic shock. The lipid-rich liquid nutrition contained 50.4 energy percent (en%) fat of which 30% were phospholipids, 8.7 en% protein and 40.9 en% carbohydrates. Rats received 3 ml enteral nutrition 18 hours before shock and 0.75 ml was given at 2 hours and at 45 minutes before hemorrhagic shock. All rats were sacrificed 90 minutes after shock.

Vagal deafferentation

To determine the role of afferent vagal fibers in the activation of the nutritional anti-inflammatory pathway, rats were vagally deafferented 10-14 days prior to hemorrhagic shock as previously described [17]. In short, animals were anesthetized with isoflurane (induction 4%, maintenance 2.5%) and injected with atropine (0.5 mg/kg s.c.) to counteract the acute systemic effects of capsaicin on the respiratory and cardiovascular system. The vagal nerve was carefully dissected from the carotid artery at the cervical level bilaterally. Capsaicin (10 mg/ml. Sigma, St Louis, MO) was applied via a cotton swab and added every ten minutes for a total of 30 minutes. The total amount of capsaicin applied to each rat did not exceed 1 mg. Surrounding tissue was protected with parafilm to prevent leakage. Sham animals were treated with vehicle (90% olive oil, 10% Tween 80).

Assessment of deafferentation

The deafferentation procedure was functionally verified by CCK satiety test [18]. Rats (n = 8) were challenged with i.p. injection of either sulphated CCK-8 (CCK8s. Bachem AG, Weil am Rhein, Germany; 4 µg/kg) or saline after 4 hours starvation. Two minutes after i.p. injection, rats were given access to a pre-weighed amount of standard rat chow. Food consumption was measured after a 30 minute interval. The

difference in food intake after saline or CCK8s injection was calculated and served as a marker for CCK-induced satiety.
The deafferentation procedure was histologically confirmed by neuronal tracing of the afferent vagus [19]. One week after deafferentation or sham operation, rats (n = 6) were injected intraperitoneally with the retrograde neuronal tracer fluorogold (Invitrogen, Carlsbad, CA) dissolved in PBS (1 mg/ml). Three days after fluorogold administration rats received an overdose of pentobarbital, followed by transcardial perfusion fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Nodose ganglia were removed, post-fixed and cryoprotected in 20% sucrose/0.1 M PB. Tissues were sectioned at 16 µm using a cryostat, air dried and coverslipped with 80% glycerol in TBS. Sections were examined for the presence and distribution of fluorogold in the perykaria of afferent vagal neurons using fluorescence microscopy. Photomicrographs were recorded with a DCC camera. Per rat, three coronal sections of nodose ganglia were used as separate values for quantitative analyses of fluorogold staining. The number of fluorogold positive cells per square micrometer in the nodose ganglion was counted using the computer assisted digital analysis program Leica Qwin (Leica Microsystems Imaging solutions Ltd, Cambridge, UK).

Receptor antagonists
Rats (n=8) were fed lipid-rich nutrition and treated intravenous with receptor antagonists 30 minutes prior to induction of shock to investigate involvement of CCK-receptors in the nutritional anti-inflammatory pathway. To discriminate between a CCK-1 or CCK-2 receptor dependent mechanism, either the CCK-1 receptor antagonist devazepide or the CCK-2 receptor antagonist L365,260 (gifts from ML Laboratories PLC, Nottingham, UK; both 500 µg/kg) or vehicle (90% saline, 5% Tween 20, 5% DMSO) were administered. Involvement of peripherally localized CCK-1 receptors was investigated using A70104 [20], also known as A65186 (100 µg/kg, kindly provided by Abbott Laboratories, Abbott Park, IL) or vehicle (99% saline, 1% DMSO).

CCK administration and measurement
Activation of the nutritional anti-inflammatory pathway by peripherally acting CCK was studied by administration of PEG-CCK9, which solely acts on peripheral CCK-1 receptors, or vehicle [21]. PEG-CCK9 was dissolved in sterile saline and administered intravenous (6 µg/kg; bolus injection) in fasted rats 30 minutes prior to shock. The involvement of peripheral CCK-1 receptors in activation of the vagal anti-inflammatory pathway by PEG-CCK9 was investigated by co-administration of A70104 with PEG-CCK9.

To determine whether continuous infusion of exogenous CCK reaching high physiologic arterial levels could mimic the anti-inflammatory response, CCK8s was dissolved in sterile saline and infused at 500 pmol/kg/h intravenous (based on previous studies [22-24] and own experiments; data not shown). The infusion-protocol started 30 minutes before shock and was maintained until sacrifice. Systemic levels of CCK were measured in arterial plasma at time of shock and at sacrifice using a CCK-radioimmunoassay (Euro-diagnostica, Malmö, Sweden).

Cytokine analysis

TNF-α and IL-6 concentrations in arterial blood were measured using a standard ELISA for rat TNF-α (kindly provided by HBT, Uden, the Netherlands) and IL-6 (BD Biosciences, Franklin Lakes, NJ).

Intestinal permeability assay and bacterial translocation

Intestinal permeability for macromolecules was assessed by measuring translocation of the 44-kD enzyme horseradish peroxidase (HRP) by the everted gut sac method as previously described [25]. Segments of the distal ileum (8 cm) were washed, everted, filled with 1 mL of Tris buffer (125 mmol/L NaCl, 10 mmol/L fructose, 30 mmol/L Tris, pH 7.5) and ligated at both ends. The filled segments were incubated in Tris buffer containing 40 μg/ml of HRP. After incubation at room temperature for 45 min, the ileum was removed from the buffer and the content was carefully collected in a 1-mL syringe. HRP-activity was measured spectophotometrically at 450 nm after addition of tetramethyl benzidine as a substrate for HRP.

Bacterial translocation to distant organs was assessed as described [11]. In short, mesenteric lymph nodes, the mid-section of the spleen and a liver-segment (IV) were collected aseptically in pre-weighed thioglycolate broth tubes (Becton Dickinson, Franklin Lakes, NJ). Tissue-fragments were weighed, homogenized and the entire suspension was transferred to agar plates (Columbia III blood agar base supplemented with 5% vol/vol sheep blood (BBL, Franklin Lakes, NJ) and Chocolate PolyviteX agar (BioMérieux, Marcy l'Etoile, France). After 48 h of incubation, colonies were counted, determined using conventional techniques, adjusted to tissue weight, and expressed as number of CFU's per gram of tissue.

Statistical analysis

Data are represented as median, range and interquartile range. A Mann-Whitney U test was used for between-group comparisons. Differences were considered statistically significant at $P < 0.05$. Statistical analysis was performed using Graphpad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

The nutritional anti-inflammatory pathway is mediated by afferent vagal fibers

Involvement of the afferent vagus in the nutritional anti-inflammatory pathway was investigated in rats after perineural application of capsaicin (deafferentation). Prolonged exposure of nerves to capsaicin typically destroys afferent fibers, while leaving efferents intact [26]. Before entering the experiment, the efficacy of deafferentation was determined using a CCK satiety test. In line with previous reports [18, 27], intraperitoneal administration of sulphated CCK8 (CCK8s) reduced 30-min food intake after 4-hour starvation with 51 ± 2% in sham operated rats compared with 26 ± 3% in deafferented rats ($p < 0.05$; Figure 1A), indicating a successful deafferentation.

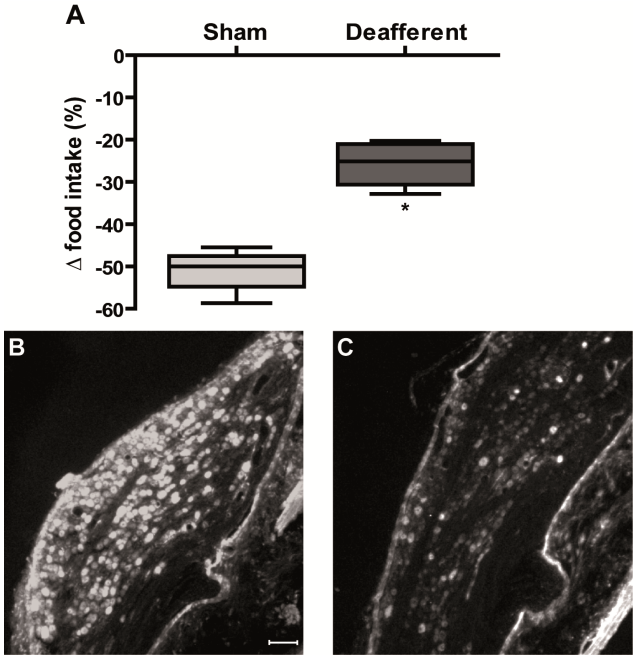

Figure 1: Intraperitoneal administration of CCK8s (4 µg/kg; n = 8) potently reduced food intake compared with saline administration in sham operated rats (A). Destruction of afferent vagal fibers by perineural application of capsaicin significantly inhibited the satiety response induced by intraperitoneal administration of CCK8s. (B, C) Fluorogold was administered intraperitoneally in deafferented and sham operated rats (n = 6). Deafferentation (C) resulted in significant loss of positively-labelled afferent vagal cell bodies in the nodose ganglia (80%) compared with sham operated animals (B). Data represented as median, range and interquartile range. Scale bar represents 100 µm. * $p < 0.05$ compared with sham.

It is known that perineural application of capsaicin results in subtotal elimination of afferent vagal fibers [26]. Therefore, the amount of remnant vagal afferent fibers was histologically assessed in a number of rats (n = 6) using the retrograde neuronal tracer fluorogold [19]. Deafferentation resulted in an 80% reduction of fluorogold positive cell bodies in the nodose ganglia, i.e. 0.25 [0.25 to 0.39] positive cells/μm^2 in sham compared with 0.06 [0.03 to 0.16] positive cells/μm^2 in deafferented animals ($p < 0.0001$; Figures 1B-C). These findings demonstrate that the majority of afferent fibers were destroyed.

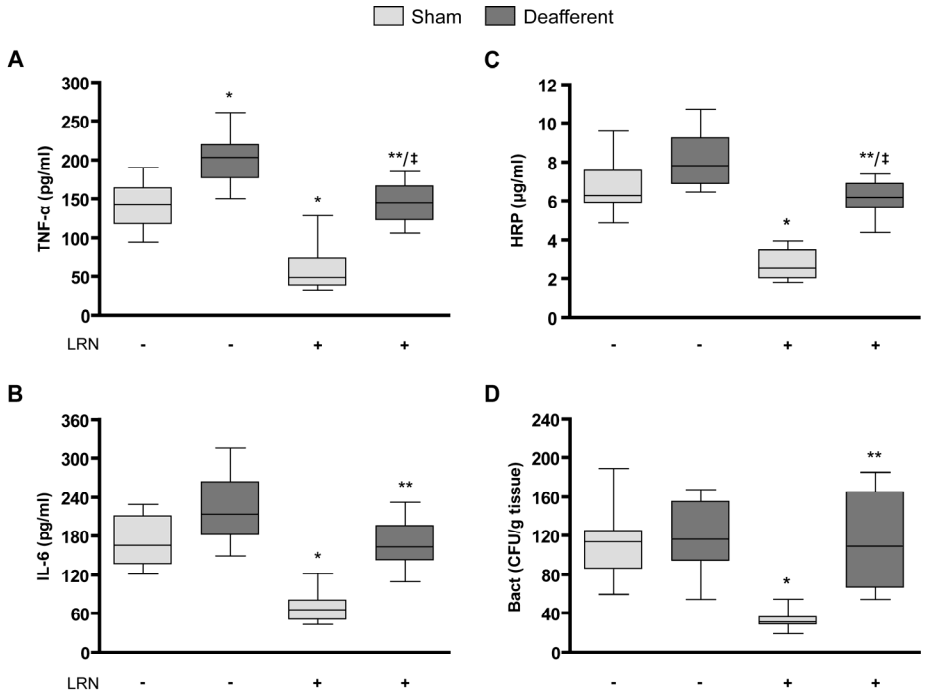

Figure 2: Administration of lipid-rich enteral nutrition in sham operated rats (light bars) inhibited shock-induced systemic levels of TNF-α (A) and IL-6 (B) compared with fasted rats. Furthermore, intestinal permeability to HRP (C) and bacterial translocation (D) were effectively reduced by lipid-rich nutrition in sham rats. Deafferentation (dark bars) negated the inhibitory effect of lipid-rich enteral nutrition on systemic inflammation and gut barrier failure. Furthermore, deafferentation increased systemic TNF-α in fasted rats. Administration of lipid-rich nutrition reduced TNF-α release and intestinal permeability to HRP in these animals. LRN; Lipid-rich nutrition. Data represented as median, range and interquartile range, n = 8. * $p < 0.01$ compared with fasted sham, ** $p < 0.01$ compared with lipid-rich sham, ‡ $p < 0.05$ compared with fasted deafferent.

The protective effects of lipid-rich nutrition on systemic inflammation and intestinal integrity were replicated in sham operated animals, indicating that these rats effectively respond to enteral lipid-rich nutrition. Next, we observed that deafferentation in fasted rats resulted in higher shock-induced TNF-α levels compared with fasted sham rats ($p < 0.05$; Figure 2A). More interestingly, the protective effects of lipid-rich nutrition on shock-induced systemic inflammation (plasma levels of TNF-α and IL-6; both $p < 0.001$; Figures 2A-B) and loss of intestinal integrity (bacterial translocation and leakage of HRP; both $p < 0.01$; Figures 2C-D) were inhibited by disruption of vagal afferent fibers with capsaicin. Administration of lipid-rich nutrition reduced plasma concentration TNF-α and leakage of HRP in deafferent animals to some extent, which is likely attributable to the remnant afferent vagal fibers. Taken together, these findings point at an important role for afferent vagal fibers in the nutritional anti-inflammatory pathway.

Both the CCK-1 and CCK-2 receptor are involved in the nutritional anti-inflammatory pathway

Previously, we demonstrated that activation of the anti-inflammatory mechanism triggered by lipid-rich nutrition was dependent on CCK-receptors [12]. Separate intravenous administration of the CCK-1 receptor antagonist, devazepide or the CCK-2 receptor antagonist, L365,260 demonstrated that the beneficial effects of lipid-rich nutrition depend on both receptor subtypes (Figure 3). The CCK-1 receptor antagonist inhibited the effects of lipid-rich nutrition on systemic inflammation and intestinal integrity to a greater extent than the CCK-2 receptor. Since both receptor antagonists readily cross the blood-brain barrier, a distinction between a peripheral or central activation of the nutritional anti-inflammatory pathway could not be made.

Lipid-rich nutrition modulates inflammation via the peripheral CCK-1 receptor

To substantiate a peripheral activation of the vagal anti-inflammatory pathway by lipid-rich nutrition, rats were treated intravenously with the peripherally acting CCK-1 receptor antagonist, A70104. A70104 does not cross the blood-brain barrier [20]. Pre-treatment with A-70104 abrogated the inhibitory effect of lipid-rich nutrition on systemic inflammation (plasma levels of TNF-α and IL-6; Figure 4A) and intestinal integrity (leakage of HRP and bacterial translocation; Figure 4B), underlining a critical role for peripherally localized CCK-1 receptors in the activation of the nutritional anti-inflammatory pathway.

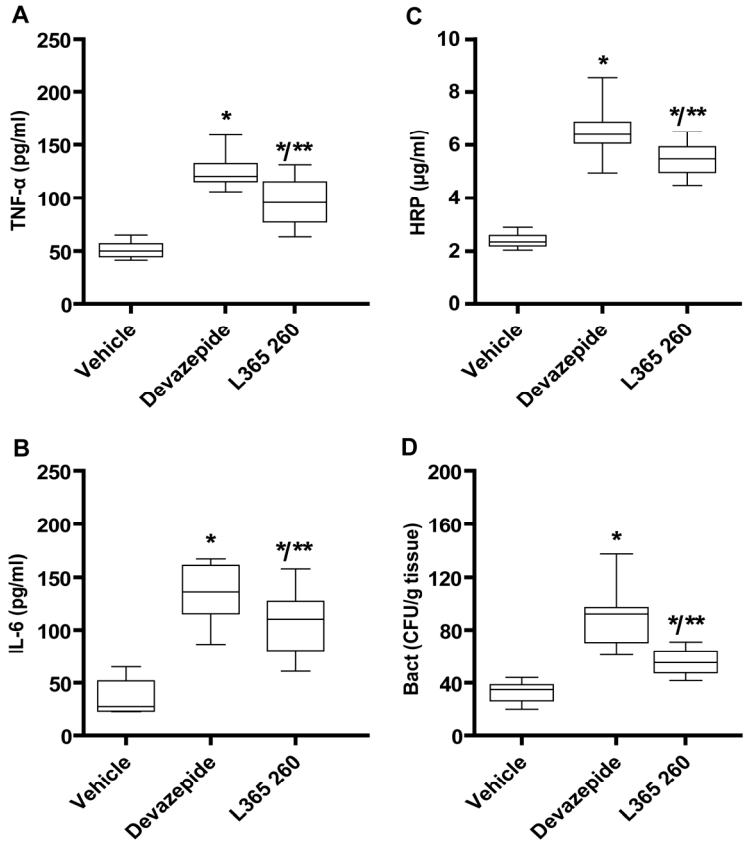

Figure 3: Administration of the CCK-1 or CCK-2 receptor antagonist inhibited the effect of lipid-rich nutrition on TNF-α (A), IL-6 (B), leakage of HRP in ileal segments (C) and bacterial translocation (D). The inhibitory effect of the CCK-1 receptor antagonist (devazepide) was stronger than the CCK-2 receptor antagonist (L365,260). Data represented as median, range and interquartile range. * $p < 0.001$ compared with vehicle, ** $p < 0.05$ compared with CCK-1 receptor antagonist (n = 8).

Peripherally acting CCK triggers the nutritional anti-inflammatory mechanism

To investigate a role for CCK-mediated activation of peripheral CCK-1 receptors in the nutritional anti-inflammatory pathway, we administered an intravenous bolus of PEG-CCK9. This CCK9 conjugate shows complete retention of biological activity and acts solely on peripheral CCK-1 receptors [21, 28]. Administration of PEG-CCK9 reduced plasma levels of TNF-α and IL-6 (Figure 5A-B) and ameliorated leakage of HRP and bacterial translocation compared with vehicle (Figure 5C-D), mimicking

the anti-inflammatory effect of lipid-rich nutrition. Co-administration of A70104 blunted the effect of PEG-CCK9 on systemic inflammation and intestinal integrity (Figure 5). Taken together, these data denote that the anti-inflammatory effects of lipid-rich nutrition are triggered via CCK-mediated activation of peripheral CCK-1 receptors.

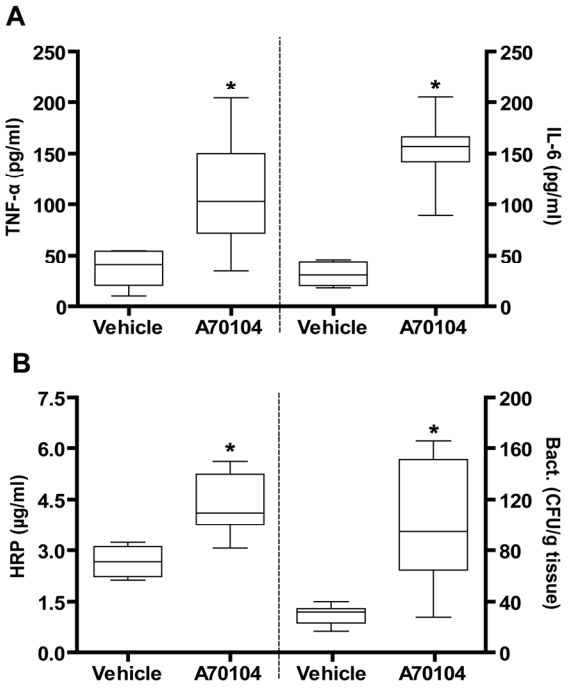

Figure 4: Pre-treatment with the solely peripherally acting CCK-1 receptor antagonist (A70104) abrogated the protective effects of lipid-rich nutrition (A-B). Data represented as median, range and interquartile range. * $p < 0.001$ compared with vehicle ($n = 8$).

Administration of supraphysiologic concentrations of CCK in clinical situations has several limitations, since it can lead to pancreatitis and anxiety [29, 30]. Therefore, we investigated whether infusion of CCK resulting in physiologic plasma concentrations activates the nutritional anti-inflammatory pathway. Infusion of CCK8s in fasted rats, reaching arterial concentrations of 11 [7 to 22] pM at shock and 20 [9 to 25] pM at sacrifice, did not reduce systemic TNF-α (170 [113 to 230] pg/ml vs vehicle: 178 [147 to 234] pg/ml) and IL-6 (196 [130 to 204] pg/ml vs vehicle: 198 [175 to 250] pg/ml). In addition, shock-induced leakage of HRP (2.9 [1.6 to 4.0] µg/ml vs vehicle: 3.6 [2.6 to 5.0] µg/ml) and bacterial translocation (97

[60 to 115] CFU/g tissue vs vehicle: 98 [90 to 123] CFU/g tissue) remained unaltered after CCK8s infusion. These data demonstrate that circulating exogenous CCK8s at physiologic arterial concentrations is unable to attenuate inflammation and cannot prevent gut barrier failure.

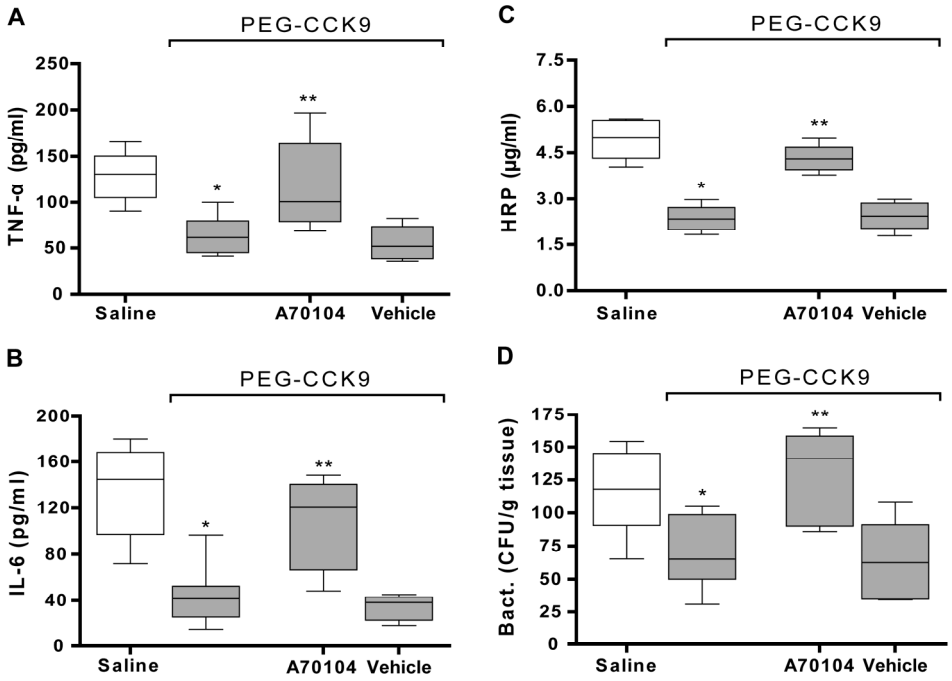

Figure 5: Bolus intravenous injection of PEG-CCK9 attenuated TNF-α (A), IL-6 (B), leakage of HRP in ileal segments (C) and bacterial translocation (D) compared with vehicle (dark bars. n = 8). The protective effect of PEG-CCK9 was abrogated by intravenous administration of A70104 (n = 6). Data represented as median, range and interquartile range. * $p < 0.01$ compared with saline, ** $p < 0.01$ compared with A70104 vehicle.

Discussion

The current manuscript demonstrates for the first time that enteral nutrients activate a previously unidentified gut-brain-immune axis. Administration of lipid-rich nutrition attenuates systemic inflammation and preserves intestinal integrity via release of CCK which activates peripheral CCK-1 receptors on the afferent vagus nerve.

The afferent vagus nerve is essential to monitor the condition of the body. Afferent vagal fibers relay a variety of signals from the periphery, such as food-related, digestive, immune and noxious stimuli [2, 3, 31]. In concordance with previous studies showing that vagal capsaicin treatment or vagotomy in rodents increased inflammation in experimentally-induced colitis [32-34], disruption of afferent vagal fibers resulted in increased shock-induced circulating TNF-α levels in fasted rats. Interestingly, deafferentation abrogated the protective effects of lipid-rich nutrition on systemic inflammation and loss of intestinal integrity, indicating that the nutritional anti-inflammatory pathway is peripherally activated. Our data are in agreement with several studies demonstrating that signal transmission from peripheral stimuli acting on the afferent vagus can be blocked by vagal deafferentation [19, 35, 36]. A limited protective effect of lipid-rich nutrition was observed in deafferent animals, which can be attributed to remnant afferent fibers [26]. Together, these findings not only underline a regulatory role for the vagus nerve in the immune response, but also implicate the afferent vagus nerve as a vital component of the nutritional anti-inflammatory pathway.

The CCK-receptor family consists of two subtypes, the CCK-1 and the CCK-2 receptor [30]. Enteral lipids activate the autonomic nervous system predominantly via CCK-1 receptors on the afferent vagus nerve, whereas certain brain areas are activated by both receptor subtypes [37, 38]. The current data demonstrate that the peripheral CCK-1 receptor is involved in the activation of the nutritional anti-inflammatory pathway, since both devazepide and the peripherally acting A70104 inhibited the anti-inflammatory effects of lipid-rich nutrition. The exact role of CCK-2 receptors in the nutritional anti-inflammatory pathway remains to be established. The observed inhibitory effect of L365,260 could be mediated via peripheral or central CCK-2 receptors, since this receptor antagonist readily crosses the blood-brain barrier [39]. However, the fact that most experimental evidence indicates that activation of brain neurons by enteral lipids and exogenous CCK is dependent on peripheral CCK-1 receptors and central CCK-2 receptors, hints at a central involvement of CCK-2 receptors in the nutritional anti-inflammatory pathway [38, 40, 41].

Ingestion of lipid-rich nutrition results in release of endogenous CCK, capable of activating both CCK-receptor subtypes [42]. There is competing evidence that CCK can activate the autonomic nervous system via the afferent vagus nerve as well as via a humoral route [1, 43-45]. Administration of the solely peripherally acting PEG-CCK9 mimicked the protective effects of lipid-rich nutrition [21]. The PEG-CCK9 induced anti-inflammatory response was shown to be dependent on peripheral CCK-1 receptors. In line with these findings, Bozkurt et al. demonstrated that exogenous CCK8s attenuates inflammation via capsaicin-sensitive vagal afferents in an experimental colitis model [35]. Taken together, these data substantiate that the

nutritional anti-inflammatory pathway is peripherally activated by a cholecystokinin-mediated stimulation of peripheral CCK-1 receptors on the afferent vagus.

Infusion of exogenous CCK is potentially a promising clinical application to treat inflammatory conditions. Administration of CCK8s at supraphysiologic concentrations has been shown to attenuate inflammation in experimental models both *in vivo* [35, 46] and *in vitro* [47, 48]. However, caution should be taken since high doses of exogenous CCK and PEG-CCK9 are known to result in pancreatic hyperplasia, pancreatitis and anxiety disorders in rodents and humans [29, 30, 49, 50]. Circulating physiologic levels of exogenous CCK8s, obtained by our infusion protocol, were unable to attenuate systemic inflammation and prevent loss of intestinal integrity. The concentration of CCK8s reaching the local environment of the vagal afferent nerve endings may have been insufficient to activate an anti-inflammatory response, since the CCK peptide is rapidly inactivated in the circulation [51, 52]. Recently, it has been shown that local CCK levels are more important to trigger the vagus nerve than circulating levels, since intestinal administration of CCK stimulates vagal afferents, without affecting plasma levels [53]. Therefore, administration of lipid-rich nutrition appears to be a more physiologic and efficient intervention to activate the CCK/CCK-1 receptor mediated vagal anti-inflammatory pathway. In addition, enteral administration of lipid-rich nutrition has been demonstrated to inhibit inflammation and attenuate organ damage in several situations, such as hemorrhagic shock and postoperative ileus [12-15], indicating that lipid-rich enteral nutrition could be a safe and subtle intervention to attenuate inflammatory conditions in the clinical setting.

In the gastrointestinal tract essential nutrients are sensed and absorbed, while potential harmful agents need to be prevented from invading the host. The mechanisms behind this dual role are largely unexplained. Uptake of nutrients inevitably exposes the host to antigenic substances [54]. A highly selective intestinal immune response is required to maintain intestinal barrier function and homeostasis in the face of constant threat. Together with previous work from our group [12], the current study reveals that the intestine directly communicates with the immune system via a nutritional vagovagal reflex (Figure 6). We hypothesize that this neural feedback loop is involved in the unresponsiveness of the dietary tract to luminal antigens.

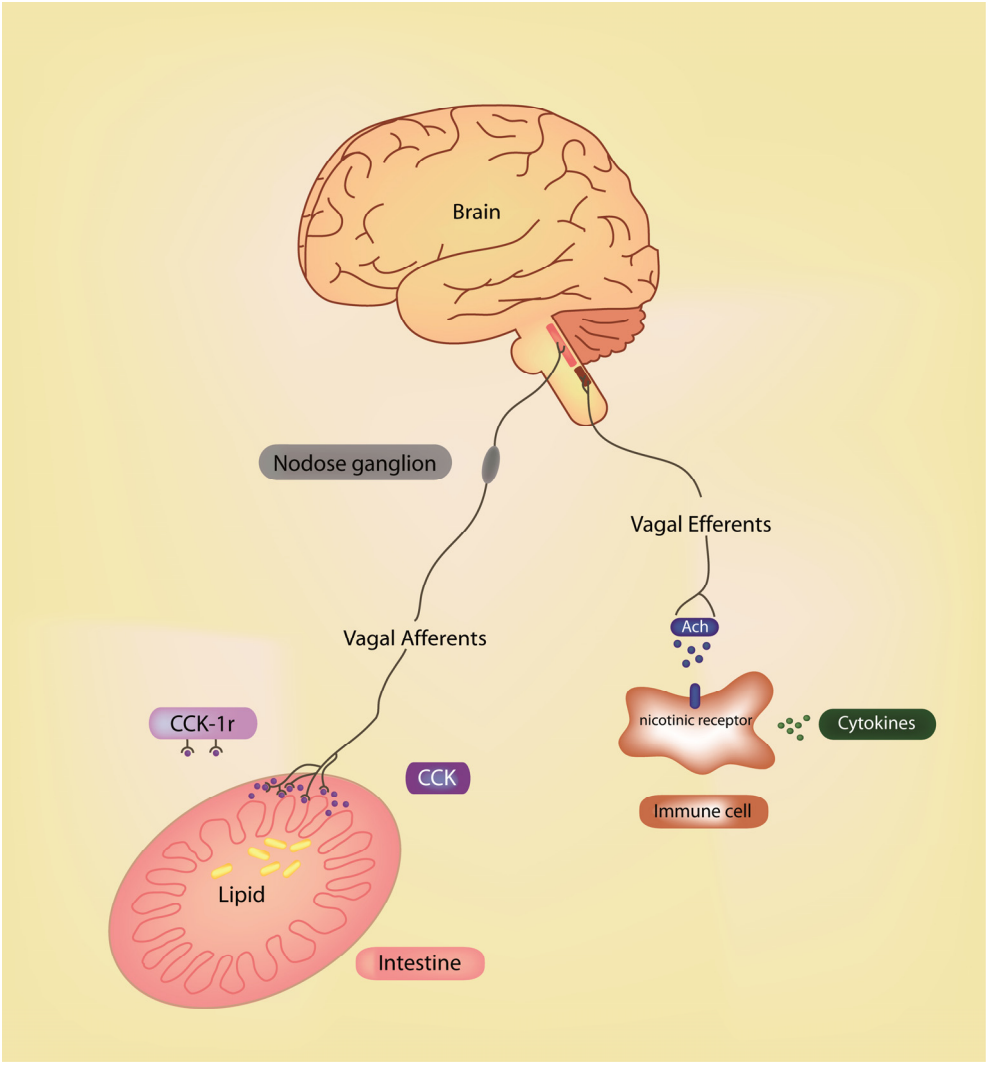

Figure 6: The enteral presence of lipid-rich nutrition results in release of cholecystokinin (CCK) from the gut wall, which binds to peripheral CCK-1 receptors (CCK-1r) located on afferent vagal nerve endings. The anti-inflammatory signal is processed in the vagal circuitry via a neural pathway in which CCK-1 and CCK-2 receptors are involved, resulting in inhibition of the inflammatory response via the efferent vagus. Production of inflammatory cytokines is attenuated by binding of acetylcholine (Ach) to nicotinic receptors on inflammatory cells.

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Chapter 2.2

Chylomicron formation and glucagon-like peptide 1 receptor are involved in the lipid-enriched nutrition -mediated anti-inflammatory pathway

Tim Lubbers
Jacco J. de Haan
M'hamed Hadfoune
Lennart Zabeau
Jan Tavernier
Yiren Zhang
David Grundy
Jan Willem M. Greve
Wim A. Buurman

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Abstract

Background: Enteral administration of lipid-enriched nutrition effectively attenuates inflammation via a cholecystokinin-mediated vagovagal anti-inflammatory reflex. Cholecystokinin release and subsequent activation of the vagus is dependent on chylomicron formation and associated with release of additional gut peptides.

Objective: The current study investigates the intestinal processes underlying activation of the cholecystokinin-mediated vagal anti-inflammatory pathway by lipid-enriched nutrition.

Methods: Rats and mice were subjected to hemorrhagic shock or endotoxemia, *respectively*. Prior to the experimental procedures, animals were fasted or fed lipid-enriched nutrition. Pluronic L-81 (L-81) was added to the feeding to investigate involvement of chylomicron formation in activation of mesenteric afferent fibers and the immune-modulating potential of lipid-enriched nutrition. Ob/Ob mice and selective receptor antagonists were used to study the role of leptin, glucagon-like peptide 1 and peptide YY in activation of the nutritional reflex.

Results: Electrophysiological analysis of mesenteric afferents in mice revealed that lipid-enriched nutrition-mediated neural activation was abrogated by L-81 ($p<0.05$). L-81 blunted the beneficial effects of lipid-enriched nutrition on systemic inflammation and intestinal integrity in both species (all parameters: $p<0.01$). Ob/Ob mice required a higher dose of nutrition compared with wildtypes to attenuate plasma levels of TNF-α and ileum-lipid binding protein, a marker for enterocyte damage (both $p<0.01$), suggesting a higher stimulation threshold in leptin-deficient mice. Administration of a glucagon-like peptide 1-receptor antagonist, but not leptin or peptide YY antagonists, suppressed the effects of lipid-enriched nutrition.

Conclusion: These data indicate that chylomicron formation triggered by lipid-enriched nutrition is essential to activate the nutritional anti-inflammatory pathway. Additionally, glucagon-like peptide 1 was identified as co-stimulatory peptide.

Introduction

Ingestion of nutrients triggers a multitude of regulatory functions in the digestive tract to maintain metabolic homeostasis [1, 2]. Nutrient sensing and intestinal feedback require release of neuropeptides from entero-endocrine cells and activation of neural pathways. The vagus nerve in particular plays a prominent role in regulation of food intake and digestive capacities of the gastrointestinal tract via the so-called gut-brain axis [3, 4].

Recently, our group described a novel feature of the gut-brain axis. Enteral administration of lipid-enriched nutrition attenuated local and systemic inflammation and prevented tissue damage via the vagus nerve [5-7]. The luminal presence of lipid-enriched nutrition triggers the brain via cholecystokinin (CCK)-mediated activation of CCK-1 receptors on afferent vagal fibers [8]. In turn, release of cytokines is inhibited through activation of nicotinic receptors on inflammatory cells via efferent vagal fibers [7].

CCK release following food intake is an important component in activation of the nutritional anti-inflammatory reflex [8]. However, little is known about the mechanisms that result in release of CCK and subsequent activation of afferent vagal fibers. Release of CCK from enterocrine-I cells is dependent on the intestinal processing of lipids, resulting in formation of chylomicrons [9, 10]. In line, chylomicrons have been shown to inhibit gastric emptying via a CCK-1 receptor mediated duodenal afferent pathway [9, 10]. In addition to CCK, glucagon-like peptide 1 (GLP-1) and protein YY (PYY), and the adipokine leptin are involved in meal-induced activation of afferent vagal fibers [1, 11] and inhibit food intake in conjunction with CCK [12-14]. The current study aims to reveal the intestinal processes triggered by lipid-enriched nutrition that result in activation of the CCK-mediated nutritional anti-inflammatory reflex.

Materials and methods

Animals and experimental groups

Male Sprague-Dawley rats, weighing 300-350 g, C57bl6 mice and Ob/Ob mice, both 10-12 weeks old, were purchased from Charles River Laboratories (Maastricht, the Netherlands) and housed under controlled conditions of temperature and humidity. Prior to the experiments, the animals were fed standard rodent chow ad libitum and had free access to water. The experimental protocols were approved by the Animal Ethics Committee of the Maastricht University Medical Centre+.

In rats, a non-lethal hemorrhagic shock (HS) model was used, as previously described [5]. In short, rats were anesthetized with isoflurane and the femoral artery

was cannulated. At time of shock, 2.1 ml blood per 100 g body weight was withdrawn. In all experiments, rats were fasted overnight (18 h) or fed lipid-enriched nutrition by oral gavage at -18 hours (3 ml), -2 hours (0.75ml) and -45 min (0.75 ml) prior to HS (Figure 1).

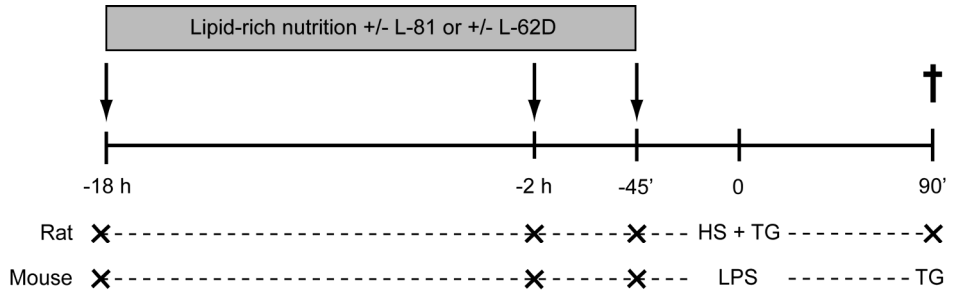

Figure 1: Experimental protocol. All animals were deprived of food 18 hours prior to HS or LPS administration. A liquid lipid-enriched (LR) diet was administered at three time points (-18 hours, -2 hours, -45 minutes) in the fed groups. L-81 or L-62D was added to the lipid-enriched nutrient investigate involvement of chylomicron formation in activation of the nutritional anti-inflammatory reflex. In rats, 30-40% of the circulating volume was withdrawn at t = 0 to induce hemorrhagic shock (HS). In the withdrawn blood, plasma triglyceride (TG) levels were determined. Mice received an intraperitoneal bolus of LPS (2 mg/kg) at t = 0. All animals were killed at 90 minutes, at which time the plasma TG levels in mice were analyzed.

In mice, systemic inflammation was induced by intraperitoneal injection of 2 mg/kg lipopolysaccharide (LPS derived from Escherichia coli 055;B5, Sigma, St. Louis, MO) in sterile phosphate-buffered saline (PBS; pH 7.4). Prior to endotoxemia, C57bl6 and Ob/Ob mice were fasted or fed lipid-enriched nutrition by oral gavage at -18 hours (0.3 ml), -2 hours (0.2 ml) and -45 min (0.2 ml). Next to this dose, a separate group of Ob/Ob mice received a higher dose (-18 hours: 0.4 ml, -2 hours: 0.3 ml and -45 min: 0.3 ml). All animals were killed at 90 minutes following either hemorrhagic shock or LPS administration (Figure 1).

The liquid lipid-rich diet contained 50.4 energy percent (en%) fat (30 en% are phospholipids), 8.7 en% protein and 40.9 en% carbohydrates. The lipid source was lecithin. Omega 3 and omega 6 fatty acids constituted less than 5 weight percent (< 5g/100 ml). Proteins were derived from lean milk powder, containing 80% casein and 20% whey protein. The carbohydrate source was a mixture of sucrose and maltodextrins (Glucidex 19DE).

Prevention of chylomicron formation
The formation of chylomicrons was prevented by adding Pluronic L-81 (6 mg/ml; kindly provided by BASF, Brussels, Belgium) to the lipid-enriched nutrition. L-81 is a hydrophobic surfactant that inhibits the formation of chylomicrons, without affecting digestion, uptake, or reesterification of absorbed lipid. Pluronic L-62D (BASF; comparable to Pluronic L-63), which is chemically similar to L-81, but does not prevent chylomicron formation, served as control [9].

Quantification of plasma triglycerides
The concentration of circulating triglycerides was measured in arterial plasma of rats collected at time of shock (t = 0) and venous plasma of mice at time of sacrifice (t = 90 min) using a triglyceride determination kit (Sigma) following the manufacturer's instructions (Figure 1).

Receptor antagonists
Lipid-enriched nutrition fed rats were treated with antagonists to the Y2-receptor (BIIE 0246 formate; 2 mg/kg in PBS) and GLP-1 receptor (Exendin-3; 500 µg/kg in 30% polyethylene glycol saline solution; both Tocris Bioscience, Ellisville, MO) 30 minutes prior to shock to investigate involvement of PYY and GLP-1 release, *respectively* in activation of the nutritional anti-inflammatory pathway.

Leptin receptor specific nanobody
Mice were injected i.p. with a blocking nanobody directed against the leptin receptor on two consecutive days prior to endotoxemia to delineate involvement of leptin in the anti-inflammatory potential of lipid-rich nutrition. The leptin receptor specific nanobody was generated by immunization of lamas with the extracellular part of the mouse leptin receptor. It was genetically fused to a nanobody directed against mouse serum albumin to prolong the half-life *in vivo*. This bi-specific nanobody was produced in E. coli and purified up to 95% purity. LPS contamination was less than 0.1 EU/mg protein as measured using the amoebocyte lysate in combination with a chromogenic substrate (Cambrex, New Jersey, NY). This nanobody acts as a potent leptin receptor antagonist both *in vitro* and *in vivo* (Zabeau *et al.*, *manuscript in preparation*).

Cytokine and I-LBP analysis
IL-6 and TNF-α concentrations in arterial blood were measured using standard ELISA's for rat IL-6 and rat and mouse TNF-α (all BD Biosciences, Franklin Lakes, NJ). Murine ileum-lipid binding protein (I-LBP) was determined in plasma using a specific ELISA (Hycult Biotech, Uden, the Netherlands).

Intestinal permeability assay and bacterial translocation

Intestinal permeability for macromolecules was assessed by measuring translocation of the 44-kD enzyme horseradish peroxidase (HRP) by the everted gut sac method [8]. Bacterial translocation (BT) to distant organs was determined as previously described [7]. In short, mesenteric lymph nodes, spleen and a liver-segment (IV) were collected aseptically. Tissue-fragments were homogenized and transferred to agar plates. After 48 h of incubation, colonies were counted, determined using conventional techniques, adjusted to tissue weight, and expressed as number of CFU per gram tissue.

Mesenteric afferent discharge

Mice were killed by cervical dislocation in accordance with the UK Animals Scientific Procedures Act (1986). Intestinal tissue was prepared for nerve recording as previously described [20]. In short, proximal jejunal segments (2 – 3 cm) were dissected so that a non-bifurcating mesenteric bundle could be identified. The isolated segments were placed in oxygenated Krebs solution at 34^{o}C. A single nerve bundle was drawn into a suction electrode for afferent recording. The jejunum was cannulated at each end and intraluminal pressure was recorded via a pressure recorder. The lumen was perfused with saline at 0.2 ml/min except during distension, when the outlet tap was closed allowing pressure to rise up to 55 mmHg and released by opening the tap.

Following a 60 minute stabilization period, intestinal segments were distended at 15 minutes intervals and mean afferent firing rate (spikes/s) was displayed as peri-stimulus rate histogram. Once reproducible responses were obtained, the effect of nutrient was tested by switching luminal perfusion to lipid-enriched nutrition alone or lipid-enriched nutrition with 3% L-81 (both 1 ml) with free-drainage. The nutrient remaining within the lumen was trapped for 15 minutes following closure of the outlet port with termination of the continuous perfusion of saline. One period of distension was achieved by perfusion with saline, which also served to flush out luminal contents when the outlet tap was opened. Saline perfusion and repeat distensions at 15 minutes continued until the response had recovered to baseline.

Statistical analysis

All experimental groups consisted of 8 animals, unless otherwise indicated. A Mann-Whitney U test was used for between-group comparisons. Whole nerve afferent discharge was calculated from the number of spikes crossing a pre-set threshold and expressed as spikes/s. Baseline discharge was calculated as the mean firing in the 1 minute period preceding distension. Discharge during distension was expressed as increase above baseline, calculated as the mean firing frequency in 5 sec periods at each level of distending pressure. Low threshold nerve afferent

discharge was expressed as the difference between the baseline discharge and discharge at the 20mmHg of the distending pressure. Data are expressed as mean +/- SEM. Data were compared statistically using repeated measure ANOVA with Dunnett post-test analysis. Prism 5.02 for Windows (GraphPad Software Inc., San Diego, CA) was used for computations. Differences were considered significant at $p < 0.05$.

Results

Pluronic L-81 decreases plasma triglyceride levels after ingestion of lipid-enriched nutrition

First, we verified the effectiveness of L-81 to prevent chylomicron formation in rats and mice by measuring postprandial plasma concentrations of triglycerides. Ingestion of lipid-enriched nutrition resulted in increased circulating triglycerides in rats and mice compared with fasted animals (both $p < 0.01$. Figure 2A-B).

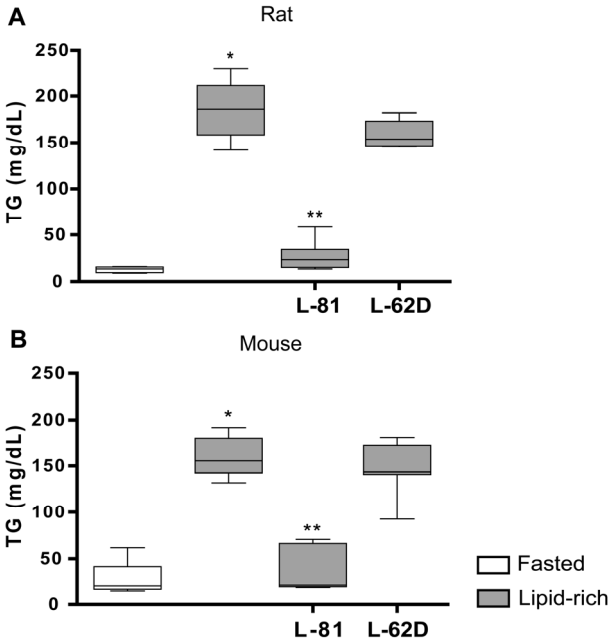

Figure 2: L-81 prevents lipid-enriched nutrition-induced rise in plasma triglycerides. Ingestion of lipid-enriched nutrition increases plasma levels of triglycerides. Supplementation of the nutrient with L-81 reduces the amount of circulating triglycerides compared with control (Pluronic L-62D) in both rats (A) and mice (B). Data are represented as median, range and interquartile range. * $p < 0.05$ compared with fasted, ** $p < 0.05$ compared with L-62D.

Addition of L-81 to the lipid-enriched nutrition reduced the amount of plasma triglycerides in both species compared with lipid-enriched nutrition plus control Pluronic L-62D (both $p < 0.01$), indicating that chylomicron formation is successfully prevented by L-81 in both species.

The inhibitory effect of lipid-enriched nutrition on systemic inflammation and loss of gut barrier is abrogated by Pluronic L-81

To investigate the role of chylomicron formation in activation of the anti-inflammatory pathway of lipid-enriched nutrition, we subjected rats to hemorrhagic shock. Hemorrhagic shock resulted in markedly elevated plasma levels of TNF-α and IL-6, as well as increased ileal permeability and bacterial translocation. These shock-induced changes were significantly attenuated by lipid-enriched nutrition, conform previous findings (Figure 3)[7]. Prevention of chylomicron formation using Pluronic L-81 reduced the effect of lipid-enriched nutrition on plasma levels of TNF-α and IL-6 compared with vehicle (both $p < 0.01$. Figures 3A-B). Moreover, L-81 reversed the protective effect of lipid-enriched nutrition on ileal leakage of HRP and bacterial translocation compared with vehicle (both $p < 0.05$. Figures 3C-D). These findings indicate that formation of chylomicrons is vital to activate the nutritional anti-inflammatory pathway in rats.

Prevention of chylomicron formation inhibits activation of mesenteric afferents and anti-inflammatory potential of lipid-enriched nutrition in mice

In order to investigate the role of chylomicron formation in activation of the autonomic nervous system by lipid-enriched nutrition, we determined mesenteric afferent discharge in response to lipid-enriched nutrition. First, we verified that L-81 inhibited the anti-inflammatory actions of lipid-enriched nutrition in mice similar to rats. Administration of lipid-enriched nutrition prior to LPS challenge attenuated plasma levels of TNF-α and I-LBP compared with fasted controls (both $p < 0.01$. Figures 4A-B). L-81 abrogated the effect of lipid-enriched nutrition on systemic inflammation and enterocyte damage compared with vehicle (both $p < 0.01$), indicating that chylomicron formation also plays a crucial role in mice. Next, we investigated firing of jejunal mechanosensitive afferents in response to lipid-enriched nutrition. The intrajejunal presence of lipid-enriched nutrition (treatment) enhanced vagal afferent discharge in response to luminal distension compared to the discharge before treatment and after treatment (both $p < 0.05$ from 8-55 mmHg. Figure 5A).

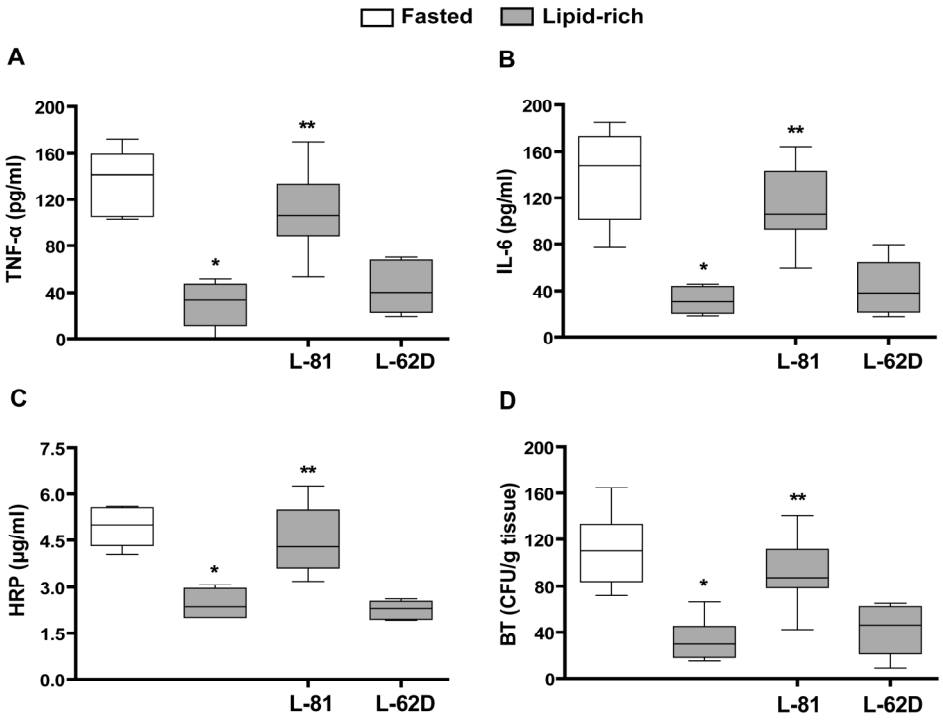

Figure 3: Lipid-enriched nutrition-induced chylomicron formation is critical to reduce shock-induced inflammation and loss of intestinal integrity in rats. Hemorrhagic shock results in systemic inflammation and loss of intestinal integrity. Pretreatment with lipid-enriched nutrition attenuates systemic plasma levels of TNF-α (A) and IL-6 (B). Furthermore, lipid-enriched nutrition reduced ileal leakage of HRP (C) and bacterial translocation (D). Addition of L-81 to lipid-enriched nutrition reduced the inhibitory effect of the nutrition on systemic inflammation and loss of intestinal integrity, while L-62D did not affect these parameters. Data are represented as median, range and interquartile range. * $p < 0.01$ compared with fasted, ** $p < 0.01$ compared with lipid-enriched nutrition plus L-62D.

Addition of L-81 suppressed the increase in afferent discharge associated with lipid-enriched nutrition (Figure 5B). Since previous studies demonstrated that low-threshold afferents preferentially project via vagal pathways [15], we specifically quantified the increase in afferent firing rate over the pressure range from 0 to 20 mmHg. Lipid-enriched nutrition enhanced afferent discharge to distension compared with the discharge before treatment and after treatment ($p < 0.001$. Figure 5C). In the presence of L-81, the increase in afferent discharge triggered by lipid-enriched nutrition was prevented (Figure 5D). Furthermore, L-81 reduced the discharge compared with the increase of lipid-enriched nutrition alone (treatment

group in 5C; $p < 0.05$), indicating that chylomicron formation is an essential step in the lipid-enriched nutrition mediated activation of vagal afferents.

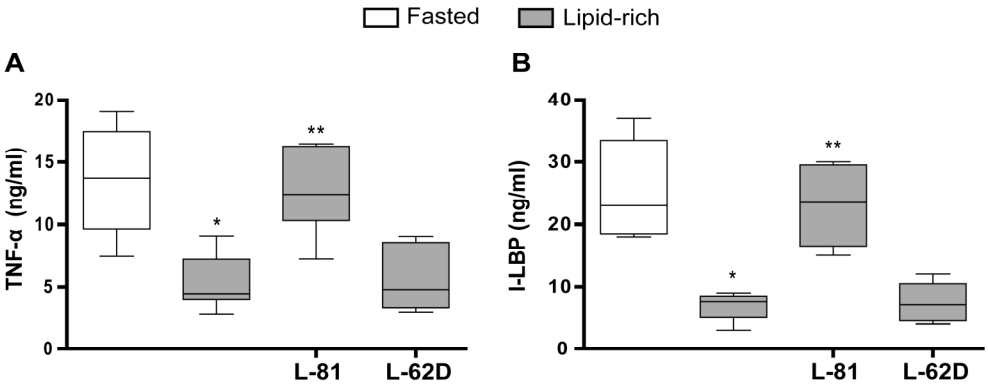

Figure 4: Prevention of chylomicron formation blunts the anti-inflammatory and gut-protective potential of lipid-enriched nutrition in endotoxemic mice. Intraperitoneal administration of endotoxin results in marked systemic levels of TNF-α and I-LBP (A-B). The systemic inflammation and enterocyte damage were effectively reduced by lipid-enriched nutrition. L-81 abrogates the inhibitory effect of lipid-enriched nutrition on plasma levels of TNF-α and I-LBP, while supplementation with L-62D did not affect the anti-inflammatory response (A-B). Data are represented as median, range and interquartile range. * $p < 0.05$ compared with fasted, ** $p < 0.05$ compared with L-62D.

Leptin is not involved in the nutritional anti-inflammatory pathway

Leptin is known to activate afferent vagal fibers and reduce food intake in combination with CCK [16]. Here, we investigated involvement of leptin in activation of the anti-inflammatory reflex by lipid-enriched nutrition in Ob/Ob mice. Administration of the standard dose of lipid-enriched nutrition did not affect the endotoxin-induced systemic inflammatory response and enterocyte damage in Ob/Ob mice (Figure 6A), indicating a potential role for leptin in activation of the nutritional anti-inflammatory pathway. However, providing a more potent stimulus via administration of a higher dose of lipid-enriched nutrition resulted in attenuated plasma levels of TNF-α ($p < 0.01$) and I-LBP ($p < 0.01$. Figure 6B). To further elucidate the role of leptin, we blocked the leptin receptor in wildtype mice using blocking nanobodies directed against the leptin receptor. Pretreatment of wildtype mice with nanobodies did not affect the anti-inflammatory potential of lipid-enriched nutrition compared with vehicle treatment (Figure 6C), indicating

that activation of the nutritional anti-inflammatory pathway is independent of leptin receptors.

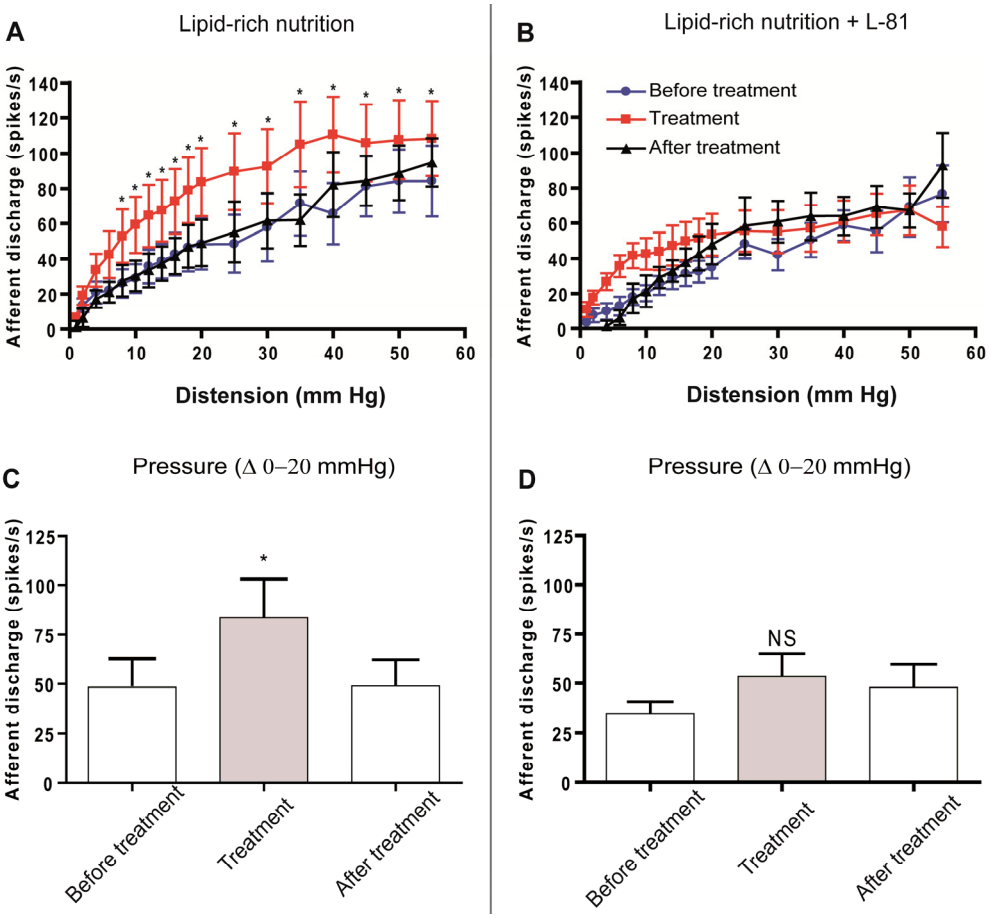

Figure 5: Activation of mesenteric afferent fibers is dependent on chylomicron formation. Lipid-enriched nutrition enhances discharge of mesenteric afferents to distension (A). Prevention of chylomicron formation with L-81 abrogates the increased firing of mesenteric afferents by lipid-enriched nutrition (B). The nutrition augmented mesenteric afferent discharge in a low-threshold experiment (C), whereas L-81 prevented activation (D). Data represented as mean +/- SEM. * $p < 0.05$ compared with control, n = 6.

GLP-1 receptor antagonists reduce the immuno-modulatory properties of lipid-enriched nutrition

The intestinal peptides GLP-1 and PYY inhibit food intake in response to luminal fat and their release has been related to CCK-release [14, 17]. In rats,

pretreatment with the GLP-1 receptor antagonist, exendin-3 suppressed the effects of lipid-enriched nutrition on TNF-α, IL-6, leakage of HRP and bacterial translocation to a limited extent (all parameters; $p < 0.05$; Figure 7A-B). These findings suggest that activation of the GLP-1 receptor by lipid-enriched nutrition is involved in the vagal anti-inflammatory reflex.

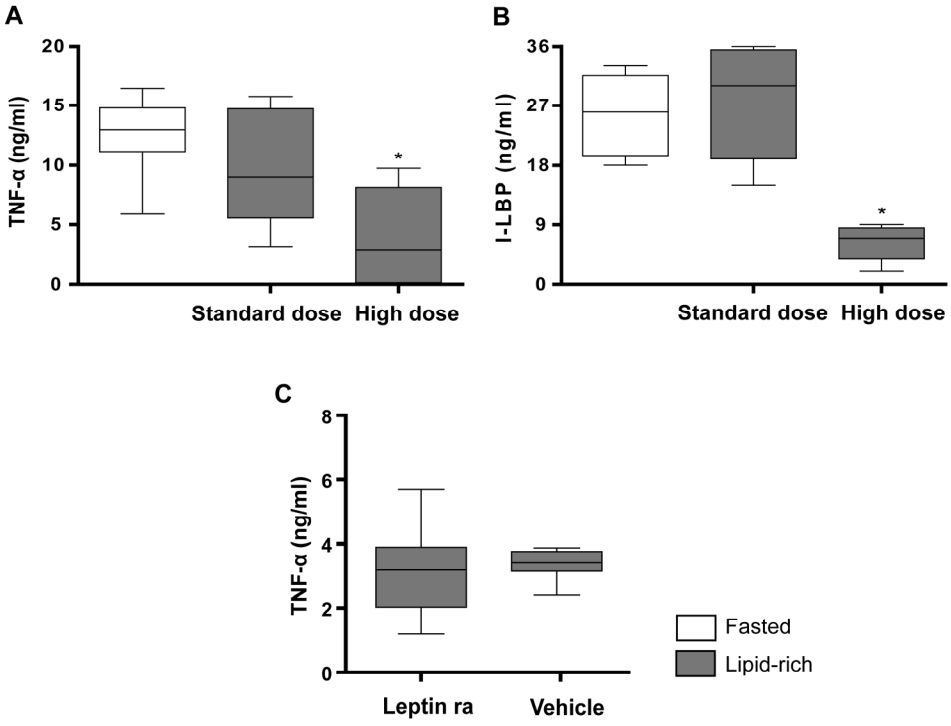

Figure 6: Endotoxemia results in marked systemic inflammation and enterocyte damage in Ob/Ob mice. Administration of lipid-enriched nutrition did not reduce plasma levels of TNF-α and I-LBP (A). Increasing the dose of lipid-enriched nutrition from 0.9 kCal (standard dose) to 1.3 kCal (high dose) attenuated plasma levels of TNF-α and I-LBP (B). The anti-inflammatory potential of lipid-enriched nutrition was unaffected in wildtype mice treated with leptin receptor blocking nanobodies (leptin ra; C). Data are represented as median, range and interquartile range.* $p < 0.01$ compared with fasted.

In contrast, administration of the selective antagonist to the PYY-receptor failed to reduce the inhibitory actions of lipid-enriched nutrition on hemorrhagic shock-induced plasma levels of TNF-α (64 [31 to 87] pg/ml vs vehicle: 62 [38 to 95] pg/ml) and IL-6 (23 [17 to 53] pg/ml vs vehicle: 30 [16 to 53] pg/ml). Furthermore, loss of

intestinal permeability (HRP: 2.4 [2.0 to 4.2] µg/ml vs vehicle: 3.2 [1.9 to 3.8] µg/ml) and bacterial translocation (38 [14 to 42] CFU/g tissue vs vehicle: 33 [37 to 42] CFU/g tissue) were not affected, indicating that activation of the PYY-receptor is not involved in the nutritional anti-inflammatory reflex.

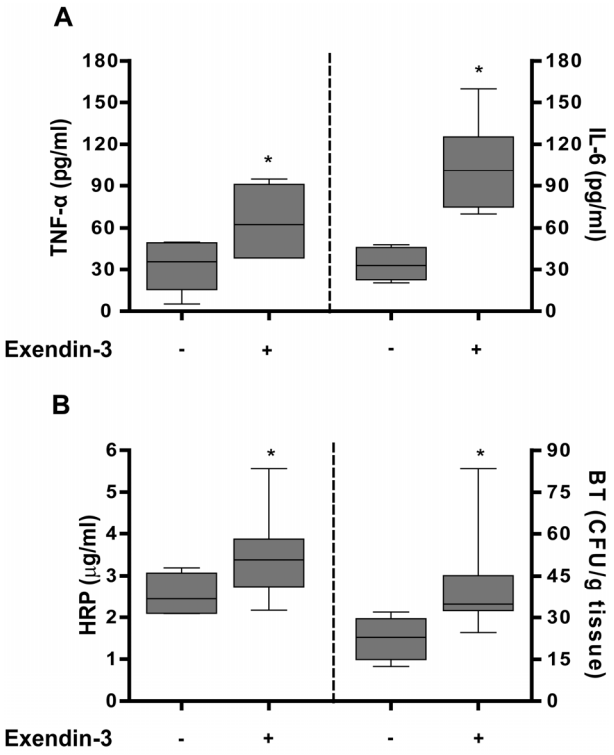

Figure 7: GLP-1 is involved in the immuno-modulatory effects of lipid-enriched nutrition. Pretreatment with the GLP-1 receptor antagonist, Exendin-3 reduced the effect of lipid-enriched nutrition on shock-induced systemic inflammation (A) and intestinal integrity (B). Data are represented as median, range and interquartile range.* $p < 0.01$ compared with vehicle.

Discussion

The current study provides insight in the mechanisms that occur at the level of the intestine, resulting in activation of the CCK-mediated nutritional anti-inflammatory reflex. First, we demonstrate that formation of chylomicrons induced by absorption of lipid-enriched nutrition plays a vital role in activation of the autonomic nervous system and the anti-inflammatory pathway in both rats and

mice. Secondly, we show that the intestinal peptide GLP-1, in addition to CCK, is involved in the immune-modulating properties of lipid-enriched nutrition.

Absorption of luminal nutrients exposes the host to antigenic components, which are able to activate the immune system [18, 19]. Postprandial chylomicron formation and subsequent systemic dissemination have been shown to lead to an inflammatory response, due to their high affinity for dietary antigens, such as endotoxin [19, 20]. Additionally, prevention of chylomicron formation attenuates LPS release from enterocytes and avoids postprandial endotoxemia [21]. Interestingly, we reveal that assembly and secretion of chylomicrons is an important step in activation of the nutritional anti-inflammatory vagovagal reflex.

The formation of chylomicrons, induced by ingestion of long-chain fatty acids and phospholipids, has been shown to activate the autonomic nervous system in a CCK-dependent manner [10, 22, 23]. Using a preparation of murine jejunum, we ascertained that lipid-enriched nutrition enhanced mesenteric afferent discharge to distension. Co-administration of L-81 reduced mesenteric afferent firing in the low-threshold range, providing evidence that activation of afferent vagal fibers depends on chylomicron formation [15]. It is noteworthy that this effect was observed within 15 minutes of exposure to L-81, suggesting that chylomicron assimilation rapidly leads to afferent activation.

As established in previous studies [5-7, 24], lipid-enriched nutrition reduced inflammation and preserved intestinal integrity. Prevention of chylomicron formation reduced the effects of lipid-enriched nutrition on systemic inflammation and intestinal integrity in both hemorrhagic shock rats and endotoxemic mice. These findings indicate that intestinal uptake and intracellular processing of lipids are important steps in initiation of the nutritional anti-inflammatory reflex in rodents. Previously, our group demonstrated that bile duct-ligation (BDL) in rats did not affect the anti-inflammatory potential of lipid-enriched nutrition, suggesting that uptake of lipids is not essential [25]. However, BDL does not completely obstruct lipid uptake. Specifically the uptake of linoleic acid, which is a strong inducer of chylomicron formation and potent CCK secretagogue, remains largely unaffected [26-28]. Since CCK release and activation of vagal afferents are dependent on absorption of lipids and chylomicron formation [22, 29], it is likely that the remaining lipid uptake in BDL rats results in sufficient chylomicron formation and CCK-release to activate the anti-inflammatory pathway. Taken together, our data demonstrate that the intracellular processing of lipids plays a dominant role in activation of the anti-inflammatory reflex, since L-81 blocks assembly and secretion of chylomicrons, but does not affect lipid uptake [30]. Moreover, these data implicate that the nutritional compositions, aimed at modulating the immune response, should be rich in long-chain fatty acids and phospholipids. As reviewed by Calder in detail, dietary supplementation with specific fatty acids, such as long-chain n-3

polyunsaturated fatty acids results in profound immune-modulating effects by influencing metabolic processes, such as eicosanoid production and other as of yet unidentified mechanisms [31]. In addition, our data indicate that enteral nutrition enriched with lipids directly activates an anti-inflammatory reflex.

Previous findings from our group demonstrated that the immuno-modulatory effects of lipid-enriched nutrition are dependent on CCK-receptors, suggesting that the pathway is largely CCK driven [6, 7]. However, this does not exclude a role for other intestinal peptides. CCK has been termed "gatekeeper of the afferent vagus" and nutrient-induced release of several intestinal peptides depend on activation of CCK-1 receptors [11, 14]. Leptin, GLP-1 and PYY are released in response to ingestion of dietary fat and function in combination with CCK to regulate satiety via afferent vagal fibers [14, 17, 32, 33].

Involvement of leptin in the immune response was described more than a decade ago [34-36]. In the current study, lipid-enriched nutrition failed to reduce systemic inflammation and enterocyte damage in Ob/Ob mice. Increasing the nutrient dose, which enhances the anti-inflammatory potential [24], effectively reduced TNF-α and I-LBP plasma levels, suggesting that Ob/Ob mice have a higher stimulation threshold and implying a co-stimulatory role for leptin. In line, leptin has been described to ameliorate the anti-inflammatory and satiation potential of CCK [12, 37]. However, a co-stimulatory role of leptin could not be established, since pharmacologic inhibition of leptin receptors in wildtype mice did not influence the effects of lipid-enriched nutrition. Considering that a higher dose of nutrition was needed in Ob/Ob mice, our data might indicate that the vagus nerve is less sensitive to lipid-enriched nutrition. This is supported by the fact that overfeeding, which is typical for leptin-deficient mice, desensitizes the afferent vagal pathway, resulting in defective intestinal lipid signaling [38, 39]. To unravel these findings, future studies are needed to evaluate the effect of long-term high-fat intake on activation of the nutritional pathway.

There is little evidence thus far that GLP-1 and PYY are involved in regulation of the inflammatory response [40, 41]. However, a role for both peptides in nutrient-dependent activation of vagal afferents has been clearly established [42]. Moreover, release of GLP-1 and PYY is dependent on activation of CCK-1 receptors [14, 17]. Here, we demonstrate that activation of GLP-1 receptors by lipid-enriched nutrition is involved in activation of the anti-inflammatory reflex. Administration of a GLP-1 receptor antagonist suppressed the anti-inflammatory potential of lipid-enriched nutrition, but did not abolish the effect. A role for the PYY could not be demonstrated, as pretreatment with the Y2-receptor antagonist did not affect activation of the nutritional anti-inflammatory pathway. These findings are supported by Raybould et al., who demonstrated that PYY-neutralizing antibodies do not affect lipid-induced gastric emptying, which is known to be regulated by

vagal afferents [43, 44]. Taken together, the current findings suggest a co-stimulatory role for GLP-1 in nutritional activation of the CCK-mediated anti-inflammatory vagovagal reflex.

In surgical and critically-ill patients, nutritional support is vital to meet metabolic demand and prevent immunodeficiency [36, 45]. Enteral administration of nutrients is preferred in these patient groups, since enteral nutrition reduces morbidity and length of hospital stay compared with parenteral nutrition [45, 46]. The current study demonstrates that enteral nutrition not only delivers essential nutrients, but also triggers a potent intestinal immune feedback system. We believe that this endogenous anti-inflammatory mechanism evolved to counteract exposure of the interior milieu to antigenic substances, such as endotoxin, resulting in postprandial inflammation and potentially aggravating chronic inflammatory conditions [19, 20]. Development of specific nutritional compositions, that effectively stimulate the vagovagal anti-inflammatory reflex, and well-timed administration could result in promising interventions to control inflammation and reduce organ damage during non-physiologic inflammatory conditions, such as surgical interventions and sepsis.

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Chapter 3.1

Post-shock intervention with lipid-enriched enteral nutrition reduces inflammation and tissue damage

Jacco J. de Haan
Tim Lubbers
M'hamed Hadfoune
Misha D.P. Luyer
Cornelis H. Dejong
Wim A. Buurman
Jan-Willem M. Greve

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Abstract

Background: An excessive inflammatory response following severe trauma is associated with poor clinical outcome. Currently, therapies directed at attenuation of an ongoing inflammatory cascade are lacking. Administration of lipid-enriched enteral nutrition prior to hemorrhagic shock has been shown to effectively inhibit early and late proinflammatory cytokines by activation of the autonomic nervous system via cholecystokinin (CCK)-receptors.

Objective: To investigate the effects of lipid-enriched enteral nutrition in a setting of developing inflammation and tissue damage initiated by hemorrhagic shock.

Methods: A rat model of hemorrhagic shock was used in which animals were either treated with enteral lipid-enriched or control low-lipid enteral nutrition or fasted. CCK-receptor antagonists were administered before feeding. Tissues and plasma were collected to assess inflammation and intestinal integrity.

Results: Administration of lipid-enriched enteral nutrition after shock significantly reduced plasma interferon-gamma (IFN-γ) and IL-10 compared to fasted animals (both $P < 0.001$). Furthermore enterocyte damage, measured as circulating ileal lipid binding protein (I-LBP), was prevented by high-lipid intervention ($P < 0.001$). Lipid-rich intervention preserved intestinal integrity in comparison to fasted animals, as assessed by bacterial translocation (BT) to distant organs ($P < 0.001$) and ileal permeability to horseradish peroxidase (HRP) ($P < 0.001$). The protective effects of high-lipid intervention were abrogated by CCK-receptor antagonists (IFN-γ; IL-10; BT; and HRP; $P < 0.05$).

Conclusion: Lipid-enriched enteral nutrition given post-shock reduces inflammation and tissue damage via a CCK-receptor dependent mechanism. These findings implicate that intervention with high-lipid enteral nutrition following events such as severe trauma is a potential therapy to attenuate the developing inflammatory response.

Introduction

An uncontrolled inflammatory response as result of severe trauma followed by a second hit such as surgery or infection is associated with poor clinical outcome [1-4]. Despite important improvements in the treatment of trauma patients over the last decades, morbidity and mortality remain high [5-7]. Our understanding of the inflammatory cascade triggered by severe trauma has advanced rapidly, however, until now there is no effective clinical therapy that controls excessive inflammation [8]. Consequently, current treatment of patients with severe trauma is aimed at rapid hemodynamic stabilization, damage control and sustained support of organ and endocrine systems [9, 10].

Improved insight into the complex inflammatory response following trauma has led to the development of diverse anti-inflammatory strategies, usually directed at single inflammatory mediators. Although such strategies were highly promising in various experimental models of trauma and sepsis, most interventions lacked effectiveness in clinical trials [8]. The intervention applied in the current study might overcome this problem by activating a potent endogenous pathway that modulates the immune system.

In recent studies, our group demonstrated that high-lipid enteral nutrition strongly attenuated the inflammatory response and preserved tissue integrity in a model of hemorrhagic shock [11, 12]. The protective effects of lipid-enriched enteral feeding are based on nutritional activation of the autonomic nervous system via cholecystokinin (CCK)-receptors leading to activation of nicotinic receptors located on inflammatory cells [13-15].

In previous studies high-lipid enteral nutrition was administered prior to shock. However, in many clinical settings as trauma, the inflammatory response is already developing before treatment can be initiated. Therefore, the aim of this study was to investigate the protective effects of lipid-enriched enteral nutrition administered in a setting in which the inflammatory cascade is unfolding.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 300-350 g were purchased from Charles River Laboratories (Maastricht, the Netherlands). Animals were housed under standardized conditions of temperature and humidity and had free access to standard chow and water. The study was approved by the Animal Ethics Committee of Maastricht University Medical Center.

Experimental design

Non-lethal hemorrhagic shock was induced as previously described [11, 12, 16]. In short, rats were anesthetized with isoflurane (induction 4%, maintenance 1.5%). The femoral artery was dissected and cannulated with a polyethylene tubing (PE-10) containing heparinized saline (10 IU/ml). At time of shock (t=0), 2.1 ml blood per 100 g body weight (representing 30-40% of circulating volume) was withdrawn at a rate of 1 ml/minute. Anesthesia was stopped at 60 minutes after shock. Prior to shock, all rats were fasted for 18 hours. Following shock, rats were fasted or submitted to either an early or a delayed intervention with high-lipid or control low-lipid enteral nutrition. In the early feeding regime, animals received 1.5 ml nutrition at 30 minutes after shock by way of orogastric tube (PE-50) and 0.75 ml at 3 hours post-shock by way of oral gavage (n=8 for each group). Animals in the early feeding protocol were sacrificed at 4 hours after shock. In the delayed feeding regime, animals received nutrition at 80 minutes (1.5 ml), 3 and 6 hours (both 0.75 ml) following shock. After the last feeding moment, they were given free access to standard chow and sacrificed at 24 hours after shock (n=10 for each group, Figure. 1). CCK-A and -B receptor antagonists (Devazepide and L365, 260 resp.; kind gifts from ML Laboratories PLC, Nottingham, United Kingdom) (both 500 µg/kg) were given 10 minutes before the first and second high-lipid feeding intravenously resp. intraperitoneally (n=6). A control group received high-lipid nutrition and vehicle (90% NaCl, 5% Tween 20, 5% DMSO).

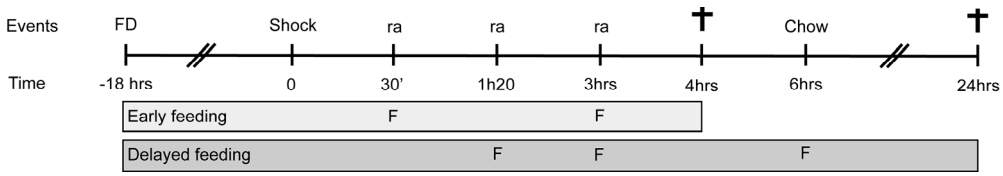

Figure 1: Experimental design of post-shock intervention with high-lipid enteral nutrition. Rats were deprived of food (FD) 18 hours prior to shock. At t=0, shock was induced. Subsequently, rats were fasted or received a liquid high-lipid or control low-lipid enteral nutrition in either an early or a delayed feeding regime. In the early feeding regime, nutrition (F) was administered at 30 minutes by gastric tube and at 3 hours by oral gavage (1.5 and 0.75ml resp.) and rats were sacrificed after 4 hours (†). In the delayed feeding protocol, nutrition was given at 80 minutes, 3 and 6 hours following shock (1.5ml, 0.75ml and 0.75ml *respectively*) by oral gavage. Next, rats were given free access to standard chow (chow) and sacrificed after 24 hours (†). In both feeding protocols, CCK-receptor antagonists were administered 10 minutes prior to the first and second high-lipid feeding (ra).

Composition of nutrition

The lipid-enriched liquid enteral diet contained 50.4 energy percent (en%) fat, of which 30% constituted of phospholipids, 8.7en% protein and 40.9en% carbohydrates. The low-lipid nutrition contained 16.0en% fat, 8.7en% proteins and 75.3en% carbohydrates. The high-lipid nutrition was isocaloric and isonitrogenous to the low-lipid nutrition and the amount of fat in the low-lipid diet was isocaloric to that present in standard rodent chow. The types of carbohydrates, proteins and fat used in both diets were identical. The lipid source was lecithin. Omega 3 and 6 fatty acids constituted <5% in both feedings. Proteins were derived from lean milk powder, containing 80% casein and 20% whey protein. The carbohydrate source was a mixture of sucrose and maltodextrins (Glucidex 19DE).

Blood analysis

Interferon-gamma (IFN-γ), ileal lipid binding protein (I-LBP) and IL-10 concentrations in arterial blood were determined using standard ELISA for rat IFN-γ and rat-I-LBP (both kindly provided by Hycult Biotechnology (Hbt), Uden, the Netherlands) and rat IL-10 (Biosource, Camarillo, CA).

Myeloperoxidase quantification

Per rat, sacrificed at 4 hours after shock, three segments of ileum were snap frozen in liquid nitrogen. Segments were homogenized in lysisbuffer (300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, en Pepstatin A, Leupeptin, Aprotinin (all 20 ng/ml); pH 7.4), centrifuged and supernatants stored at -20^{0}C until analysis. Myeloperoxidase (MPO) was quantified using ELISA. In brief, a microtiter plate was coated with mAb 8F4, cross reactive with rat MPO (Hbt, Uden, the Netherlands) overnight at 4 ^{0} C and blocked with 1% BSA in PBS. Binding was detected with biotinylated rabbit-α-human MPO (DAKO, Glostrup, Denmark) and visualized with 3,3',5,5'-Tetramethylbenzidine (TMB). The results were recorded using an ELISA plate reader at 450 nm. MPO content per sample was calculated, after correction for total extracted protein per sample.

Intestinal permeability

Intestinal permeability was assessed by measuring permeability to horseradish peroxidase (HRP) in isolated segments of ileum as previously described [11, 17]. In short, 8-cm segments of the distal ileum were washed, everted, and filled with 1 ml of Tris buffer (125 mmol/l NaCl, 10 mmol/l fructose, 30 mmol/l Tris; pH 7.5) and ligated at both ends. The filled segment was incubated in Tris buffer containing 40 mg/ml of HRP (Sigma, St. Louis, MO). After incubation at room temperature for 45 minutes, segments of ileum were removed from the buffer and the content was

carefully collected in a 1-ml syringe. HRP activity was measured spectrophotometrically at 405 nm after addition of TMB as substrate for HRP.

Microbiological methods

Bacterial translocation to distant organs was assessed as described [11-13]. In short, mesenteric lymph nodes, the mid-section of the spleen and liver-segment IV were collected aseptically in preweighed thioglycolate broth tubes (Becton Dickinson, Franklin Lakes, NJ) in all rats. Tissue-fragments were weighed and homogenized and subsequently, the suspension was transferred to agar plates (Columbia III blood agar base supplemented with 5% vol/vol sheep blood (BBL, Franklin Lakes, NJ) (duplicate plates) and Chocolate PolyviteX agar (BioMérieux, Marcy L'Etoile, France)). After 48 hours of incubation, the number of colonies were counted and determined using conventional techniques. Subsequently, the numbers were adjusted to tissue weight, and expressed as number of colony forming units (CFU) per gram of tissue.

Immunohistochemistry

Ileum sections (4μm) of rats sacrificed 4 hours after hemorrhagic shock and healthy controls were stained for tight junction protein Zonula Occludens protein 1 (ZO-1). After a 1 hour incubation with Rabbit anti-ZO-1 (61-7300, Zymed Laboratories, San Francisco, CA), the sections were incubated for 1 hour with Texas red conjugated goat anti-rabbit antibody (Jackson, West Grove, PA). This was followed by 2 minutes incubation with 4',6-diamino-2-phenyl indole (DAPI), mounted in Fluorescence Mounting Solution (Dakocytomation). The distribution of tight junctions was recorded at a magnification of 200x using the Metasystems Image Pro System (black and white charge-couple device camera; Metasystems, Sandhausen, Germany) mounted on a Leica DM-RE fluorescence microscope (Leica, Wetzler, Germany). All images were taken at equal time-exposures after being normalized to negative control sections without primary antibody, to exclude for non-specific binding of the secondary antibody or autofluorescence. At least 25 microscopic fields for each tissue section were examined.

Statistical analysis

All data are expressed as mean ± SEM. A Mann-Whitney U test was used for between-group comparisons. Differences were considered statistically significant at $p \leq 0.05$.

Results

Lipid-enriched enteral intervention following hemorrhagic shock reduces inflammation

Two feeding protocols were chosen (Figure 1). An early feeding regime started at 30 minutes following shock, the moment at which the first signs of intestinal tight junction loss were observed (data not shown). A delayed feeding protocol was initiated at 80 minutes post-shock at which timepoint systemic inflammation is detectable [18].

Early intervention with high-lipid enteral feeding after shock significantly decreased circulating IFN-γ compared with low-lipid treated and fasted animals (0.4 ± 0.1 ng/ml vs. 0.8 ± 0.1 ng/ml; $P < 0.01$ and 1.4 ± 0.1 ng/ml; $P < 0.001$). Also low-lipid nutrition resulted in lower IFN-γ levels compared with fasted animals ($P < 0.01$). CCK-receptor antagonists (CCK-ra) were administered 10 minutes before treatment with high-lipid nutrition to assess the role of CCK-receptor mediated activation of the autonomic nervous system. CCK-ra inhibited the effects of high-lipid nutrition on plasma IFN-γ (1.2 ± 0.2 ng/ml vs. vehicle: 0.6 ± 0.1 ng/ml; $P < 0.05$, Figure 2A). Also plasma levels of the anti-inflammatory cytokine IL-10 were markedly decreased by treatment post shock with high-lipid enteral nutrition in the early feeding regime compared with animals that were fasted after shock (109 ± 7 pg/ml vs. 156 ± 4 pg/ml; $P < 0.001$). A protective trend was seen in high-lipid treated animals compared to low-lipid controls (129 ± 7 pg/ml, $P = 0.06$). Moreover, low-lipid nutrition reduced IL-10 levels compared to fasted animals ($P < 0.01$). Administration of CCK-ra blocked the effects of high-lipid feeding on IL-10 plasma levels compared with vehicle treated animals (150 ± 9 pg/ml vs. 107 ± 10 pg/ml; $P < 0.05$, Figure 2B).

Local intestinal inflammation was assessed by detection of myeloperoxidase (MPO) levels in ileum. In line with systemic inflammatory parameters, a protective trend towards reduced tissue MPO concentrations were observed in rats treated with high-lipid enteral feeding compared with low-lipid feeding (1.5 ± 0.3 ng/µg protein vs. 2.3 ± 1.0 ng/µg protein; $P = 0.06$). Post-shock treatment with low-lipid nutrition did not sort any effect on MPO tissue levels in comparison with animals that were fasted (2.5 ± 1.2 ng/µg protein; $P = 1.0$, Figure 3).

Administration of high-lipid enteral nutrition after shock reduces intestinal damage

Hemorrhagic shock resulted in an elevation of plasma ileal lipid binding protein (I-LBP), a 14kD cytosolic protein expressed by mature enterocytes that is rapidly released following cellular damage. Both the early and delayed feeding regimes with a high-lipid enteral diet significantly lowered I-LBP-levels in plasma compared

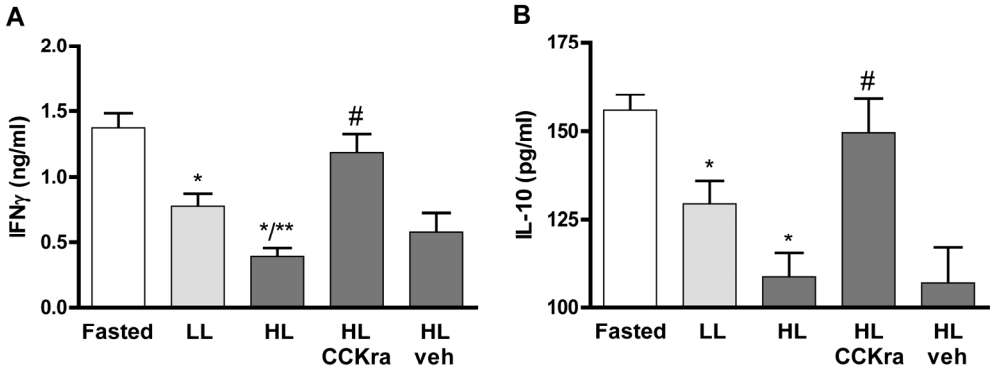

Figure 2: High-lipid enteral intervention in the early feeding regime following hemorrhagic shock reduces plasma IFN-γ and IL-10. High-lipid (HL) enteral diet after shock significantly reduced plasma IFN-γ concentrations at 4 hours after shock compared with low-lipid (LL) treated or fasted animals (A; * $P < 0.01$ and ** $P < 0.01$ resp.). Administration of low-lipid nutrition resulted in decreased IFN-γ levels compared with fasted animals (* $P < 0.01$). Administration of CCK-receptor antagonists (CCKra) abrogated the anti-inflammatory effects of high-lipid nutrition on IFN-γ (# $P < 0.05$ to high-lipid + vehicle). High-lipid enteral feeding following shock significantly decreased plasma levels of IL-10 compared with fasted controls (* $P < 0.01$) and a trend of IL-10 reduction was observed of high-lipid feeding compared with low-lipid treated controls (B). Next, low-lipid treated animals resulted in lower IL-10 levels compared with fasted animals (* $P < 0.01$). CCKra blunted the effect of high-lipid nutrition on IL-10 (# $P < 0.05$ to high-lipid + vehicle).

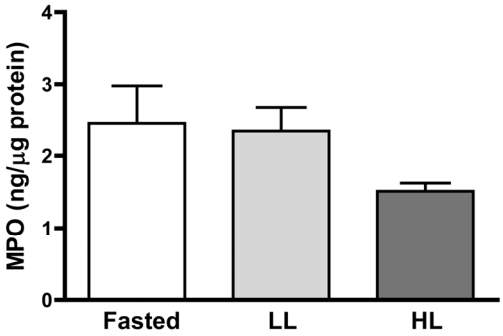

Figure 3: Protective effects of high-lipid enteral nutrition on local inflammation. A protective trend towards decreased myeloperoxidase (MPO) concentrations in ileum was seen in high-lipid (HL) treated animals compared with low-lipid (LL) control groups ($P = 0.06$). Low-lipid feeding did not exert any effect on tissue MPO levels compared with fasted animals.

with fasted animals (early feeding: 3.7 ± 0.3 ng/ml vs. 8.0 ± 1.1 ng/ml; $P < 0.001$ and delayed feeding: 1.3 ± 0.2 ng/ml vs. 6.3 ± 0.7 ng/ml; $P < 0.001$, Figure 4A and B). Moreover, I-LBP levels were significantly reduced in high-lipid treated animals compared with the low-lipid group in the early feeding regime (4.9 ± 0.5 ng/ml; $P = 0.05$). Low-lipid feeding resulted in decreased I-LBP levels compared with fasted animals (early and delayed feeding; $P < 0.05$).

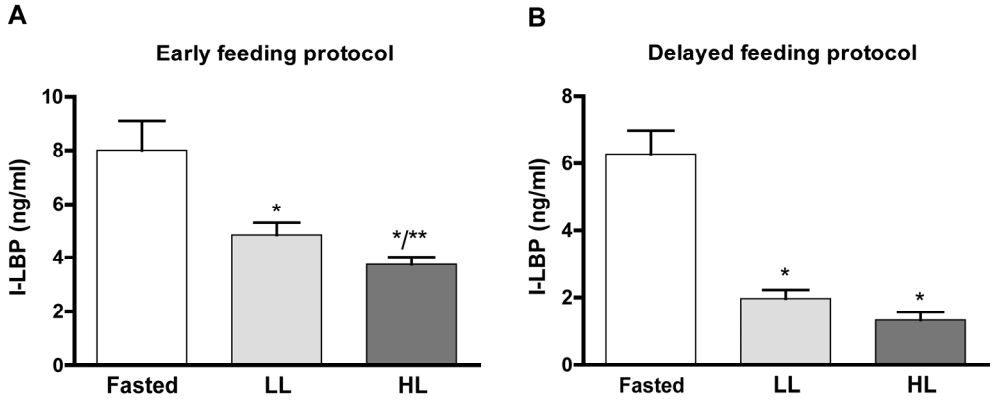

Figure 4: Administration of high-lipid enteral nutrition after shock decreases intestinal damage. Early high-lipid (HL) enteral feeding resulted in lower plasma ileal lipid binding protein (I-LBP) levels compared with low-lipid (LL) feeding (A; ** $P < 0.05$). I-LBP plasma levels after early nutritional intervention (A) and the delayed intervention (B) were significantly reduced in high-lipid and low-lipid treated animals compared with fasted controls (* $P \leq 0.05$).

Improved intestinal barrier function

Bacterial translocation to distant organs and leakage of HRP in ileum were determined as parameters for loss of intestinal integrity following hemorrhagic shock. An early enteral regime of high-lipid feeding after shock resulted in a significantly reduced number of colony forming bacteria in distant organs compared with fasted animals (69.7 ± 6.4 CFU/gr tissue vs. 237.6 ± 16.4 CFU/gr tissue; $P < 0.001$) and to animals on a low-lipid diet (100.9 ± 9.2 CFU/gr tissue; $P < 0.05$). Also low-lipid feeding reduced bacterial translocation compared to fasted animals ($P < 0.01$, Figure 6A). Furthermore, early intervention after shock with lipid-enriched enteral feeding significantly reduced leakage of HRP compared with fasted animals (9.0 ± 0.8 µg/ml vs. 26.4 ± 2.1 µg/ml; $P < 0.001$). Moreover, low-lipid feeding reduced ileal permeability to HRP in comparison with fasted animals (11.8 ± 1.1 µg/ml; $P < 0.05$, Figure 6B). The delayed feeding regime resulted in a significant reduction of bacterial translocation at 24 hours after shock in animals given high- lipid feeding (43.0 ± 3.6 CFU/g tissue vs. fasted: 133.1 ± 16.8 CFU/g

tissue; $P < 0.001$ and vs. low-lipid: 69.0 ± 4.9 CFU/g tissue; $P < 0.01$, Figure 6C). Furthermore, low-lipid feeding reduced bacterial translocation compared to fasted animals ($P < 0.001$). Also HRP leakage was reduced after the delayed feeding regime with high-lipid intervention compared with low-lipid treated and fasted controls (6.6 ± 0.2 µg/ml vs. 7.3 ± 0.3 µg/ml; $P < 0.05$ and 9.1 ±0.3 µg/ml; $P < 0.001$, Figure 6D). Next, low-lipid feeding reduced HRP permeability compared with fasted animals ($P < 0.05$).

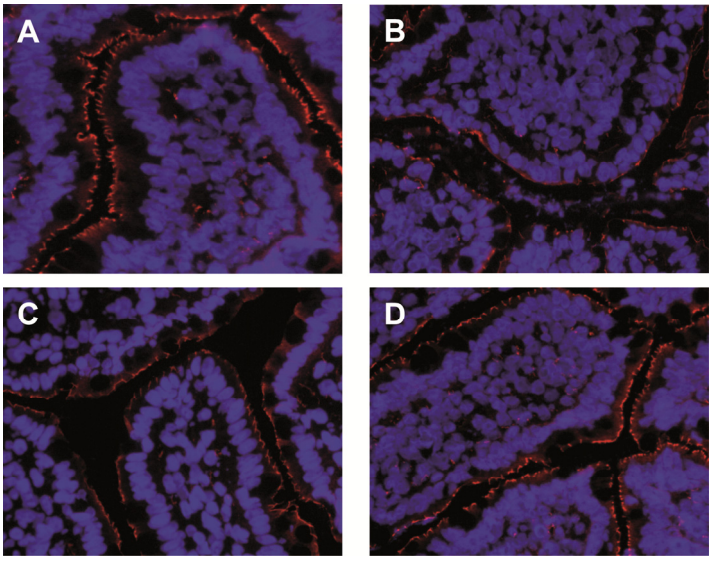

Figure 5: High-lipid feeding improves tight junction integrity in the intestine. In healthy control animals, ZO-1 expression on ileal villi at 200x magnification was intact and regular (A). Fasting after shock resulted in ZO-1 villus lining in animals that was irregular and discontinuous (B). In animals receiving high-lipid nutrition following shock, ZO-1 expression was largely preserved, although small irregularities were observed (C-D). The protective effects were present to a lesser extent following treatment with low-lipid nutrition. The histology shown is representative for all tissue samples studied.

Administration of CCK-receptor antagonists (CCKra) abrogated the protective effects of high-lipid nutrition on intestinal integrity. CCKra inhibited the reduction of bacterial translocation in high-lipid treated animals compared to high-lipid treated animals given vehicle in both feeding regimes (early feeding: 173.3 ± 12.6 CFU/g tissue vs. 110.3 ± 14.0 CFU/g tissue; $P < 0.05$, Figure 6A and delayed feeding: 165.5 ± 14.4 CFU/g tissue vs. 54.2 ± 11.8 CFU/g tissue; $P < 0.05$, Figure 6B).

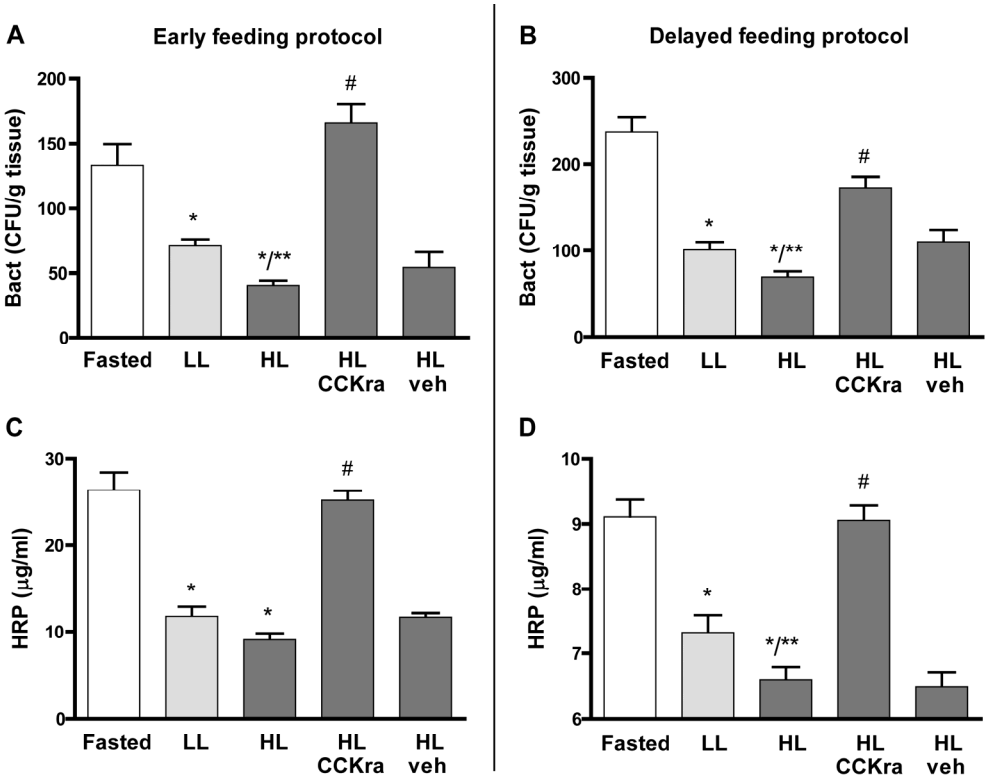

Figure 6: Bacterial translocation was significantly reduced in animals on an early high-lipid (HL) regime compared to low-lipid (LL) treated and fasted controls (A; * $P < 0.05$ and ** $P < 0.01$ resp.). Furthermore, low-lipid enteral feeding decreased bacterial translocation compared to fasted animals (* $P < 0.01$). Early intervention with lipid-enriched feeding after shock significantly reduced leakage of horseradish peroxidase (HRP) in comparison to fasted animals (B; * $P < 0.05$). Also low-lipid feeding resulted in a significant reduction of HRP permeability compared to fasted animals (* $P < 0.05$). Delayed intervention with high-lipid nutrition reduced bacterial translocation significantly (C; * $P < 0.01$ vs. fasted and ** $P < 0.001$ vs. low-lipid). Moreover, bacterial translocation was reduced in low-lipid animals compared to the fasted group (* $P < 0.01$). A significant decrease of permeability to HRP was seen after delayed enteral feeding with high-lipid nutrition compared to low-lipid treated (D; ** $P = 0.05$) and fasted animals (* $P < 0.05$). Also low-lipid feeding resulted in a significant reduction of HRP permeability compared to fasted animals (* $P < 0.05$). In both feeding protocols CCK receptor antagonists abrogated the effects of high-lipid nutrition on bacterial translocation (early and delayed feeding: # $P < 0.05$ compared with HL + vehicle). Administration of CCK receptor antagonists blunted the protective effects of high-lipid feeding on ileal permeability to HRP in both the early and delayed feeding regime (# $P < 0.05$ compared with HL + vehicle).

Blockage of CCK-receptors also abrogated the reduction of HRP leakage by high-lipid nutrition compared with vehicle treated animals (early feeding: 25.2 ± 2.5 µg/ml vs. 11.7 ± 1.0 µg/ml; $P < 0.05$ and delayed feeding: 9.1 ± 0.2 µg/ml vs. 6.5 ± 0.2 µg/ml; $P < 0.05$).

Discussion

The inflammatory response to trauma has to be regulated carefully [19]. Uncontrolled inflammation occurring after severe trauma may result in a systemic inflammatory response syndrome (SIRS) or sepsis that are associated with poor clinical outcome [2, 8, 20-22]. Therefore, control of the inflammatory cascade following trauma is considered pivotal to prevent the development of detrimental inflammatory syndromes [3, 8, 23, 24]. To mimic the clinical setting of trauma in which the inflammatory response is unfolding only a post-treatment approach is feasible, therefore the intervention in this study was started after induction of hemorrhagic shock. In this study the short-term effects (within 24 hours) of inhibition of inflammation by nutritional intervention were investigated.

This is the first study to show that post-shock treatment with high-lipid enteral feeding reduces the shock-induced developing inflammatory response in a CCK-receptor dependent mechanism. Hemorrhagic shock resulted in significantly increased though moderately high circulating IFN-γ levels (1.4 ng/ml). Early administration of lipid-enriched feeding following shock strongly decreased these plasma levels of interferon-gamma (IFN-γ), a potent late inflammatory mediator with multiple actions on both innate and adaptive immune system [25]. In various experimental models including hemorrhagic shock elevated IFN-γ levels have been implicated in the development of inflammation, tissue injury and gut barrier dysfunction [26-29]. Also clinical studies demonstrated a crucial role of IFN-γ in the post-traumatic immune response [30, 31]. Recent experimental studies indicated that reduction of late inflammatory mediators such as IFN-γ is essential to attenuate an ongoing inflammatory response induced by major trauma, [28, 32]. The reduced levels of plasma IFN-γ following post-shock high-lipid feeding were accompanied by reduced circulating levels of anti-inflammatory cytokine IL-10. Elevated IL-10 plasma levels indicate a hyper inflammatory state and are associated with an increased risk to develop septic complications . In the current study, the reduction of both pro- and anti-inflammatory cytokines (IFN-γ and IL-10 resp.) indicates a broad inhibiting effect on developing inflammation by post-shock treatment with high-lipid enteral feeding.

Next, post-shock high-lipid enteral intervention strongly improved intestinal integrity. Translocation of bacteria and permeability to horseradish peroxidase

(HRP) in high-lipid treated animals were significantly reduced compared to low-lipid and fasted controls. These effects are in line with the effects of nutritional intervention given prior to shock [11, 12, 18]. Intestinal barrier dysfunction is widely postulated to play an important role in the development of inflammatory complications following surgery or trauma [33-39]. On the other hand, inflammatory cytokines such as IFN-γ impair intestinal barrier function by disruption of epithelial tight junctions [25, 29]. In accordance, our study shows that a reduction of systemic (IFN-γ and IL-10) and local inflammation (MPO) by high-lipid intervention parallels a preserved expression of tight junction protein ZO-1. Furthermore, the findings that elevated ileal lipid binding protein (I-LBP) plasma levels following shock were reduced by lipid-enriched nutrition indicate a protective effect on intestinal epithelial cell damage as well [40-43]. These protective effects on intestinal integrity were observed in two feeding protocols. The early feeding regime started at 30 minutes following shock, a time point at which the first signs of shock-induced intestinal tight junction loss are present (data submitted for publication). The delayed feeding protocol was initiated at 80 minutes post-shock at which time point systemic inflammation is already detectable [13]. This study demonstrates that, next to an extensive anti-inflammatory effect, post treatment with high-lipid enteral feeding results in a strong preservation of intestinal integrity.

The results show that the protective effects of high-lipid enteral nutrition given post-shock are mediated by CCK-receptors. These findings are in accordance with our previous study which unraveled a neuro-endocrine pathway underlying the anti-inflammatory actions of high-lipid nutrition [13]. In this mechanism, CCK-receptor dependent stimulation of the autonomic nervous system inhibits inflammation via binding of acetylcholine to alpha-7-nicotinergic receptors on macrophages [13-15]. The current study is the first to show that high-lipid nutritional stimulation of the autonomic nervous system strongly reduces the development of inflammation and intestinal barrier dysfunction in a setting of inflammation induced by hemorrhagic shock.

Severe trauma followed by a second hit such as surgery or infection provokes an excessive systemic inflammation that may result in organ dysfunction. Therefore, therapies are needed that reduce the inflammatory response of the primed immune system [1, 3, 4]. The findings of this study that post-shock high-lipid enteral nutrition has strong anti-inflammatory effects are supported by previous studies demonstrating that activation of nicotinic receptors on immune cells is protective in settings of an ongoing inflammation. Nicotinic receptor activation via electrical vagus stimulation or administration of the alpha7nicotinergic agonist GST21 increased survival rates when applied after induction of septic peritonitis in mice [44 45]. The current study demonstrates that nutritional activation of the anti-inflammatory vagus-cholinergic pathway via CCK-receptor stimulation is a simple

and physiological alternative. Moreover, feeding with a high lipid content was shown to be more effective than low-lipid feeding intervention.

In summary, administration of enteral high-lipid nutrition attenuates a developing inflammatory response and preserves intestinal integrity via a CCK-receptor dependent mechanism. This study implicates early enteral administration of high-lipid nutrition as a novel approach to attenuate the complex inflammatory cascade that unfolds in clinical settings such as severe trauma.

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Chapter 3.2

Lipid-rich enteral nutrition reduces postoperative ileus in rats via activation of cholecystokinin-receptors

Tim Lubbers
Misha D.P. Luyer
Jacco J. de Haan
M'hamed Hadfoune
Wim A. Buurman
Jan Willem M. Greve

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Abstract

Introduction: Postoperative ileus is a major clinical problem, in which inflammation of the intestinal muscularis plays a key pathogenic event. Previously, administration of lipid-rich nutrition has been shown to reduce inflammation by activation of the autonomic nervous system via cholecystokinin-receptors. This study investigates the effect of lipid-rich nutrition on the local inflammatory response and gastrointestinal hypomotility in a rat model of postoperative ileus.

Methods: Postoperative ileus was induced by manipulation of the small intestine in rats. Peritoneal lavage fluid, plasma and jejunal segments were collected at several time points to determine inflammatory mediators in fasted rats and rats fed a lipid-rich or control nutrition. Gastrointestinal transit was measured 24 hours after surgery.

Results: Administration of lipid-rich nutrition markedly reduced the manipulation-induced local inflammatory response compared to rats treated with control nutrition. The intervention with lipid-rich nutrition significantly reduced plasma levels of rat mast cell protease-II ($p<0.05$) and peritoneal levels of tumor necrosis factor-alpha ($p<0.01$) and interleukin-6 ($p<0.05$). Furthermore, the influx of neutrophils, expressed as tissue level myeloperoxidase was significantly prevented by lipid-rich nutrition ($p<0.05$). Above all administration of lipid-rich enteral nutrition resulted in a significant improvement of gastrointestinal transit compared to control nutrition ($p<0.05$). Blocking of cholecystokinin-receptors prevented the anti-inflammatory and motility promoting effect of lipid-rich feeding.

Conclusion: Our data demonstrate that nutritional stimulation of the autonomic nervous system with enteral lipids reduces postoperative ileus by inhibition of inflammation. Clinically, lipid-rich enteral nutrition may be a new therapeutic option in the treatment of postoperative ileus.

Introduction

Postoperative ileus is a pathologic condition commonly seen after abdominal surgery with intestinal manipulation. The condition is characterized by generalized hypomotility of the gastrointestinal tract and delayed gastric emptying, leading to increased morbidity and prolonged hospitalization [1, 2]. The pathogenesis of postoperative ileus consists of a biphasic process in which neuronal and inflammatory mechanisms are involved. Neural pathways and release of neuropeptides play a dominant role in the early phase of ileus, lasting minutes to hours [3-5], whereas inflammation results in the sustained phase that lasts hours to days [5-7]. In rats as well as humans, manipulation of the gut during surgical interventions leads to a marked inflammatory response within the intestinal muscularis. The degree of inflammation is directly proportional to the level of postoperative gastrointestinal hypomotility [7-10]. Currently there is no effective treatment for postoperative ileus and interventions rely on supportive measures [6, 11].

Recently, it has been demonstrated in a murine model of intestinal manipulation that electric or pharmacologic stimulation of the cholinergic anti-inflammatory pathway effectively decreased the inflammatory response in the intestinal muscularis via activation of the nicotinic acetylcholine receptor alpha7 subunit (α7 nAChR) on inflammatory cells and attenuated hypomotility of the gastrointestinal tract [12-14].

A physiologic approach to activate the cholinergic anti-inflammatory vagal pathway is administration of lipid-rich enteral nutrition [15]. In a model of non-lethal hemorrhagic shock, the intervention with lipid-rich nutrition very effectively reduces systemic inflammation [16, 17] by activation of the autonomic nervous system via cholecystokinin (CCK)-receptors [15]. In the present study, our aim was to determine whether postoperative ileus can be attenuated by an intervention with lipid-rich nutrition.

Methods

Animals and experimental groups

Healthy male Sprague Dawley rats, weighing 300-350 gram were purchased from Charles River Laboratories (Maastricht, the Netherlands). Animals were housed under standardized conditions of temperature and humidity and had access to standard food and water ad libitum. Experiments were performed in agreement with the Animal Ethics Committee of the Maastricht University Medical Center.

Postoperative ileus was induced by gentle surgical manipulation of the small bowel, as previously described [9]. In short, rats underwent a laparotomy via a midline abdominal incision under sterile conditions. The small intestine was placed on moist gauze pads outside the abdomen, without manipulating cecum and colon. The small intestine was manipulated with moist cotton swabs for five minutes. This procedure was used to simulate surgical inspection of the bowel during abdominal surgery. After manipulation, the small intestine was moistened and placed in the abdomen. The abdomen was closed in two layers with continues sutures. Animals were sacrificed at 20 minutes, 3 hours and 24 hours after manipulation (Figure 1). In all experimental designs, rats were either fasted, to mimic the clinical situation in which patients are fasted prior to surgery or fed lipid-rich or a control enteral nutrition by way of oral gavage before and after manipulation. All animals, sacrificed at 24 hours, were given free access to standard rodent chow from 6 hours after manipulation onwards.

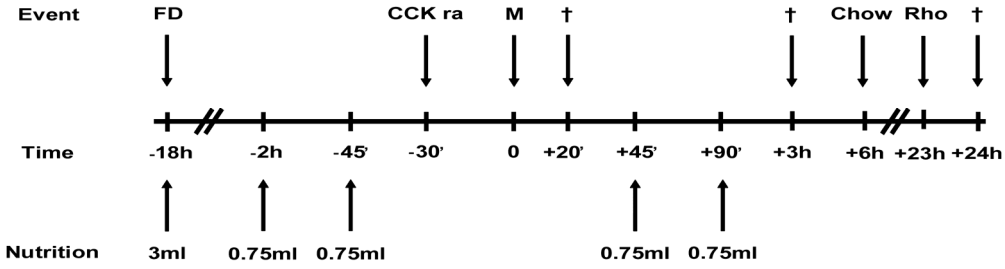

Figure 1: Experimental protocol. Rats were deprived of food (FD) 18 hours prior to manipulation. At t = 0, rats were anesthetized and underwent intestinal manipulation (M). Animals were sacrificed at 20 minutes, 3 hours or 24 hours (†). CCK-receptors antagonists were applied 30 minutes prior to manipulation (CCK ra). Gastrointestinal transit was measured by oral administration of rhodamine one hour before sacrifice (Rho). Rats, sacrificed at 24 hours, were given free access to standard rodent chow 6 hours after manipulation (Chow). A liquid lipid-rich or control nutrition was administered by oral gavage at -18 hours (3ml; other time points 0.75 ml), -2 hours, -45 minutes, +45 minutes, +90 minutes in the fed group.

The lipid-rich liquid enteral nutrition contained 50.4 en% fat of which 30% were phospholipids, 8.7 energy percent (en%) protein and 40.9 en% carbohydrates; the control nutrition was composed of 16.0 en% fat, 8.7 en% protein and 75.3 en% carbohydrates. The protein and carbohydrate composition of the two feedings were identical. The amount of fat in the control nutrition was isocaloric to that present in standard rodent chow and the lipid-rich nutrition was isocaloric and isonitrogenous to the control nutrition. Rats received 3 ml enteral nutrition 18

hours before manipulation and 0.75 ml at 2 hours and 45 minutes before manipulation as well as 45 minutes and 90 minutes after manipulation (Figure 1). This feeding regime was based on previous studies from our group and pilot experiments.

Protease and cytokine assays

The mast cell degranulation marker, mast cell protease-II (MCP-II) was measured 20 minutes postoperatively in plasma (n = 10 for each group) and inflammatory cytokines, TNF-α and IL-6 in peritoneal lavage fluid at three hours (n = 6 for each group, Figure 1). Peritoneal lavage fluid was obtained by ip injection of 10 ml sterile PBS. After one minute of massaging, the abdomen was opened and fluid was aspirated. Lavage fluid was centrifuged and supernatant stored at -20 ^{0}C until analysis. MCP-II, TNF-α and IL-6 concentrations were measured using a standard ELISA for rat TNF-α (kindly provided by Hycult Biotechnology, Uden, the Netherlands), IL-6 (BD Biosciences, Franklin Lakes, NJ) and MCP-II (Moredun Scientific, Edinburgh, UK).

Myeloperoxidase quantification

Per rat, sacrificed at 24 hours, three segments of jejunum were snap frozen in liquid nitrogen (n = 6 for each group). Segments were homogenized in lysisbuffer (300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, en Pepstatin A, Leupeptin, Aprotinin (all 20 ng/ml); pH 7.4), centrifuged and supernatants stored at -20 °C until analysis.

Myeloperoxidase (MPO) was quantified using ELISA. In brief, a microtiter plate was coated with mAb 8F4, cross reactive with rat MPO (kindly provided by Hycult Biotechnology, Uden, the Netherlands) overnight at 4 ^{0} C and blocked with 1% BSA in PBS. Binding was detected with biotinylated rabbit-α-human MPO (DAKO, Glostrup, Denmark) and visualized with TMB. The results were recorded using an ELISA plate reader at 450 nm. MPO content per sample was calculated, after correction for total extracted protein per sample.

MPO immunohistochemistry

Formalin fixed jejunum was sectioned and stained for MPO. Sections were rehydrated and endogenous peroxidases blocked with H_2O_2. Sections were washed in TBS, blocked with 20% normal pig serum and incubated with rabbit-α-human MPO. After rinsing with TBS, sections were incubated with secondary antibody, biotinylated pig-α-rabbit IgG. The staining was visualized with Vectastain ABC/Elite (Vector Laboratories, Burlingame, CA) and AEC as chromogen. Sections were coverslipped with DAKOCytomation and photomicrographs were recorded using a Nikon E800 microscope.

Gastrointestinal transit

Gastrointestinal transit was measured in control and manipulated animals 24 hours postoperatively by evaluating the gastrointestinal distribution of rhodamine-B-labeled dextran (70,000 molecular weight; Molecular Probes, Carlsbad, CA) as previously described (n = 8 for each group) [8]. In brief, animals were administered rhodamine (200 µl of 6.25 mg/ml solution in PBS) via oral gavage. One hour after administration gastrointestinal transit was assessed in the stomach and the small bowel, which was divided in 10 equal parts. Segments were opened and mixed vigorously in 2 ml PBS to obtain the rhodamine-containing gut content. Solutions were centrifuged and clear supernatant was quantified in a multiwell fluorescence plate reader (excitation 530/20 nm and emission 590/50 nm). Total recovered rhodamine was calculated and each segment was expressed as percentage of total rhodamine. A histogram of fluorescence distributed along the gastrointestinal tract was plotted for transit analysis (% rhodamine per segment). The intestinal passage of rhodamine per rat was expressed as geometric center for statistical analysis. Geometric centers were calculated from each experiment as (Σ % FITC per segment X segment number)/100 [18].

CCK-receptor antagonists

To investigate whether the anti-inflammatory pathway induced by administration of lipid-rich nutrition is activated by CCK, rats were treated with a combination of CCK-receptor antagonists, Devazepide and L365, 260 (both 500 µg/kg; kind gifts from ML Laboratories PLC, Nottingham, UK) or vehicle (90% NaCl, 5% Tween 20, 5% dimethyl sulfoxide) administered intraperitoneally 30 minutes before manipulation of the gut (n = 6 for each group, Figure 1).

Statistical analysis

Data are represented as mean +/- SEM. A Mann-Whitney U test was used for between-group comparisons. Differences were considered statistically significant at $p < 0.05$.

Results

Mast cell degranulation following intestinal manipulation

Previous studies have indicated that intestinal manipulation initiates an inflammatory response in the intestinal muscularis, which results from activation and degranulation of mast cells [19]. When mast cells are activated and degranulate, preformed mast cell protease-II (MCP-II) is released [20]. MCP-II levels increased markedly in plasma 20 minutes following intestinal manipulation (10.9 ± 1.6 ng/ml)

compared to laparotomy (1.4 ± 0.2 ng/ml; $p < 0.01$. Figure 2). Administration of lipid-rich enteral nutrition resulted in a significant reduction of MCP-II levels (5.8 ± 0.4 ng/ml) compared to animals treated with control nutrition (7.8 ± 0.8 ng/ml) and fasted animals (both; $p < 0.05$). These findings indicate that lipid-rich nutrition prevents mast cell degranulation in response to manipulation of the intestine.

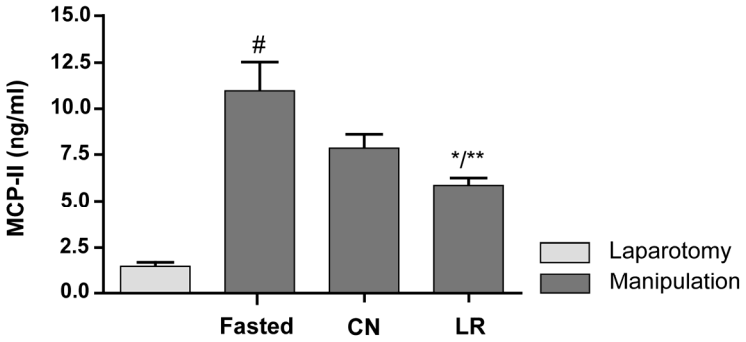

Figure 2: Manipulation of the small intestine results in a marked increase of MCP-II plasma levels 20 minutes after manipulation. Administration of lipid-rich (LR) decreased plasma levels of MCP-II compared to animals treated with control nutrition (CN) and fasted animals. Data represented as mean ± SEM. # $p < 0.01$ compared to laparotomy, * $p < 0.05$ compared to fasted, ** $p < 0.05$ compared to CN

Inhibition of manipulation-induced peritoneal TNF-α and IL-6 levels

The role of activated resident macrophages has been widely demonstrated in the pathogenesis of ileus [10, 21-23]. Levels of macrophage-derived cytokines, TNF-α and IL-6 were measured in peritoneal lavage fluids at 3 hours after intestinal manipulation (Figures 3A-B).

Intestinal manipulation resulted in a peritoneal TNF-α level of 84 ± 11 pg/ml and IL-6 level of 155 ± 25 pg/ml, whereas both cytokines could not be detected in the laparotomy group. Administration of lipid-rich nutrition significantly attenuated release of TNF-α (31 ± 4 pg/ml) and IL-6 (53 ± 12 pg/ml) into the peritoneal cavity compared to animals fed a control nutrition (55 ± 8 pg/ml; $p < 0.01$ and 91 ± 11 pg/ml; $p < 0.05$, *respectively*) and fasted animals ($p < 0.01$), indicating an inhibitory action of lipid-rich nutrition on resident macrophages.

Prevention of neutrophil influx in muscularis of manipulated intestine

Jejunal tissue levels of MPO were quantified 24 hours after manipulation to assess infiltration of MPO-positive cells (Figure 4A). Manipulated animals

demonstrated a significant increase in tissue level MPO (123 ± 11 pg/µg protein) compared to control laparotomy animals (34 ± 4 pg/µg protein; $p < 0.01$). Next, the jejunal influx of MPO-positive cells was immunohistochemically verified. MPO-containing cells, morphologically identified as rat neutrophils were predominantly located between the longitudinal and circular muscle layer of the small intestine (Figure 4B).

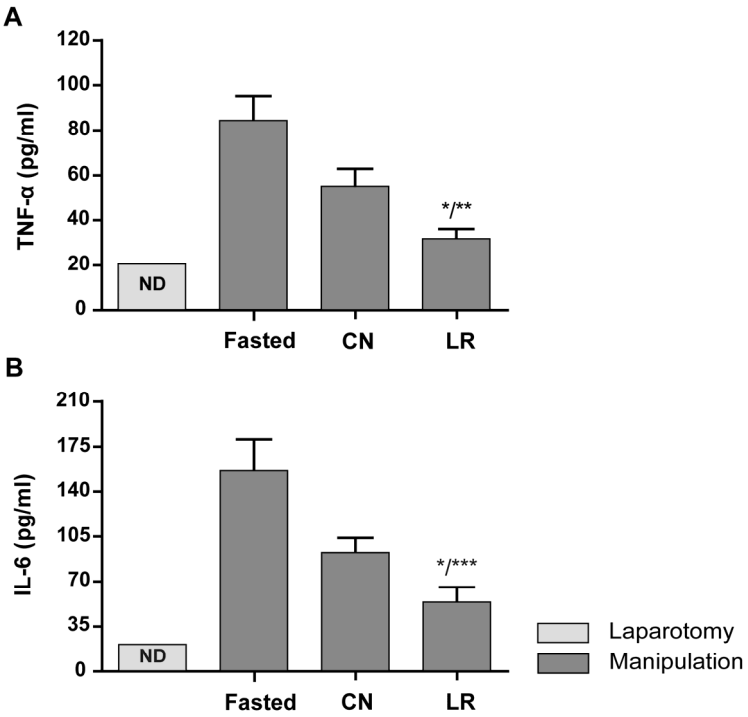

Figure 3: Lipid-rich nutrition inhibits the inflammatory response of resident macrophages. Intestinal manipulation results in increased peritoneal levels of TNF-α (A) and IL-6 (B) three hours after surgery. Lipid-rich nutrition (LR) inhibits the manipulation-induced release of TNF-α and IL-6 compared to rats treated with control nutrition (CN) and fasted animals. Data represented as mean ± SEM. ND; not detectable, * $p < 0.01$ compared to fasted, ** $p < 0.01$ compared to CN, *** $p < 0.05$ compared to CN.

The intervention with lipid-rich feeding significantly prevented manipulation-induced influx of MPO-positive cells in the intestinal muscularis (Figure 4A). Tissue MPO levels were significantly reduced by lipid-rich nutrition (81 ± 8 pg/µg protein) compared to control nutrition (109 ± 9 pg/µg protein; $p < 0.05$) and animals in the

fasted state ($p < 0.05$). Figure 4C demonstrates a representative photomicrograph of animals treated with lipid-rich nutrition.

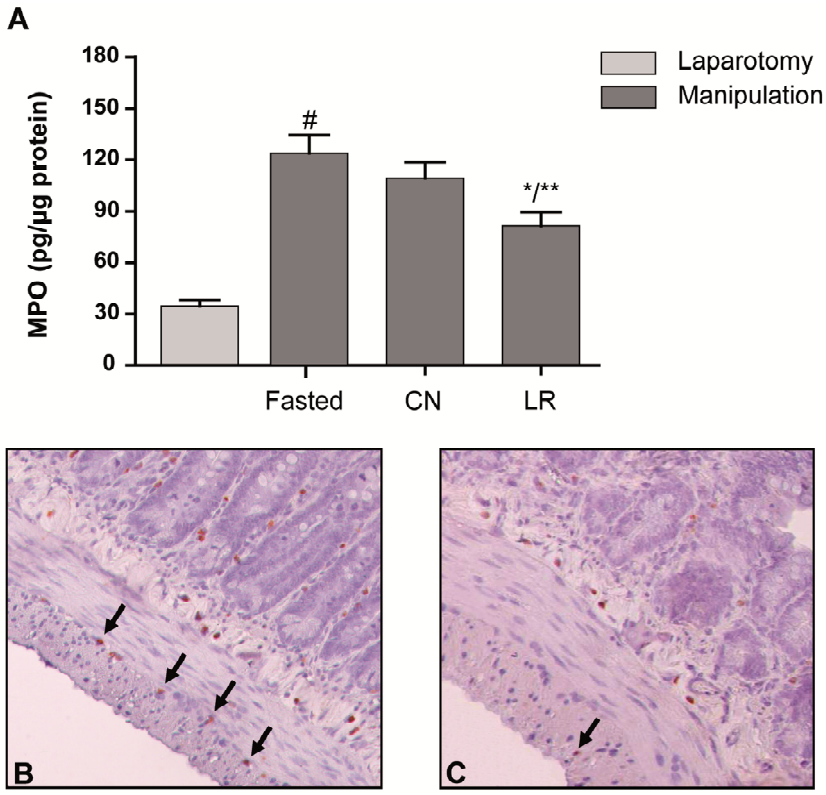

Figure 4: Lipid-rich nutrition prevents influx of neutrophils in intestinal muscularis. Manipulation results in a marked influx of neutrophils, expressed as jejunal tissue MPO levels compared to the laparotomy group (A). Administration of lipid-rich nutrition (LR) significantly prevents influx compared to control nutrition (CN) fed and fasted rats. This is histologically confirmed by a reduction in MPO-positive cells (→) in the intestinal muscularis of rats treated with lipid-rich nutrition (C) compared to fasted rats (B). Data represented as mean ± SEM. $^{\#}$ $p < 0.01$ compared to laparotomy, * $p < 0.05$ compared to fasted, ** $p < 0.05$ compared to control nutrition.

Improved gastrointestinal transit following intestinal manipulation

Gastrointestinal transit was measured over a period of one hour using the fluorescent transit marker rhodamine at 24 hours after manipulation. Manipulation of the intestine resulted in a significant reduction in intestinal transit of rhodamine, expressed as geometrical center (GC: 5.4 ± 0.3; $p < 0.01$) compared to laparotomy

(GC: 6.9 ± 0.2) (Figure 5A). Administration of lipid-rich nutrition significantly enhanced intestinal passage of rhodamine (GC: 6.8 ± 0.3) compared to animals treated with control nutrition (GC: 5.7 ± 0.3; $p < 0.05$) and fasted animals ($p < 0.01$).

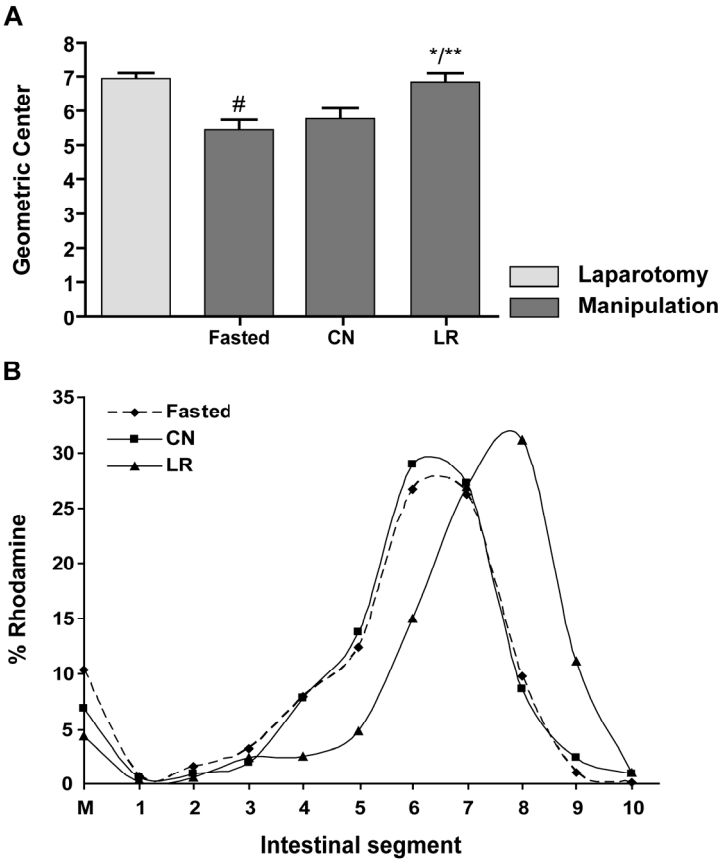

Figure 5: Intervention with lipid-rich nutrition improves gastrointestinal transit in manipulated animals. Manipulation of the gut results in a reduction of geometrical center (GC) compared to laparotomy, indicating a loss of gastrointestinal transit (A). Administration of lipid-rich nutrition (LR) improves GC compared to animals receiving control nutrition (CN) and fasted animals. Distribution of rhodamine in the stomach (S) and along 10 equal segments of small intestine (1: proximal duodenum to 10: terminal ileum) (B). LR accelerates gastric emptying and enhanced intestinal transit compared to rats treated with control nutrition and fasted rats. GC represented as mean ± SEM, distribution of rhodamine as mean. $^{\#}$ $p < 0.01$ compared to laparotomy, * $p < 0.01$ compared to fasted, ** $p < 0.05$ compared to CN.

Figure 5B visualizes the improvement of gastrointestinal transit in rats treated with lipid-rich nutrition compared to animals treated with control nutrition and fasted animals. The content of rhodamine in the stomach of animals fed a lipid-rich nutrition was lower compared to the intervention with control nutrition and fasted rats. Furthermore, rhodamine was transported more distally in the small intestine in the lipid-rich intervention group.

Blocking CCK-receptors blunts the protective effect of lipid-rich feeding on the inflammatory infiltrate and aggravates gastrointestinal hypomotility

CCK-receptor antagonists were administered to investigate the involvement of the CCK-mediated anti-inflammatory pathway [15] in the inhibitory effect of lipid-rich nutrition on manipulation-induced influx of MPO-positive cells and gastrointestinal hypomotility. Blockage of CCK-receptors significantly prevented the inhibitory effect of lipid-rich nutrition on tissue levels of MPO (142 ± 16 pg/µg protein; $p < 0.01$), whereas vehicle treatment demonstrated no effect (90 ± 10 pg/µg protein; Figure 6A). These findings support that the inhibitory effect of lipid-rich nutrition on influx of MPO-positive cells is mediated through a CCK-dependent mechanism.

In addition, application of CCK-receptor antagonists abrogated the promoting effect of lipid-rich nutrition on gastrointestinal transit of rhodamine (GC: 5.2 ± 0.4; $p < 0.01$), whereas transit remained unaltered in vehicle-treated rats (GC: 7.0 ± 0.2. Figure 6B). Taken together, both the prevention of neutrophil influx and improvement of gastrointestinal transit by lipid-rich enteral nutrition were shown to be CCK-dependent.

Discussion

This is the first study to show that a nutritional intervention with lipid-rich nutrition reduces postoperative ileus following intestinal manipulation. We found that administration of lipid-rich nutrition blunted plasma levels of MCP-II and reduced intraperitoneal levels of TNF-α and IL-6. In addition, lipid-rich feeding prevented influx of neutrophils in the intestinal muscularis and improved gastrointestinal transit via activation of CCK-receptors.

Intestinal manipulation has been accepted as a valid model of postoperative ileus [9, 24, 25]. Gentle manipulation of the small intestine results in an inflammatory response in the intestinal muscularis and hypomotility of the gastrointestinal tract. Key elements in the pathogenesis of postoperative ileus are activation and degranulation of mast cells, activation of resident macrophages and influx of neutrophils in the intestinal muscularis [5, 7, 19].

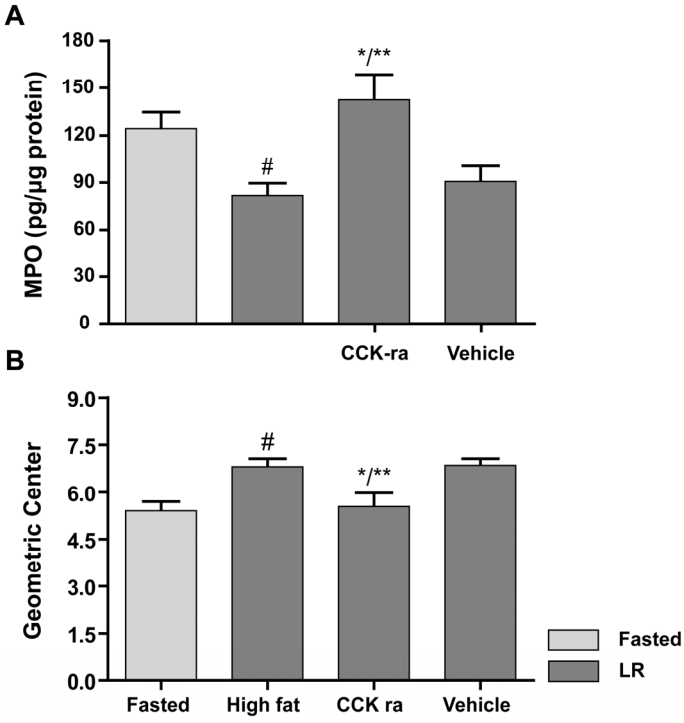

Figure 6: CCK-receptor antagonists abrogate the inhibitory effect of lipid-rich nutrition on the manipulation-induced inflammatory response and gastrointestinal hypomotility. Jejunal tissue MPO levels in manipulated animals (A). Administration of CCK-receptor antagonists (CCK-ra) reverse the anti-inflammatory effect of lipid-rich nutrition (LR), while vehicle did not. CCK-ra abrogate the effect of lipid-rich nutrition on gastrointestinal transit (B). Vehicle treatment did not affect the improvement in gastrointestinal transit. MPO data represented as mean ± SEM, GC represented as mean ± SEM. # $p < 0.05$ compared to fasted, * $p < 0.01$ compared to lipid-rich, ** $p < 0.05$ compared to vehicle.

Previously, we have demonstrated in a rodent model of hemorrhagic shock that lipid-rich enteral nutrition effectively attenuates systemic inflammation and gut barrier failure by activation of the vagus nerve via CCK-receptors [15]. The protective properties of our nutritional intervention have been demonstrated to be specific for the lipid content in enteral nutrition and are not related to caloric intake [16, 17, 26]. Furthermore, electric or pharmacologic stimulation of the vagal pathway has been demonstrated to effectively resolve local inflammation and consequent impaired motility [12, 13].

Activation and degranulation of mast cells has been reported to play an important role in the initiation of postoperative ileus by activating resident macrophages [19]. Intestinal mast cells are in close contact with vagal nerve endings and electric stimulation of the vagus nerve has been demonstrated to influence mast cells [27, 28]. Therefore, we investigated whether lipid-rich nutrition reduces mast cell activation via nutritional stimulation of the vagal anti-inflammatory pathway.[15] Administration of lipid-rich nutrition reduced release of rat MCP-II, suggesting that dietary fat prevents manipulation-induced degranulation of mast cells. The pathways responsible for this inhibitory effect needs further elucidation, since release of endogenous CCK and stimulation of rat mast cells with acetylcholine has been shown to result in degranulation [29, 30].

Manipulation of the intestine has been shown to activate resident macrophages in the intestinal muscularis, either via mast cell-derived mediators [19, 31] or via exposure to invading luminal antigens during a period of increased intestinal permeability [32, 33]. Activation of resident macrophages has been demonstrated by local production of macrophage-derived TNF-α and IL-6 and release of these pro-inflammatory cytokines in peritoneal fluid [7, 10, 12, 34]. In line with previous reports from our group, describing that lipid-rich nutrition significantly reduces systemic levels of TNF-α and IL-6 following hemorrhagic shock [15, 16], we investigated the effect of our nutritional intervention on local inflammation. Administration of lipid-rich nutrition inhibited TNF-α and IL-6 levels in peritoneal lavage fluid following intestinal manipulation. Our data are supported by the study of De Jonge *et al*, who demonstrated in a mouse model of postoperative ileus that stimulation of the vagus nerve significantly attenuates peritoneal levels of TNF-α and IL-6 [12].

Following activation of resident inflammatory cells, intestinal manipulation results in influx of inflammatory cells [35]. We confirm influx of neutrophils in the intestinal muscularis, expressed as enhanced tissue levels of MPO and increased number of MPO-positive cells in the intestinal muscularis after manipulation. These inflammatory infiltrates inhibit gastrointestinal motility and trigger inhibitory spinal pathways leading to generalized paralysis of the gastrointestinal tract [5, 7, 36]. Prevention of the formation of an inflammatory infiltrate in the muscularis by blocking ICAM-1 was shown to attenuate postoperative ileus [7, 35]. Here, we demonstrate that administration of lipid-rich nutrition prevented the influx of MPO-positive cells in the muscularis after manipulation. These findings indicate that lipid-rich nutrition not only attenuates systemic inflammatory responses, but is also able to inhibit inflammation at tissue level. Our observations are supported by The *et al*, who demonstrated that pharmacologic stimulation of the cholinergic pathway with AR-R17779 attenuates influx of MPO-positive cells in the intestinal muscularis to a similar extent and resolves the impairment in gastric emptying [13].

Manipulation of the small intestine resulted in a significant decrease in gastrointestinal transit. The degree of postoperative ileus following intestinal manipulation in our study is in line with data of Wehner et al. [21]. The extent of gastrointestinal hypomotility was shown to be proportional to the level of intestinal inflammation [7-10], while prevention or reduction of the manipulation-induced inflammatory response attenuated hypomotility [7, 12, 21, 35]. In line, we demonstrate that administration of lipid-rich nutrition effectively reduced the manipulation-induced decrease of gastrointestinal transit by attenuation of the local inflammatory response, indicating that a nutritional intervention with high-lipid content ameliorates postoperative ileus.

Previously, we described that activation of the autonomic nervous system by dietary fat, leading to inhibition of systemic inflammation is dependent on CCK-receptors [15]. CCK as well as CCK-receptors are known to affect motility [37-39]. To eliminate the influence of CCK and CCK-receptor antagonists on gastrointestinal transit, we performed our measurements 24 hours after manipulation when the release of CCK induced by our nutritional intervention and the direct effect of the receptor antagonists are expected to have extinguished [40-42]. In the present study, administration of CCK-receptor antagonists abrogated the anti-inflammatory action of lipid-rich nutrition and prevented the improvement of postoperative ileus. Our data indicate that the nutritionally mediated vagal anti-inflammatory pathway is responsible for the attenuation of postoperative ileus. Our findings are supported by recent reports describing that electric stimulation of the efferent vagus or pharmacologic activation of the α7 nAChR ameliorates postoperative ileus by inhibition of the local inflammatory response [12, 13]. Although very effective, electric stimulation of the vagus nerve is an invasive procedure and generalized stimulation of the nicotinic acetylcholine receptor might have a wide scope of side effects by activation of non-relevant cells and cell systems [43-45]. Nutritional activation of the vagal anti-inflammatory pathway with lipid-rich nutrition is a physiologic approach to reduce local inflammation and ameliorate postoperative ileus following intestinal manipulation.

Postoperative ileus is associated with increased morbidity, length of hospital stay and health care costs [6, 11, 46]. The current treatment for postoperative ileus is supportive in nature and comprised of nothing per mouth, nasogastric suction and bowel rest [6, 11]. The cellular and molecular changes underlying postoperative ileus are difficult to treat at this stage, since the inflammatory cascade is already ongoing. Patients at risk of developing postoperative ileus may therefore benefit from a simple and safe intervention with lipid-rich enteral nutrition to prevent the manipulation-induced inflammatory response and consequent hypomotility of the gastrointestinal tract. Early administration of enteral nutrition has already been demonstrated to be beneficial in surgical patients and is successfully implemented

in "fast-track" programs [47, 48]. Therefore, clinical applicability of lipid-rich nutrition to treat postoperative ileus calls for further investigation.

In summary, we show that an intervention with lipid-rich nutrition attenuates postoperative ileus by inhibiting the local inflammatory response via activation of CCK-receptors in rats. These data indicate that an intervention with lipid-rich nutrition can be a valuable tool in the prevention and treatment of postoperative ileus.

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Chapter 3.3

Controlling postoperative ileus by vagal activation

Tim Lubbers
Wim A. Buurman
Misha D.P. Luyer

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Abstract

Postoperative ileus is a frequently occurring surgical complication, leading to increased morbidity and hospital stay. Abdominal surgical interventions are known to result in a protracted cessation of bowel movement. Activation of inhibitory neural pathways by nociceptive stimuli leads to an inhibition of propulsive activity, which resolves shortly after closure of the abdomen. The subsequent formation of an inflammatory infiltrate in the muscular layers of the intestine results in a more prolonged phase of ileus. Over the last decade, clinical strategies focusing on reduction of surgical stress and promoting postoperative recovery have improved the course of postoperative ileus. Additionally, recent experimental evidence implicated antiinflammatory interventions, such as vagal stimulation, as potential targets to treat postoperative ileus and reduce the period of intestinal hypomotility. Activation of nicotinic receptors on inflammatory cells by vagal input attenuates inflammation and promotes gastrointestinal motility in experimental models of ileus. A novel physiological intervention to activate this neuroimmune pathway is enteral administration of lipid-rich nutrition. Perioperative administration of lipid-rich nutrition reduced manipulation-induced local inflammation of the intestine and accelerated recovery of bowel movement. The application of safe and easy to use antiinflammatory interventions, together with the current multimodal approach, could reduce postoperative ileus to an absolute minimum and shorten hospital stay.

Introduction

Postoperative ileus is a common pathological condition in the surgical ward and presents as an inability to tolerate enteral nutrition, nausea, abdominal distension, and lack of flatus and defecation. Although all surgical patients are at risk of developing postoperative ileus, the condition is mostly observed after abdominal surgery with manipulation of the gastrointestinal tract [1]. Cessation of bowel movement and delayed gastric emptying, which can be up to five days after colorectal surgery, results in increased morbidity and a prolonged hospital stay [2-4]. The duration of postoperative ileus has a major financial impact, adding an average of 6.300 US$ to hospital costs per patient who develops ileus [5]. The additional health care costs in the US have been estimated to be 1.5 billion US$ annually[6]. Increased insight into the pathophysiology and discovery of novel treatment options could diminish the length of postoperative ileus, decrease patient morbidity, and reduce hospital costs.

Pathophysiology of postoperative ileus

The pathophysiology underlying postoperative ileus is complex and multifactorial, consisting of endogenous and pharmacological characteristics. Recent experimental studies have demonstrated that the pathogenesis of the endogenous component of postoperative ileus can be grossly divided in two distinct phases[1]. The first phase, or neural phase, results from activation of mechanoreceptors and nociceptors by stimuli, such as incision of the skin and, more importantly, by direct manipulation of the intestine [7]. Activation of these receptors initiates a neural reflex, which is dependent on release of mediators, such as α-calcitonin gene-related peptide and substance P, which inhibit gastrointestinal motility and result in generalized intestinal hypomotility [8-10]. The neural phase of postoperative ileus lasts minutes to hours and resolves after closure of the wound when the noxious stimuli have ceased [9,11,12]. The motility of the colon in particular depends heavily on input from the autonomic nervous system, which might explain colonic susceptibility to isolated and prolonged ileus [13].

The second, more protracted, inflammatory phase is caused by formation of an inflammatory infiltrate in the muscular layers of the intestine [7,14,15]. Manipulation of the intestine initiates an inflammatory cascade starting with activation and degranulation of mast cells [16-18]. Subsequently, resident macrophages are activated either via mast cell-derived mediators or by luminal antigens [17,19,20]. These activated macrophages produce cytokines and chemokines, which attract neutrophils to the muscular layer of the intestine. Invaded neutrophils directly impair intestinal

smooth muscle cell contractility *via* release of nitric oxide and prostaglandins [21,22]. The formation of an inflammatory infiltrate not only impairs motility in the manipulated areas, but also leads to generalized hypomotility of the gastrointestinal tract *via* activation of inhibitory adrenergic neural pathways. There is emerging evidence that inflammation also plays a vital role in postoperative ileus in humans, therefore a major focus of current research has been directed at the development of antiinflammatory treatments [18,23,24]. In experimental models of intestinal manipulation, it was demonstrated that administration of antiinflammatory agents, such as mast cell stabilizers [17], non-steroidal antiinflammatory drugs [25,26], and IL-10 [27], prevent development of postoperative ileus. In addition, it was recently shown in patients undergoing major abdominal surgery that an intervention with the mast cell stabilizer, Ketotifen, reduced gastroparesis [24].

Clinical strategies to treat postoperative ileus

A number of strategies for preventing postoperative ileus are combined in the so-called fast-track program. The goals of fast-track surgery are reduction of perioperative surgical stress and promotion of postoperative recovery. Adequate pain relief, minimal invasive surgery and early enteral nutrition are important to achieve these goals [28]. Adequate pain relief can attenuate postoperative ileus in two important ways. First, intraoperative spinal anesthesia and postoperative epidural analgesia with local anesthetics during abdominal surgery reduce the neural phase of ileus by interruption of neural transmission. Second, local anesthetic interventions minimize the use of opioid-derivatives [29,30]. Both endogenous opioids, released in response to noxious stimuli, and exogenous opioids are notorious for their inhibitory effect on gastrointestinal motility, thereby aggravating postoperative ileus [31]. Blocking the μ-opioid receptor with Alvimopan, a selective, peripherally active antagonist, has been demonstrated to accelerate recovery of bowel function and decrease hospital stay, without affecting the analgesic effects of opioids [32,33]. In addition, non-steroidal antiinflammatory drugs seem promising for their opioid-sparing and antiinflammatory effects [26,34]. However, caution should be taken as the use of cyclo-oxygenase-2 inhibitors after colonic surgery has been associated with increased anastomotic leakage [35].

Surgical trauma and direct manipulation of the intestine are major factors in the occurrence of postoperative ileus. The degree of gastrointestinal hypomotility correlates with the degree of manipulation and intestinal inflammation [19]. The introduction of minimally invasive techniques, such as laparoscopy, significantly reduced the duration of postoperative ileus and length of hospital stay [36]. This

improvement is probably due to minimization of trauma, resulting in less pain and a diminished release of neurotransmitters and inflammatory mediators [18,28,37].

Finally, enteral nutrition is found to be essential for enhanced recovery after surgery. Ingestion of nutrients elicits various reflexes and releases several neuropeptides that promote gastrointestinal motility [38,39]. Traditionally however, a nil-by-mouth regime is often enforced starting from several hours before surgery until days postoperatively. Recent studies have demonstrated that early enteral nutrition is safe and well tolerated after abdominal surgery. In addition, early enteral nutrition reduces postoperative ileus and length of hospital stay [40,41]. Unfortunately, studies investigating the effect of early enteral nutrition on postoperative ileus remain difficult to interpret, as the studies often lack essential information on the type of analgesia that was used [2]. Enteral nutrition is a promising intervention to treat ileus; however, future well-designed studies are needed to evaluate the effect of early enteral nutrition on intestinal motility. When implementing early enteral nutrition routinely, caution should be taken, as there is a small chance that enteral nutrition could lead to intestinal ischemia in the circulatory compromised patient [42,43].

The implementation of fast-track regimes in the surgical field has improved the course of postoperative ileus. However, despite these efforts, it still remains an important clinical challenge. Inhibition of the inflammatory phase, by targeting the cellular and molecular changes underlying postoperative ileus is another focus of treatment.

Experimental strategies to control postoperative ileus

The inflammatory phase dominates the course of postoperative ileus. Novel experimental interventions aimed at preventing the activation of inflammatory cells, such as administration carbon monoxide [44,45], pretreatment with blocking antibodies to intracellular adhesion molecule-1 and lymphocyte function-associated antigen-1 [7,46], inactivating macrophages [47], and preventing mast cell activation [17], have displayed promising results in reducing gastrointestinal hypomotility. Borikova, *et al.* [48] described a novel approach for modulating the inflammatory response; electrical stimulation of the vagus nerve attenuates systemic inflammation in a murine endotoxin model. Stimulation of the vagus nerve modulates inflammation via release of acetylcholine that binds to nicotinic receptors on inflammatory cells, hence the term "cholinergic anti-inflammatory pathway" [49,50]. In addition, the vagus nerve has recently been identified as an important modulator of intestinal health; loss of vagal integrity aggravates intestinal inflammation and augments loss of gut barrier function [51,52].

In a murine model of intestinal manipulation, electrical stimulation of the vagus nerve ameliorates postoperative gastrointestinal hypomotility *via* inhibition of local intestinal inflammation. Vagal stimulation activates the alpha7 nicotinic acetylcholine receptor on intestinal macrophages and attenuates release of pro-inflammatory cytokines *via* the Jak2-Stat3 signaling pathway [53]. Furthermore, administration of the selective alpha-7 receptor agonist, AR-R17779, prevented postoperative ileus in mice [54]. Although very effective in preventing postoperative ileus in animal models, caution should be taken when implementing electric vagus stimulation and pharmacologic interventions in patients. Electrical stimulation remains an invasive procedure, while pharmacologic stimulation of nicotinic receptors might cause unwanted stimulation of different cell types and organs [55,56].

A more physiological way to activate the vagal antiinflammatory pathway is by administration of enteral nutrition enriched with lipids. Administration of lipid-rich nutrition prior to, or following, hemorrhagic shock attenuates systemic inflammation and preserves intestinal integrity [57,58]. These positive effects of lipid-rich nutrition on gut barrier function and systemic inflammation are specific for the amount of lipids in the nutrition, as a low-lipid control feeding did not exert these protective effects. The enteral presence of lipids activates the autonomic nervous system *via* cholecystokinin receptors. Subsequently, inflammation is inhibited through activation of nicotinic receptors on inflammatory cells *via* the efferent vagus [59]. Enteral administration of lipid-rich nutrition was demonstrated to reduce postoperative ileus in a rodent model of intestinal manipulation [16]. Enteral nutrition enriched with lipids prevented degranulation of mast cells, inhibited release of macrophage-derived TNF-α and Il-6, and prevented influx of neutrophils into the intestinal muscularis to a greater extent than the control, low-lipid, nutrition. More importantly, the beneficial effect of lipid-rich nutrition on manipulation-induced local inflammation promoted gastrointestinal transit in a CCK-receptor-dependent manner [16]. These findings indicate that lipid-rich nutrition reduces postoperative ileus *via* activation of the nutritional antiinflammatory pathway. Luminal lipids are known to activate the autonomic nervous system *via* cholecystokinin-mediated stimulation of peripheral CCK-1 receptors on afferent vagal fibers, resulting in several regulatory digestive functions, such as satiety [60]. Therefore, the antiinflammatory potential of lipid-rich enteral nutrition could rely on activation of a nutritional CCK-dependent vagovagal reflex.

Interestingly, sham feeding is another physiological technique that activates the cephalic vagal axis by mimicking food intake, thereby stimulating bowel motility [38,39]. Furthermore, activation of the cephalic phase elicits digestive functions *via* vagovagal cholinergic reflexes [61]. Sham feeding by chewing gum has been shown to improve bowel movement and reduce time to first flatus and first defecation after open gastrointestinal surgery, and demonstrates a trend towards a

reduced hospital stay [62,63]. However, the exact mode of action remains to be investigated.

Conclusion

Surgical interventions, and abdominal surgery in particular, are frequently accompanied by the occurrence of postoperative ileus. Postoperative ileus is a multifactorial surgical complication that requires a multifactorial treatment approach. Minimal invasive surgery to reduce surgical stress, epidural analgesia to block inhibitory reflexes, minimizing opioid use, and attenuation of intestinal inflammation by antiinflammatory interventions should reduce postoperative ileus to a minimum. The development of safe and easy-to-use treatments to prevent intestinal inflammation will play a key role in controlling postoperative ileus and deserves further investigation. Stimulation of the vagal antiinflammatory pathway, by interventions such as enteral administration of lipids, is one of the promising interventions contributing to a further reduction of postoperative ileus.

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Chapter 4.1

Lipid-enriched enteral nutrition controls the inflammatory response in murine gram-negative sepsis

Tim Lubbers
Jacco J. De Haan
M'Hamed Hadfoune
Yiren Zhang
Misha D. Luyer
David Grundy
Wim A. Buurman
Jan Willem M. Greve

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Abstract

Background: Controlling the inflammatory cascade during sepsis remains a major clinical challenge. Recently, it has become evident that the autonomic nervous system reduces inflammation via the vagus nerve.

Objectives: The current study investigates whether nutritional stimulation of the autonomic nervous system effectively attenuates the inflammatory response in murine gram-negative sepsis.

Methods: Mice were subjected to an intraperitoneal bolus of lipopolysaccharide (LPS) derived from Escherichia Coli. Prior to LPS administration, mice were fasted or enterally fed either lipid-rich nutrition or low-lipid nutrition. Antagonists to cholecystokinin receptors or nicotinic receptors were administered 30 minutes before LPS challenge. Blood and tissue samples were collected at 90 minutes. In separate experiments, mesenteric afferent discharge was determined *ex vivo* in response to both nutritional compositions.

Results: Both lipid-rich and low-lipid nutrition dose-dependently reduced LPS-induced TNF-α release (high dose: both 1.4 ± 0.4 ng/ml) compared with fasted mice (3.7 ± 0.8 ng/ml; $p<0.01$). The anti-inflammatory effect of both nutritional compositions was mediated via cholecystokinin receptors ($p<0.01$), activation of mesenteric vagal afferents ($p<0.05$) and peripheral nicotinic receptors ($p<0.05$). Lipid-rich nutrition attenuated the inflammatory response at lower dosages than low-lipid nutrition, indicating that enrichment of enteral nutrition with dietary lipid augments the anti-inflammatory potential. Administration of lipid-rich nutrition prevented endotoxin-induced damage to the small intestinal epithelium and reduced inflammation in liver and spleen compared with fasted (all $p<0.01$) and low-lipid nutrition treated animals (all $p<0.05$).

Conclusions: The current study demonstrates that lipid-rich nutrition attenuates intestinal damage and systemic as well as organ-specific inflammation in murine gram-negative sepsis via the nutritional vagal anti-inflammatory pathway. These findings implicate enteral administration of lipid-enriched nutrition as a promising intervention to modulate the inflammatory response during septic conditions.

Introduction

An exaggerated inflammatory response following surgery, trauma and burns is a dreaded complication, that can ultimately lead to sepsis and septic shock [1,2]. A key characteristic in sepsis is the fierce systemic inflammatory response to bacterial toxins, which is mediated by excessive release of numerous pro-inflammatory mediators [3, 4]. The subsequent hyper-inflammatory response results in multiple organ dysfunction and correlates with adverse outcome [5, 6]. Despite recent advances in medical care, sepsis remains life-threatening in the intensive care unit [7]. Although experimental studies demonstrated promising results of anti-inflammatory strategies aimed at inhibition of single pro-inflammatory mediators, clinical implementation has largely failed to improve survival [8]. Enhanced insight in disease pathology and development of novel treatments, which broadly affect the inflammatory response, are critical to reduce sepsis-induced mortality [9].

Recent experimental evidence has revealed an important neuroimmune pathway [9]. Circulating cytokines are sensed by afferent vagal fibers, resulting in fever and an anti-inflammatory response via the hypothalamic-pituitary-adrenal axis [10, 11]. Moreover, electric stimulation of the vagus nerve attenuates inflammation via activation of nicotinic acetylcholine receptors on inflammatory cells [12,13]. Activation of this so-called "cholinergic anti-inflammatory pathway" improved outcome in several systemic inflammatory models [14, 15].

Recently, our group demonstrated in rat models of postoperative ileus and non-lethal hemorrhagic shock that nutritional activation of the autonomic nervous system modulates the inflammatory response [16-18]. Enteral administration of lipid-rich nutrition activates the autonomic nervous system via cholecystokinin (CCK)-receptors. Cytokine release is subsequently inhibited via a vagally-mediated stimulation of nicotinic receptors on inflammatory cells [19]. These findings implicate lipid-rich nutrition as a physiologic anti-inflammatory intervention in settings of controlled inflammation. The current study aims to investigate the anti-inflammatory potential of lipid-rich nutrition in murine endotoxemia. Additionally, involvement of the vagal pathway in the immune-modulating effect of enteral nutrition was studied.

Materials and methods

Animals

Male C57bl6 mice, aged 8-12 weeks were purchased from Charles River Laboratories (Maastricht, the Netherlands) or bred at the University of Sheffield and housed under controlled conditions of temperature and humidity with ad libitum

access to standard rodent chow and water. The experimental protocols were approved by the Animal Ethics Committee of the Maastricht University Medical Center and University of Sheffield.

Experimental design and procedure

Mice received an intraperitoneal dose of lipopolysaccharide (LPS from Escherichia coli 055;B5, Sigma-Aldrich, Zwijndrecht, the Netherlands) 2 mg/kg in sterile phosphate-buffered saline (PBS; pH 7.4) to induce gram-negative sepsis. Prior to LPS, mice were randomly assigned to a fasted group or one of the nutritional intervention groups. The fasted group was starved 18 hours prior to LPS administration, whereas all nutritional groups were fed per oral gavage at 18 hours, 2 hours and 45 minutes prior to LPS administration. The nutritional groups were fed lipid-rich or control low-lipid nutrition in three different dosage regimens (see Figures 1A-B).

The liquid lipid-rich diet contained 50.4 energy percent (en%) fat, of which 30% were phospholipids, 8.7en% protein and 40.9en% carbohydrates. The low-lipid nutrition contained 16.0en% fat, 8.7en% proteins and 75.3en% carbohydrates. The carbohydrate and protein composition of both diets were identical. The lipid source was soy lecithin. Omega 3 and omega 6 fatty acids constituted <5% in both feedings [16]. The high-lipid nutrition was isocaloric and isonitrogenous to low-lipid nutrition and the amount of fat in the control diet was isocaloric to that present in standard rodent chow. Proteins were derived from lean milk powder, containing 80% casein and 20% whey protein. The carbohydrate source was a mixture of sucrose and maltodextrins (Glucidex 19DE). To investigate the role of gastrointestinal distention in activation of the nutritional anti-inflammatory pathway, mice were fed a non-caloric 30% Polyethylene Glycol (PEG) 20.000 solution in PBS (Sigma-Aldrich; see Figure 1). All animals, subjected to endotoxin, displayed reduced locomotor activity, pilo-erection, and developed diarrhea.

Receptor antagonists

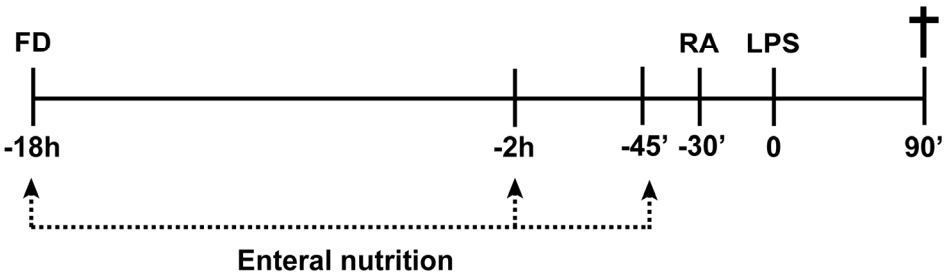
Mice received an intraperitoneal injection with antagonists to the CCK-1 receptor, Devazepide and the CCK-2 receptor, L365,260 or chlorisondamine diiodide 30 minutes before induction of endotoxemia. Devazepide and L365, 260 (both 500 µg/kg; kind gifts from ML Laboratories PLC, Nottingham, UK) were dissolved in 90% saline, 5% Tween 20, 5% DMSO. Chlorisondamine diiodide (125 µg/kg; Tocris Bioscience, Bristol, UK) was dissolved in saline.

Mesenteric afferent discharge

Mice were killed by cervical dislocation in accordance with the UK Animals Scientific Procedures Act (1986). Intestinal tissue was prepared for nerve recording as

previously described [20]. In short, proximal jejunal segments (2 – 3 cm) were dissected so that a non-bifurcating mesenteric bundle could be identified. The isolated segments were placed in oxygenated Krebs solution at 34°C. A single nerve bundle was drawn into a suction electrode for afferent recording. The jejunum was cannulated at each end and intraluminal pressure was recorded via a pressure recorder. The lumen was perfused with saline at 0.2ml/min except during distension, when the outlet tap was closed allowing pressure to rise up to 55 mmHg and released by opening the tap. Following a 60 minute stabilization period, intestinal segments were distended at 15 minutes intervals and mean afferent firing rate (spikes/s) was displayed as peri-stimulus rate histogram.

A



B

	-18 h	-2 h	-45 min	N
Low dose	0.3 ml	0.1 ml	0.1 ml	8
Intermediate dose	0.3 ml	0.2 ml	0.2 ml	8
High dose	0.4 ml	0.3 ml	0.3 ml	8
CCK- or nicotinic RA	0.4 ml	0.3 ml	0.3 ml	6
PEG-solution	0.4 ml	0.3 ml	0.3 ml	6

Figure 1: Experimental protocol and groups. Mice were deprived of food (FD) 18 hours prior to LPS administration. In the nutritional intervention groups, mice were fed lipid-rich or low-lipid nutrition per oral gavage at three time points (-18 hours, -2 hours and -45 minutes) prior to LPS. Antagonists to the CCK or peripheral nicotinic receptor (RA) were administered 30 minutes before induction of endotoxemia. Mice were killed at 90 minutes (A). The feedings were given in three dosage regimes, namely the low-dose (5% of normal daily intake (NDI), the intermediate dose (7% NDI) and the high dose (10% NDI) (B). Animals that received RA were fed the high dose. Polyethylene glycol (PEG) solution was administered in the high dose to fasted rats.

Once reproducible responses were obtained, the effect of nutrient was tested by switching luminal perfusion to either lipid-rich or low-lipid nutrition (both 1 ml) with free-drainage. The nutrient remaining within the lumen was trapped for 15 minutes following closure of the outlet port with termination of the saline perfusion. One period of distension was achieved by perfusion with saline, which also served to flush out luminal contents when the outlet tap was opened. Saline perfusion and repeat distensions at 15 minutes continued until the response had recovered to baseline.

Cytokine analysis

TNF-α levels were determined in plasma and tissue harvested at 90 minutes following LPS challenge. Hepatic and splenic tissues were snap frozen in liquid nitrogen, after which they were homogenized in lysisbuffer (300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, en Pepstatin A, Leupeptin, Aprotinin (all 20 ng/ml); pH 7.4), centrifuged and supernatants stored at -20 ^{0} C until analysis. TNF-α was measured using a standard enzyme-linked immunosorbent assay (ELISA) for mice TNF-α (R&D systems Europe, Oxon, UK).

Determination of intestinal epithelial cell damage

Localization of ileum-lipid binding protein (I-LBP) was visualized by immunohistochemistry on 4 µm cut paraffin sections of ileum. Sections were incubated for 50 minutes with rabbit anti-mouse I-LBP (Hycult Biotech, Uden, the Netherlands). Thereafter, sections were incubated for 30 minutes with biotin labeled swine anti-rabbit IgG conjugate (Dako, Glostrup, Denmark), followed by 30 minutes incubation with the AB-complex and AEC staining. Nuclear staining was performed using haematoxylin. Pictures were taken using the Metasystems Image Pro System (Metasystems, Sandhausen, Germany) mounted on a Leica DM-RE microscope (Leica, Wetzler, Germany). Magnifications of 200x were used to display I-LBP expression. I-LBP was quantified in plasma using a specific ELISA (Hycult Biotech).

Statistical analysis

Data are expressed as median, range and interquartile range, unless otherwise indicated. A two-tailed Mann-Whitney U test was used for between-group comparisons of plasma TNF-α and I-LBP. Spearman's correlation was used to assess the association between plasma levels of I-LBP and TNF-α. Whole nerve afferent discharge was calculated from the number of spikes crossing a pre-set threshold and expressed as spikes/s. Baseline discharge was calculated as the mean firing in the 1 minute period preceding distension. Discharge during distension was

expressed as increase above baseline, calculated as the mean firing frequency in 5 sec periods at each level of distending pressure. Depicted data represent the last saline distension prior to nutrient perfusion, distension with nutrient in the lumen and first distension after washout. Discharge data were compared statistically using repeated measure ANOVA with Dunnett post-test analysis. Prism 5.02 for Windows (GraphPad Software Inc., San Diego, CA) was used for computations. All experimental groups consisted of 8 animals, unless otherwise indicated.

Results

Lipid-rich nutrition reduces systemic inflammation more efficiently than low-lipid nutrition

In order to investigate the anti-inflammatory potential of lipid-rich nutrition in murine gram-negative sepsis, we administered lipid-rich and low-lipid nutrition to endotoxemic mice in three dosage regimens (Figure 1). Low dose administration of both feeds prior to LPS challenge did not affect circulating levels of TNF-α compared with fasted mice (Figure 2). Pretreatment with an intermediate dose of lipid-rich nutrition suppressed systemic inflammation compared with fasted mice ($p < 0.01$) and low-lipid nutrition ($p < 0.05$). Administration of a high dosage of both feeds effectively inhibited circulating TNF-α (both $p < 0.001$), indicating that both feeds are capable of attenuating systemic inflammation. To explore a role for gastrointestinal distention in the anti-inflammatory effect of enteral nutrition, we administered a high dose PEG solution. This non-caloric volume load did not affect plasma TNF-α levels, signifying that enteral nutrition attenuates endotoxin-induced systemic inflammation in a dose-dependent manner and that nutrition enriched with lipids is more efficient than low-lipid nutrition.

The anti-inflammatory potential of enteral nutrition is mediated via the nutritional anti-inflammatory pathway

To clarify whether the anti-inflammatory effects of both feeds were exerted via the vagal anti-inflammatory pathway, we first investigated whether a high dose of both lipid-rich and low-lipid nutrition triggered CCK-receptors. Pretreatment of mice with a combination of antagonists to the CCK-1 and CCK-2 receptor reduced the inhibitory effect of lipid-rich and low-lipid nutrition on plasma levels TNF-α compared with vehicle (both $p < 0.01$. Figure 3A).

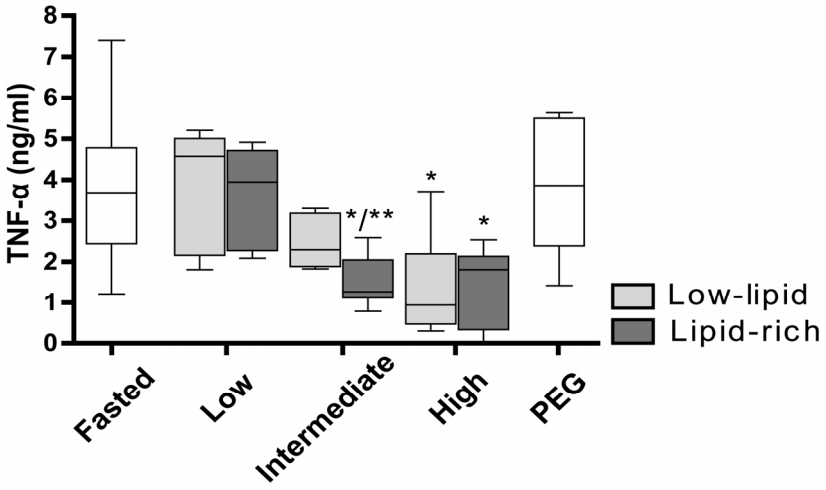


Figure 2: Enteral nutrition dose-dependently attenuates systemic inflammation in murine gram-negative sepsis. Intraperitoneal injection of endotoxin results in marked plasma levels of TNF-α in fasted mice. Pretreatment with lipid-rich nutrition attenuated systemic inflammation in the intermediate and high dose. Low-lipid nutrition reduced TNF-α levels only in the highest dose. Gastrointestinal distension using bolus administration of PEG-solution did not affect systemic inflammation (n = 6). * $p < 0.05$ compared with fasted. ** $p < 0.05$ compared to intermediate dose of low-lipid.

Next, we examined whether luminal presence of lipid-rich and low-lipid nutrition trigger firing of jejunal mechanosensitive afferents *ex vivo*. Since previous studies demonstrated that low-threshold afferents preferentially project via vagal pathways [21], we quantified increase in afferent firing rate over the pressure range from 0 to 20 mmHg. Lipid-rich nutrition (treatment = T) enhanced afferent discharge to distension compared with discharge before treatment (BT) and after treatment (AT: $p < 0.001$. Figure 4A). Likewise, administration of low-lipid nutrition increased afferent discharge compared with BT and AT ($p < 0.05$. Figure 4B). These differences were not related to changes in jejunal compliance and not mirrored in the firing of high threshold afferents at distension pressures > 20 mmHg, which project via spinal pathways (data not shown).

Lastly, we administered chlorisonamide diiodide, a peripheral nicotinic receptor antagonist. Chlorisondamine diiodide inhibited the anti-inflammatory effect of lipid-rich and low-lipid nutrition compared with vehicle (both; $p < 0.05$. Figure 3B). These findings suggest that lipid-rich and low-lipid nutrition, at a sufficient dose, modulate endotoxemia-induced inflammation via the nutritional anti-inflammatory pathway.

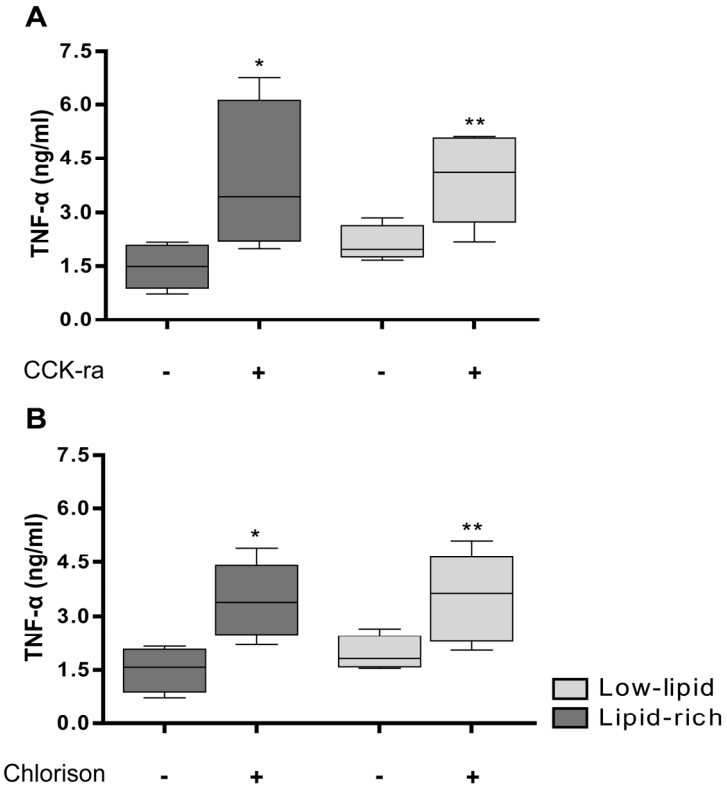


Figure 3: The anti-inflammatory effect of enteral nutrition is mediated via CCK-receptors and peripheral nicotinic receptors. Administration of CCK-receptors antagonists (ra) reversed the inhibitory effect of the highest dose of lipid-rich and low-lipid nutrition on circulating levels of TNF-α (A). Pretreatment with the nicotinic ra counteracted the beneficial effect of lipid-rich and low-lipid nutrition treated mice. (B) * $p < 0.05$ compared with lipid-rich + vehicle, ** $p < 0.01$ compared with low-lipid + vehicle, n = 6.

Lipid-rich nutrition reduces intestinal epithelial cell damage during endotoxemia

Gastrointestinal injury is implicated as a major component in the pathogenesis of sepsis and is linked with disease progression [22,23]. Lipid-rich nutrition was shown to attenuate systemic inflammation at lower quantities than low-lipid nutrition.
Therefore, we investigated whether lipid-rich nutrition also attenuated intestinal epithelial cell damage more potently than low-lipid nutrition. Intestinal epithelial cell damage was assessed as plasma levels of I-LBP, a small cytosolic protein constitutively expressed in mature ileal enterocytes, which is rapidly released

following cellular damage [16]. Endotoxemia in mice resulted in increased plasma levels of I-LBP compared with healthy control mice ($p < 0.01$. Figure 5A). Endotoxemia-induced loss of I-LBP was immunohistochemically verified. Figure 5C represents the ileal distribution and localization of I-LBP in healthy controls. Figure 5D demonstrates endotoxin-induced loss of I-LBP from enterocytes and sludging of villus tips. Lipid-rich nutrition at an intermediate dose significantly reduced circulating levels of I-LBP compared with fasted mice ($p < 0.01$) and low-lipid nutrition ($p < 0.05$). Pretreatment with antagonists to CCK-receptors prevented the protective effects of lipid-rich nutrition on enterocyte damage ($p < 0.05$). TNF-α has been demonstrated to influence intestinal barrier function [23, 24]. In the current model, we confirm that TNF-α is significantly correlated with I-LBP ($r^2 = 0.74$, $p < 0.001$. Figure 5B).

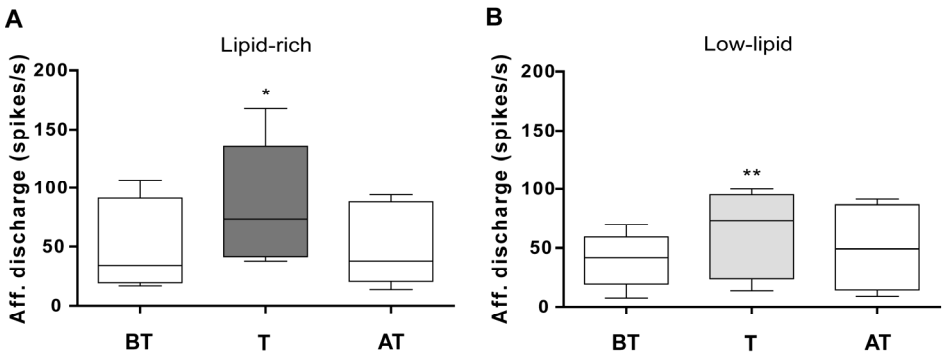


Figure 4: Enteral nutrition enhances afferent vagal discharge to distension. Intrajejunal administration (T) of lipid-rich nutrition augments mesenteric afferent firing to luminal distension in the low-threshold range (0-20 mm Hg) compared with distension before treatment (BT) and after treatment (AT) (A). Administration of low-lipid nutrition resulted in a similar activation of the mesenteric afferent discharge (B). * $p < 0.001$ for the lipid-rich treatment, ** $p < 0.05$ for the low-lipid treatment, $n = 6$.

Lipid-rich nutrition attenuates hepatic and splenic inflammation

The systemic inflammatory response of sepsis does not only lead to circulating cytokines, but also results in inflammation at organ level. To delineate the anti-inflammatory potential of lipid-rich nutrition, we investigated the expression of TNF-α in tissue homogenates of liver and spleen. Intraperitoneal administration of LPS enhanced TNF-α protein levels in the liver (Figure 6A) and spleen (Figure 6B) compared with healthy controls (not detectable). Lipid-rich nutrition, administered at an intermediate dose reduced TNF-α content in the liver and spleen compared with fasted mice (both $p < 0.01$) and low-lipid nutrition (both $p < 0.05$). These

observations indicate that lipid-rich nutrition decreases endotoxemia-induced organ-specific inflammation.

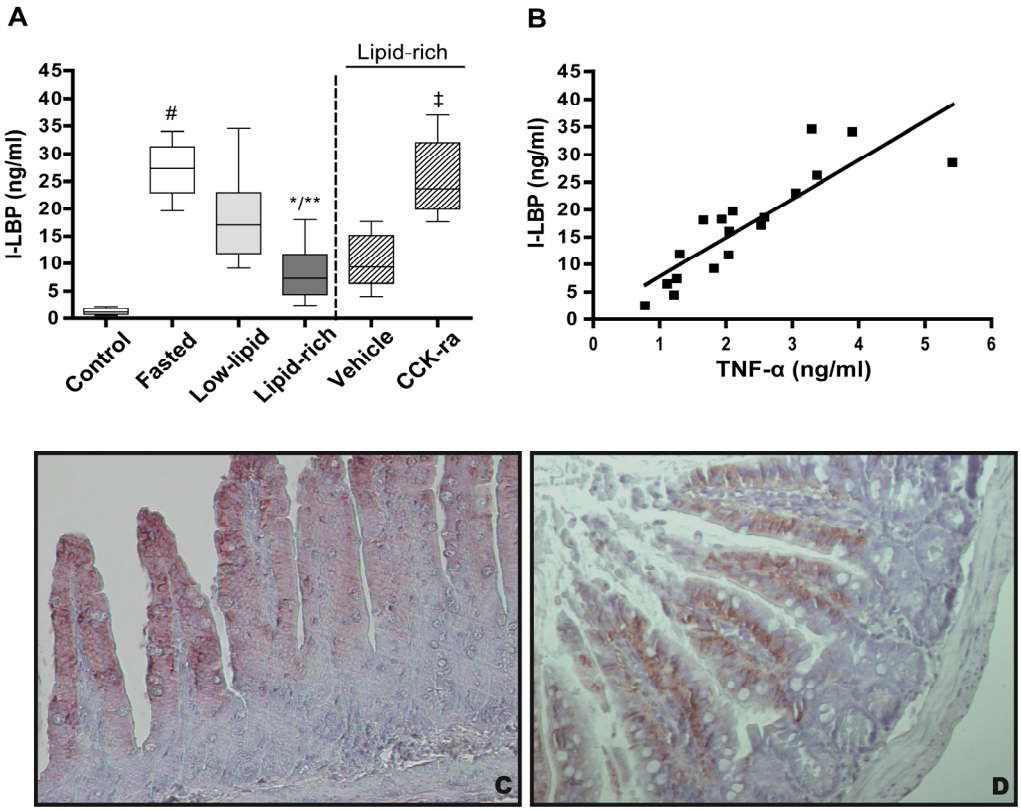


Figure 5: Lipid-rich nutrition reduces intestinal epithelial cell damage. Endotoxemia resulted in significant intestinal epithelial cell damage, measured as plasma levels of I-LBP (A) and visualized as loss of I-LBP from enterocytes (C-D). The nutritional intervention with lipid-rich nutrition in the intermediate dose prevented intestinal epithelial cell damage compared with fasted and low-lipid groups. CCK-receptor antagonist reduce the effect of lipid-rich nutrition on enterocyte damage. (B) The plasma levels of I-LBP were significantly correlated with plasma TNF-α levels (r^2: 0.74, $p < 0.001$). Data represented as single dots (B). $^{\#}$ $p < 0.05$ compared with healthy control, * $p < 0.05$ compared with fasted, ** $p < 0.05$ compared with low-lipid, ‡ $p < 0.05$ compared with vehicle.

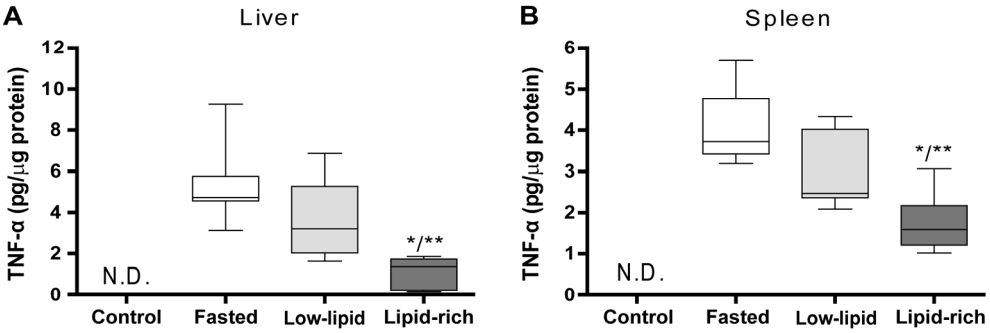


Figure 6: Lipid-rich nutrition attenuates organ-specific inflammation. Endotoxemia results in enhanced hepatic (A) and splenic (B) levels of TNF-α protein. Administration of an intermediate dose of lipid-rich nutrition attenuates the endotoxin-induced inflammation in the liver and spleen. * $p < 0.01$ compared with fasted, ** $p < 0.01$ compared with low-lipid nutrition.

Discussion

Treatment of a dysregulated inflammatory response in critically-ill and surgical patients continues to be a clinical predicament. Anti-inflammatory therapies are needed that broadly affect the inflammatory response and can be safely used in these patients. The current study identifies lipid-rich nutrition as a promising intervention to treat inflammation during septic conditions.

Administration of lipid-rich nutrition has been shown to modulate inflammation in several rat models [16-19]. In the current murine model, a dose-dependent approach was used to investigate the anti-inflammatory effect of lipid-rich nutrition on gram-negative sepsis. Administration of a high dose of low-lipid and lipid-rich nutrition reduced endotoxemia-induced systemic TNF-α levels. In rats, the luminal presence of lipid-rich nutrition reduced inflammation via a vagus nerve-dependent neuroimmune pathway. Bilateral cervical vagotomy abrogated the protective effects of lipid-rich nutrition in rats [19]. Here, we demonstrate for the first time that jejunal presence of lipid-rich or low-lipid nutrition augmented mesenteric afferent discharge of low-threshold mechanoreceptors, indicating that both feeds activate vagal afferents [21]. Previous studies have demonstrated direct effect of CCK on mucosal afferents independent of mechanical sensitivity [25]. However, an interaction between responses to distension and CCK has been shown previously for gastric afferents [26, 27], suggesting that these endings encode both the presence and composition of luminal nutrients. The current finding suggests that

the same may be true for jejunal mechanoreceptors, which appear to be sensitized by the presence of lipid.

The vagal pathway is activated via CCK-receptors and exerts its anti-inflammatory effect via peripheral nicotinic receptors [16, 18, 19]. Administration of antagonists to the CCK-receptor or peripheral nicotinic receptor both inhibited the anti-inflammatory effect of lipid-rich and low-lipid nutrition in mice, indicating that enteral nutrition activates two important receptor subtypes which are involved in the nutritional vagal anti-inflammatory pathway [19].

Distension of the gastric wall has been shown to activate the vagus nerve in a CCK-receptor dependent manner [28]. Therefore, gastrointestinal distension, provoked by ingestion of a large amount of feeding, could play a role in the observed anti-inflammatory effect. However, administration of a PEG-solution did not affect systemic inflammation, signifying that stimulation of CCK-receptors by luminal nutrients rather than gastrointestinal distension alone is essential.

In several experimental rat models, the anti-inflammatory potential of lipid-rich nutrition was superior to low-lipid nutrition [18, 19]. These findings are confirmed in mice, since lipid-rich nutrition inhibited plasma TNF-α levels at lower quantities than low-lipid nutrition. In addition, the mesenteric afferent recordings demonstrated a greater discharge for lipid-rich than low-lipid nutrition. Increasing the lipid load has results in higher CCK plasma levels and prolonged CCK-release [29], leading to a sustained activation of CCK-receptors. Release of CCK and subsequent activation of mesenteric vagal afferents is triggered by intestinal application of protein as well as fatty acids [30]. However, the potency of lipid-rich nutrition to attenuate inflammation compared with low-lipid nutrition likely depends on the lipid fraction, since both feeds contain the same amount of protein and vary in the lipid composition.

Systemic inflammation following endotoxin administration has been demonstrated to result in intestinal damage [22, 31]. TNF-α is one of the principal mediators of the pathophysiological changes during endotoxemia, including intestinal compromise [3, 32, 33]. Here, intraperitoneal administration of endotoxin resulted in marked intestinal epithelial cell damage. Accordingly, plasma levels of I-LBP showed a robust correlation with circulating TNF-α. Preservation of intestinal enterocytes has been shown to improve survival in a cecal ligation and puncture model [34]. Moreover, release of intestinal-fatty acid binding protein (I-FABP), another member of the fatty acid binding protein family, is associated with disease severity, systemic inflammation and survival in clinical settings [35-38]. The enterocyte damage in the current model was significantly reduced by lipid-rich nutrition. In addition to the current data, we demonstrated that administration of lipid-rich nutrition prior to or following hemorrhagic shock in rats diminishes intestinal

compromise [16, 17], emphasizing the use of lipid-rich nutrition to reduce intestinal damage during inflammatory conditions.

The liver and spleen are critically involved in the response to sepsis [39, 40]. Administration of bacteria or endotoxin primarily activates macrophages in both organs, leading to organ damage and malfunction via a local cytokine response in which TNF-α plays a prominent role [41-43]. The marked elevation of TNF-α protein observed in the liver and spleen upon intraperitoneal administration of endotoxin was effectively reduced by lipid-rich nutrition. These findings are supported by studies demonstrating that stimulation of the vagal anti-inflammatory pathway attenuates inflammation in the liver and spleen during septic conditions [55, 56]. Reduction of the primary inflammatory response in the liver has been shown to ameliorate hepatic injury [39, 46]. In line with the observed anti-inflammatory effect on the liver, we previously demonstrated that lipid-rich nutrition reduced liver damage in rats [47]. These findings indicate that stimulation of the nutritional anti-inflammatory pathway with lipid-rich nutrition has potent anti-inflammatory effects in the liver and spleen under septic conditions.

Taken together, the current manuscript underlines that the autonomic nervous system plays a vital role in regulation of the inflammatory response. The data demonstrate that stimulation of the parasympathetic nervous system with lipid-rich nutrition is a promising intervention to control endotoxemia-induced hyper-inflammation. Interestingly, blockage of the sympathetic nervous system with beta-adrenergic receptor antagonists has been shown to improve immune competence and outcome in critical-illness [48, 49]. Administration of enteral lipid-rich nutrition combined with beta-adrenergic blockade in critically-ill patients could be synergistic and deserves further investigation.

Conclusions

Nutritional regimens for critically-ill and surgical patients have changed significantly in the last two decades. Reduction of the preoperative fasting, preoperative enteral administration of nutrients and early nutritional support have positively influenced outcome [50]. Enteral nutrition is preferred in critically-ill and surgical patients and has been shown to be well tolerated [51-52]. The current study supports the positive effects of enteral nutrition and reveals a potential mode of action. Our data demonstrate that enteral nutrition attenuates inflammation and intestinal damage during murine gram-negative sepsis via the nutritional anti-inflammatory pathway. These findings expand our current knowledge on the applicability of enteral nutrition to treat inflammatory conditions and indicate that enrichment of enteral nutrition with lipid potentiates the beneficial effects.

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Chapter 4.2

Identification of a nutritional intervention to be used in a human proof-of-principle study

Tim Lubbers
Jacco J. De Haan
Jan Willem M. Greve
Wim A. Buurman

Abstract

Background: An exaggerated inflammatory response following injury and infection results in major morbidity and mortality in hospitalized patients. Previously, it was shown in rodents that enteral administration of lipid-enriched nutrition attenuates inflammation and associated organ damage via a cholecystokinin-mediated anti-inflammatory reflex.

Objective: The current study was initiated to select a nutritional intervention to be used as anti-inflammatory intervention in a human proof-of-principle study

Methods: After an overnight fast, healthy male (n = 6) and female (n = 6) subjects underwent the following dosing regime during 4 consecutive days: a 200 ml or 400 ml bolus of the commercially available lipid-rich Diasip© (day 1 and 2) followed by a 200 ml or 400 ml bolus of the low-lipid nutrition Respifor© (days 3 and 4). In the second part of the study, 6 healthy male subjects ingested a custom-made lipid- and protein-enriched (enriched) nutrition, designed to induce intestinal CCK release, at day 1 followed by a standard tube feed on day 2. In all experiments, blood was withdrawn via intravenous catheter before ingestion of the feed and at indicated time points thereafter. CCK levels were determined in plasma.

Results: Ingestion of 200 ml Diasip© and Respifor© resulted in peak CCK plasma levels within 15 minutes (7.0 ± 1.1 pmol/L and 8.2 ± 2.0 pmol/L, *respectively*). CCK plasma levels steadily decreased afterwards, approximating detection level at the last determined time point (t = 90 minutes). No differences in CCK plasma response were found between Diasip© and Respifor© (p = 0.44). Remarkably, the CCK plasma response following ingestion of the custom-made enriched nutrition was identical to the standard tube feed (p = 0.99). Doubling of the nutritional dose of Diasip© and Resipfor© tended to protract the CCK plasma response compared with the single dose (p = 0.05 and p = 0.06, *respectively*), but resulted in similar peak plasma levels compared with the lower dose (8.6 ± 1.8 pmol/L; p = 0.77 [Diasip©] and 7.8 ± 1.7 pmol/L; p = 0.98 [Respifor©]).

Conclusion: The current study demonstrates that determination of CCK plasma levels is an ineffective tool to select a suitable nutritional composition to be used for a human proof-of-principle study. In addition, our findings show that increasing the ingested dose did not influence peak CCK plasma levels, but resulted in a protracted CCK plasma response instead.

Introduction

An overzealous inflammatory response to injury and infection poses a major clinical dilemma. Specifically, surgical, trauma and ICU patients are at risk of developing an dysregulated immune response, resulting in systemic inflammatory response syndrome, tissue damage, sepsis and eventually death [1]. Until today, modulation of the immune response during these conditions has been proven to be extremely difficult, as promising experimental interventions failed to improve outcome in clinical trails or even aggravated the course of disease [2-5].

Recently, enteral administration of lipid-rich nutrition was shown to effectively attenuate inflammation and reduce tissue injury in several inflammatory rodent models, including hemorrhagic shock, endotoxemia and postoperative ileus [6-9]. Moreover, the beneficial effects of lipid-rich nutrition were also observed when the intervention was started following hemorrhagic shock, a setting similar to trauma where inflammation and tissue injury are already present [10]. Ingestion of lipid-rich nutrition triggers a novel gut-brain-immune axis [11]. In this axis, the luminal presence of lipid results in intestinal release of cholecystokinin (CCK), which activates afferent vagal fibers via peripheral CCK-1 receptors [11]. Subsequent activation of efferent vagus nerve reduces release of pro-inflammatory mediators via activation of nicotinic receptors on inflammatory cells [12,13]. Nutritional stimulation of the CCK-mediated vagovagal anti-inflammatory reflex could provide a promising and physiological tool to limit inflammation in the clinical setting and improve outcome.

The current pre-clinical study aims to indentify a suitable nutritional intervention to be used during a human proof-of-principle study and future clinical interventions, based on their capacity to release CCK. First, plasma CCK responses following ingestion of a commercially available lipid-rich nutrition, Diasip© and a control low-lipid nutrition, Respifor© were determined in healthy male and female subjects. In addition, the CCK plasma response was investigated following a twofold increase in the nutritional dose. Lastly, the effect of a lipid- and protein-enriched (enriched) nutrition, developed to induce intestinal release of CCK, on circulating levels of CCK was studied.

Materials and Methods

Subjects

This study was registered at ClinicalTrail.gov as NCT00468507. After approval of the local ethics committee of the Maastricht University Medical Centre+, 6 healthy male and 6 healthy female subjects between the age of 45 and 60 years gave

written informed consent to participate in the experiments in accordance with the Declaration of Helsinki 2008.

Nutritional intervention

The nutritional intervention was performed in a double-blind randomized setting with cross-over design. In the first part of the study, all subjects received Diasip© and Respifor© in a dose of 200 ml and 400 ml on four separate days. In the second part, all subjects received 200 ml of enriched nutrition and Nutrison Standard© on two days. On the evening prior to the experiment, subjects were deprived of food from 24.00 hrs onwards. On the experimental day, venous blood was withdrawn via an intravenous catheter before administration of the enteral nutrition and serially thereafter until 120 minutes. Blood was collected in pre-chilled glass tubes (BD Biosciences, Breda, the Netherlands), put on ice, centrifuged and stored at -20 degrees Celsius until analysis. On t = 0, subjects were asked to ingest 200 ml of the liquid nutrition within one minute. During the experiments in which a total of 400 ml was administered, subjects were asked to drink a second bolus within 1 minute 5 minutes following the first bolus (Figure 1).

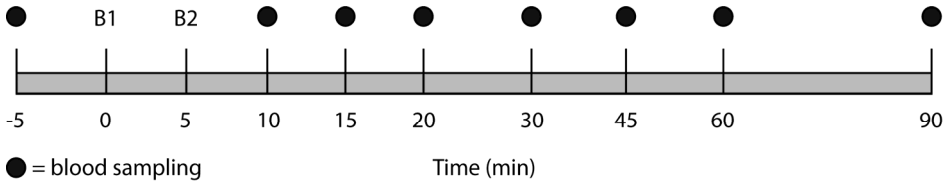

Figure 1: Experimental procedure. After an overnight fast, a blood sample was drawn from an intravenous catheter prior to nutrient ingestion (t = -5 min). At t = 0 subjects were asked to drink 200 ml (B1) of one of the nutritional compositions within 1 minute. During the experiments with the double dose, subjects were asked to drink a second bolus of 200 ml (B2) within 1 minute at t = 5 min. In all experiments, blood was withdrawn at the indicated time points until 90 minutes.

Feeding composition

See Table 1 for the composition of the commercially available products, Diasip©, Resifor© and Nutrison Standard©. The enriched nutrition contained 44 energy percent (en%) fat, 25en% protein and 31en% carbohydrates. The fat fraction contained 10% phospholipids. The protein fraction consisted of intact casein, whey protein and soy protein hydrolysate. All nutritional compositions provided 1 kcal/ml.

CCK determination

Systemic CCK levels were determined in plasma using a CCK-radioimmunoassay with a sensitivity of 0.3 pmol/L (Eurodiagnostica, Malmö, Sweden).

Statistical analysis

All values are depicted as mean ± SEM. Two-way analysis of variance was used to detect differences between groups for serial data. Differences in serial data within groups were analyzed by one-way ANOVA with Bonferroni's post-hoc test. Prism 5.02 for Windows (GraphPad Software Inc., San Diego, CA) was used for computations. A p-value less than 0.05 was considered statistically significant.

Table 1: Composition of nutritional products

	Fat (en%)	Protein (en%)	Carbohydrate (en%)
Diasip©	49	20	35
Resifor©	20	20	60
Nutrison Standard©	35	16	49
Enriched	44	25	31

Results

Bolus administration of a high-lipid and low-lipid commercially available nutrition results in comparable CCK plasma levels

Circulating CCK was undetectable in all fasted subjects. Ingestion of 200 ml of Diasip© and Resifor© resulted in a significant rise in CCK plasma levels over time (both $p < 0.0001$, Figure 2A). CCK levels peaked at 15 minutes following ingestion of high-lipid nutrition (7.0 ± 1.1 pmol/L) and at 10 minutes in the low-lipid nutrition group (8.2 ± 2.0 pmol/L). The CCK peak levels of both compositions were similar ($p = 0.98$). Following the plasma peak, circulating levels of CCK returned to baseline at 90 minutes. The plasma CCK response and area under the curve (AUC) release did not differ between the two feeds ($p = 0.44$ and $p = 0.84$, *respectively*. Figure 2C).

Remarkably, doubling the dose of both nutritional compositions did not affect peak plasma levels of CCK (Figure 2B). A double dose of Diasip© or Respifor© resulted in similar peak plasma CCK levels compared with the single dose of both compositions (8.6 ± 1.8 pmol/L; $p = 0.77$ [Diasip©] and 7.8 ± 1.7 pmol/L; $p = 0.98$ [Respifor©]). Again, there was no difference in the plasma CCK response and AUC between the higher dose of lipid-rich and the low-lipid nutrition ($p = 0.80$ and p=

0.78, *respectively*). Increasing the dose of Diasip© tended to enhance the AUC (p = 0.05). Doubling the dose of Respifor© displayed the same trend (p = 0.061). No gender differences in the plasma CCK response could be observed during the four different nutritional interventions (data not shown).

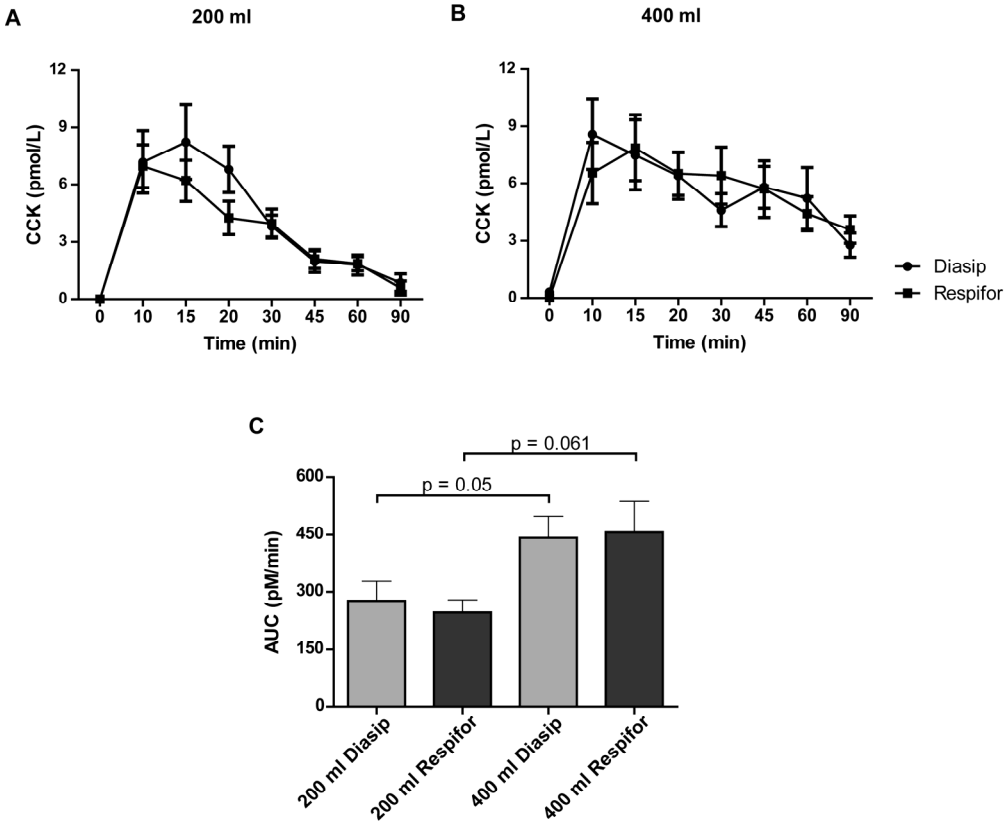

Figure 2: Diasip© and Respifor© demonstrate similar CCK plasma responses. CCK levels were undetectable in fasted subjects. Ingestion of Diasip© and Respifor© resulted in peak plasma levels within 15 minutes (A-B). Following peak plasma levels, CCK levels gradually decreased towards the last determined time point. In the low volume group, CCK plasma levels approximated fasted levels at t = 90 minutes (A). During the experiments with the higher dose, peak levels equalled those observed with the lower dose, while the CCK response and AUC tended to be increased (B-C). No differences in plasma CCK response and AUC were observed between the lipid-rich and low-lipid nutrition (A-C).

Enriched nutrition and Nutrison Standard© exhibit a similar CCK response

As no gender difference was observed in the plasma CCK response in the first part of the current study, 6 male subjects were included in this study. Peak CCK plasma levels of 7.1 ± 1.6 pmol/L for the enriched nutrition and 7.4 ± 1.7 pmol/L for Nutrison Standard© were observed at 10 minutes following administration (p = 0.82, Figure 3A). Both compositions displayed an identical plasma CCK response and AUC (p = 0.99 and p = 0.42, respectively. Figure 3).

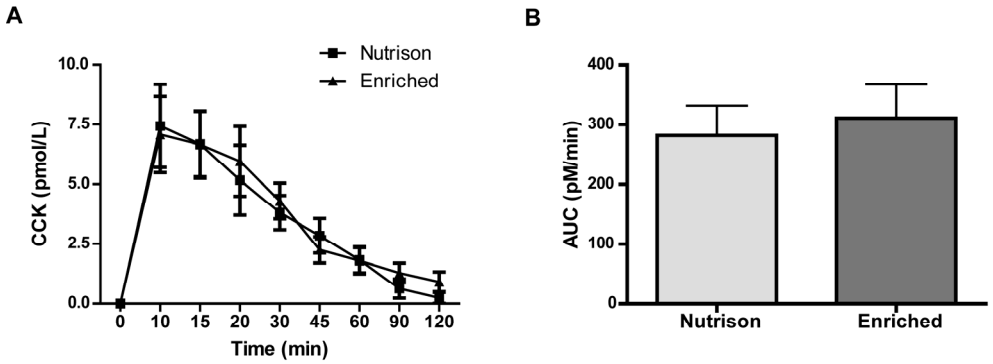

Figure 3: Enriched nutrition displays a similar plasma CCK response compared with Nutrison Standard©. Ingestion of enriched nutrition and Nutrison Standard resulted in peak plasma CCK levels at 10 minutes following administration (A). CCK levels steadily dropped until the last determined time point. No difference in CCK plasma response and AUC were demonstrated following ingestion of enriched nutrition and Nutrison Standard© (B).

Discussion

Previous rodent studies from our group demonstrated that the nutritional anti-inflammatory pathway is predominantly activated by intestinal CCK release [6-8,10,11]. Unfortunately, local intestinal CCK levels cannot be analyzed in the human setting until today. Therefore, the CCK plasma response following ingestion of various nutritional compositions was determined as surrogate marker for intestinal CCK release. In the first set of experiments, the CCK plasma response following ingestion of Diasip© and Respifor© was investigated, as these nutritional compositions are akin to our rodent feedings regarding fat, protein and carbohydrate content. Although our group demonstrated in rodents that lipid-rich nutrition displayed a superior anti-inflammatory potential compared with low-lipid nutrition [6,7], plasma CCK levels were undetectable in this setting (unpublished data). In humans on the other hand, plasma CCK levels are readily detectable following ingestion of

nutrients [15,16]. In line with previous reports, plasma CCK levels peaked within 15 minutes following administration of Diasip© and Respifor© and returned to baseline within 90 minutes [17-19]. However, no difference in circulating CCK levels was observed following ingestion of the two feeds. Remarkably, even our enriched nutrition displayed similar plasma CCK responses compared with Nutrison Standard©. This custom-made enriched nutrition contained a high lipid content supplemented with phospholipids and a high protein content containing hydrolyzed protein and whey protein. This composition and these specific components are known to induce CCK release in man [20-22]. The current data suggest that oral ingestion of isocaloric mixed meals with different macronutrient compositions likely results in comparable CCK plasma responses. In accordance with our findings, Maffeis et al. demonstrated that ingestion of a high-fat mixed meal of which 52% were lipids and a low-fat mixed meal of which 27% were lipids resulted in equivalent CCK plasma levels [15]. As all macronutrients induce CCK release in man [16,23], we hypothesize that differences in macronutrient content in a mixed meal should to be larger to detect differences in CCK plasma levels. These findings are supported by previous studies, which investigated CCK plasma levels in response to lipid as the single nutrient in man [16,24].

Following a threefold increase of the lipid infusion, CCK plasma levels were significantly enhanced compared with the lower lipid concentration. The increase in lipid load in this experiment was comparable with the Diasip and Respifor study, suggesting that the absence of differences in the CCK plasma response can be attributed to the additional presence of protein and carbohydrate in the investigated compositions.

In the current experimental approach, the plasma CCK response is also influenced by the phenomenon of gastric emptying. Gastric emptying, which is predominantly mediated via CCK, is the rate limiting step in the transport of nutrients from the stomach to the duodenum [25-27]. In our study, this is most likely visualized by the protracted CCK plasma response following the 400 ml dose of Diasip© and Respifor©. In these experiments, peak levels of CCK were similar compared with the lower dose, while the plasma CCK response was protracted. These data suggest that the amount of nutrition entering the duodenum is similar between the high and low dose, but the luminal exposure to nutrients is prolonged following the high dose. Moreover, these findings imply that the CCK-driven gastric emptying dictates CCK peak levels and subsequent plasma response following oral ingestion of nutrients. In line, it has been demonstrated that the duration of duodenal nutrient exposure determines the CCK plasma response [16,24]. Taking these consideration into account, continuous intraduodenal administration of nutrients would be a way to bypass gastric emptying, maintain a high and protracted CCK

plasma response and effectively stimulate the CCK/CCK-1 receptor mediated anti-inflammatory pathway in future studies in man.

Based on CCK plasma levels, a suitable nutritional composition to be used in a human study could not be identified in the current study. Consequently, we should bear in mind that circulating CCK levels might not reflect local CCK levels and subsequent activation of the nutritional pathway via vagal afferents, as the CCK peptide is rapidly inactivated in plasma [28,29]. Moreover, we have recently shown that activation of glucagon-like peptide-1 (GLP-1) receptors is also involved in the nutritional anti-inflammatory reflex [30]. In addition, other previously undiscovered intestinal peptides might additionally be involved, as CCK has been identified as the gatekeeper of the vagus nerve [31,32]. Based on our experimental and current findings, a nutritional intervention, aimed at attenuation of the inflammatory response in humans, should induce stable and prolonged intestinal CCK and GLP-1 release. To this end, a postpyloric administration of a lipid- and protein-enriched nutrition is proposed.

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Chapter 4.3

Lipid- and protein-enriched enteral nutrition limits inflammation in a human endotoxemia model

Tim Lubbers
Matthijs Kox
Jacco J. de Haan
Jan Willem M. Greve
Jan C. Pompe
Bart P. Ramakers
Peter Pickkers
Wim A. Buurman

Submitted

Abstract

Background: A dysregulated inflammatory response is an important cause of morbidity and mortality in critically ill patients. Enteral administration of lipid-enriched nutrition was previously shown to attenuate inflammation and organ damage via a cholecystokinin-mediated vagovagal reflex in animal studies.

Objective: The current proof-of-principle study investigates the immuno-modulatory potential of enteral lipid- and protein-enriched (enriched) nutrition during experimental human endotoxemia.

Methods: After an overnight fast, 18 healthy male subjects received an intravenous bolus of *Escherichia coli* lipopolysaccharide (LPS; 2 ng/kg). Subjects in the fasted group (n = 6) were deprived of food throughout the study, while subjects in the intervention groups were fed either enriched (n = 6) or isocaloric control nutrition (n = 6) via nasojejunal tube, starting 1 hour prior to LPS administration until 6 hours afterwards.

Results: LPS administration resulted in a marked inflammatory response. Continuous postpyloric administration of nutrition increased plasma cholecystokinin levels. Enriched nutrition attenuated circulating levels of the pro-inflammatory cytokines TNF-α and IL-6 and the IL-1 receptor antagonist compared with control nutrition (all: $p<0.01$) and fasted subjects (all: $p<0.05$). Additionally, enriched nutrition augmented the anti-inflammatory response, reflected by increased IL-10 release compared with fasted subjects ($p<0.0001$).

Conclusions: The current study establishes the anti-inflammatory potential of enriched nutrition in humans. The immediate anti-inflammatory effect of enriched nutrition suggests that the beneficial effects are mediated via a cholecystokinin-dependent vagovagal reflex. Enteral administration of enriched nutrition is a promising intervention to modulate the immune response in the early course of systemic inflammation.

Introduction

Despite diagnostic and therapeutic advances in medical care, a dysregulated systemic inflammatory response remains a major complication in surgical, trauma and critically ill patients, which is associated with increased morbidity and mortality [1, 2]. Modulation of the early excessive inflammatory response represents a potential therapeutic option to improve outcome [3]. Although experimental studies demonstrated promising results of interventions aimed at inhibition of single pro-inflammatory mediators, clinical implementation has failed to be successful [4]. Enhanced insight in disease pathology and development of novel treatment modalities which broadly affect the inflammatory response are warranted to reduce morbidity and mortality [5-7]. Recently, enteral administration of nutrients was shown to improve immune competence and clinical outcome in surgical and critically ill patients [8-10]. Although nutritional interventions are promising, the exact mechanism behind the beneficial effects remains to be elucidated.

In the past two decades, the autonomic nervous system has been identified as a potent endogenous modulator of the immune response [7]. Inflammatory cytokines are sensed by afferent vagal fibers, resulting in fever and a humoral anti-inflammatory response via the hypothalamic-pituitary-adrenal axis [11]. In turn, parasympathetic outflow suppresses release of pro-inflammatory cytokines through binding of acetylcholine to nicotinic acetylcholine receptors on inflammatory cells [12, 13]. Selective pharmacologic or electric stimulation of this neuroimmune axis, called the cholinergic anti-inflammatory pathway, improves outcome in various inflammatory models [14, 15]. Our group demonstrated that this endogenous neuroimmune axis can be activated by short-term administration of enteral lipid-enriched nutrition [16-18]. The luminal presence of lipid-enriched nutrition results in cholecystokinin (CCK) release, which activates CCK-receptors located on afferent vagal fibers [19]. Activation of these receptors triggers a vagovagal reflex, that reduces systemic and organ-specific inflammation and decreases intestinal damage via activation of peripheral nicotinic receptors [17, 18].

A well-timed nutritional stimulation of this potent gut-brain-immune axis could be a promising intervention to treat inflammatory conditions in the clinical setting. Therefore, the aim of the current proof-of-principle study was to investigate the anti-inflammatory potential of a nutritional intervention, designed to result in a marked and prolonged CCK-release, in man. Based on observations that predominantly enteral lipids and proteins trigger CCK release [20], continuous postpyloric administration of a lipid- and protein-enriched (enriched) nutrition was compared to an isocaloric low-lipid and low-protein control nutrition and to fasting. The effect of enriched nutrition on acute inflammation was studied utilizing

the experimental human endotoxemia [21]. Furthermore, the influence of enriched nutrition on endotoxin-induced sub-clinical intestinal damage was investigated.

Materials and Methods

Subjects

This study was registered at ClinicalTrail.gov as NCT01100996. After approval of the local ethics committee of the Radboud University Nijmegen Medical Centre, 12 healthy male subjects gave written informed consent to participate in the experiments in accordance with the Declaration of Helsinki. Samples of fasted subjects (n = 6) were obtained from the placebo-group that participated in another double-blind LPS study (NCT00513110). There were no differences in subject characteristics (Table 1). All subjects tested negative for HIV and hepatitis B and did not have any febrile illness in the two weeks preceding the study. The subjects did not use any prescription drugs, aspirin or nonsteroid anti-inflammatory drugs.

Table 1. Subject characteristics**.** Data are represented as mean ± SEM. NA, not applicable

	Fasted	Enriched	Control	*P* value between groups
Age (y)	24 ± 1	23 ± 1	25 ± 2	.30
BMI (kg/cm^2)	22.0 ± 0.7	23.0 ± 0.6	23.1 ± 0.9	.60
BMR (kcal)	2822 ± 44	2845 ± 79	3020 ± 137	.22
Rate of infusion (kcal/min)	NA	2.0 ± 0.1	2.1 ± 0.1	.31

Experimental human endotoxemia

Subjects were prehydrated with 1.5 L glucose 2.5%/NaCl 0.45% after which they received an intravenous bolus of 2 ng/kg body weight U.S. reference *E. coli* endotoxin (*Escherichia coli* O:113, Clinical Center Reference Endotoxin, National Institute of Health, Bethesda, MD) administered in one minute [(22)]. Blood was drawn before the start of postpyloric feeding and serially thereafter up to 24 hours after LPS administration (Figure 1). Routine hematology parameters were determined using flow cytometry (Sysmex XE-2100; Goffin Meyvis, Etten-Leur, the Netherlands).

Postpyloric feeding

On the experimental day, two groups received a nutritional intervention in a double-blind randomized fashion, while one group was fasted during the entire experiment (all groups: n = 6). The nutritional intervention groups received continuous postpyloric infusion of a liquid, enriched or an isocaloric control enteral nutrition via a self-advancing nasal-jejunal feeding tube (Tiger 2, Cook Medical, Bloomington, IN, Figure 1), which was placed on the evening before the experiment. The rate of feeding for each subject was based on their individual basal metabolic rate (BMR) multiplied by their activity level (1.55 times for all subjects) using the Harris-Benedict equation (Table 1).

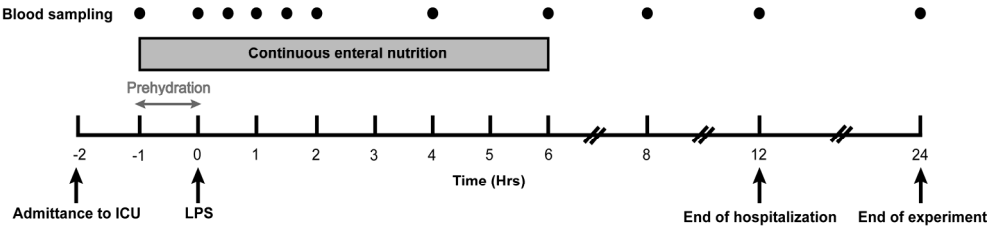

Figure 1: Experimental design. Subjects are admitted to the intensive care unit (ICU) after an overnight fast. One hour prior to LPS administration, subjects are prehydrated and the continuous administration of enteral nutrition started in the nutritional intervention groups, lasting until six hours after LPS administration. Blood is withdrawn at indicated time points during the experiment. Subjects leave the hospital 12 hours after intravenous administration of LPS and return the day after for final blood sampling.

Feeding composition and CCK measurement

The enriched nutrition contained 44 energy percent (en%) fat, 25en% protein and 31en% carbohydrates. The fat fraction contained 10% phospholipids. The protein fraction consisted of intact casein, whey protein and soy protein hydrolysate. The control nutrition contained 20en% fat, 16en% protein and 64en% carbohydrates. Both the enriched and control nutrition provided 1 kcal/ml. Systemic CCK levels were determined in plasma using a CCK-radioimmunoassay (Eurodiagnostica, Malmö, Sweden).

Determination of plasma cytokines and sub-clinical intestinal damage

TNF-α, IL-6, IL-10, and IL-1 receptor antagonists (IL-1RA) were measured batchwise using a multiplex Luminex Assay according to the manufacturer's instructions (Millipore, Billerica, MA). Intestinal-fatty acid binding protein (i-FABP) was determined in plasma using an in-house developed ELISA.

Statistical analysis

All values are depicted as mean ± SEM. Two-way analysis of variance was used to detect differences between groups for serial data. Differences in serial data within groups were analyzed by one-way ANOVA with Bonferroni's post-hoc test. Data were excluded from the analysis after being identified as significant outlier using the Grubb's test (extreme studentized deviate method). Prism 5.02 for Windows (GraphPad Software Inc., San Diego, CA) was used for computations. A p-value less than 0.05 was considered statistically significant.

Results

Hematologic and clinical response

As summarized in Table 2, administration of endotoxin resulted in changes in hematologic and clinical parameters in the fasted and nutritional intervention groups.

In all groups, mean arterial blood pressure decreased from 90 minutes after LPS administration onwards ($p < 0.001$), while a compensatory rise in heart rate was observed ($p < 0.001$). Also, endotoxemia resulted in a rise in core body temperature ($p < 0.001$) and white blood cell count ($p < 0.001$) in both the fasted and nutritional intervention groups. The LPS-induced changes in hemodynamic parameters, body temperature and white blood cell count were not affected by enteral nutrition (Table 2).

Administration of endotoxin resulted in flu-like symptoms such as headache, nausea, shivering and myalgia, which were expressed as sickness score. The sickness score of all subjects peaked at 90 minutes following LPS administration. Administration of enriched or control nutrition did not affect the sickness score compared with fasted subjects ($p = 0.43$ and $p = 0.28$, *respectively*). In the enriched nutrition group 3 out of 6 subjects vomited compared with 2 out of 6 in the control nutrition group, resulting in a higher sickness score of enriched nutrition versus control nutrition ($p < 0.01$). None of the subjects in the fasted group vomited. In line, early administration of enteral nutrition has been reported to result in increased nausea and vomiting compared with fasting [9].

Table 2: Hemodynamic parameters, blood leukocyte count and sickness score during human endotoxemia

		T = 0	*T = 1*	*T = 2*	*T = 3*	*T= 4*	*T = 8*	*T = 24*	**p value between groups**
MAP, mm Hg	Fasted	96 ± 3	94 ± 5	85 ± 5	81 ± 4	81 ± 3	88 ± 3	ND	.22
	Enriched	104 ± 5	95 ± 4	91 ± 5	89 ± 3	83 ± 2	85 ± 1	ND	
	Control	100 ± 2	98 ± 3	94 ± 5	89 ± 3	82 ± 3	87 ± 2	ND	
HR, beats/min	Fasted	68 ± 2	77 ± 4	78 ± 4	87 ± 3	85 ± 4	79 ± 2	ND	.34
	Enriched	63 ± 3	66 ± 3	75 ± 3	91 ± 2	82 ± 3	77 ± 4	ND	
	Control	68 ± 6	81 ± 5	84 ± 6	90 ± 5	90 ± 6	79 ± 5	ND	
Temperature, ^{o}C	Fasted	36.7 ± 0.2	37.0 ± 0.2	38.0 ± 0.3	38.5 ± 0.4	38.3 ± 0.3	37.5 ± 0.1	ND	.81
	Enriched	36.7 ± 0.2	37.1 ± 0.2	37.7 ± 0.2	38.3 ± 0.3	38.1 ± 0.2	37.5 ± 0.1	ND	
	Control	36.7 ± 0.1	37.0 ± 0.1	38.0 ± 0.3	38.3 ± 0.2	38.3 ± 0.2	37.4 ± 0.2	ND	
Leukocytes, x10*9/L	Fasted	5.7 ± 1.2	3.1 ± 0.9	5.2 ± 0.7	ND	8.7 ± 0.2	11.7 ± 0.7	7.5 ± 0.9	.81
	Enriched	5.9 ± 0.6	2.7 ± 0.7	5.2 ± 0.4	ND	9.7 ± 0.8	12.2 ± 0.9	7.1 ± 0.5	
	Control	5.2 ± 0.4	3.1 ± 0.6	5.6 ± 0.6	ND	9.7 ± 0.9	13.2 ± 1.3	7.9 ± 0.6	
Sickness score	Fasted	0.3 ± 0.3	1.3 ± 0.8	2.3 ± 0.6	1.8 ± 0.6	0.5 ± 0.3	0.5 ± 0.3	ND	**.04**
	Enriched *b*	0 ± 0	1.7 ± 1.3	4.0 ± 1.4	3.2 ± 1.4	1.3 ± 0.6	0.7 ± 0.5	ND	
	Control	0.3 ± 0.3	3.5 ± 0.9	1.7 ± 0.3	1.7 ± 0.4	0.8 ± 0.6	0.3 ± 0.2	ND	

T, time expressed in hours after lipopolysaccharide administration; MAP, mean arterial pressure; HR, heart rate; ND, not determined. Data expressed as mean ± SEM. *p* values are comparisons between groups over time and were determined by two-way repeated measures-analyses of variance. ^{b} represents significant difference over time with control group.

Enteral feeding with enriched nutrition modulates the cytokine profile during experimental human endotoxemia

Intravenous administration of LPS resulted in a marked pro-inflammatory response. The TNF-α values of one subject in the enriched nutrition group were removed from the analysis after being identified as significant outlier. Treatment with enriched nutrition significantly attenuated TNF-α levels compared with fasted ($p < 0.0001$) and control nutrition ($p < 0.05$; Figure 2A). Enriched nutrition lowered peak TNF-α levels with 40 ± 8% compared with fasted subjects and 29 ± 10% compared to control nutrition.

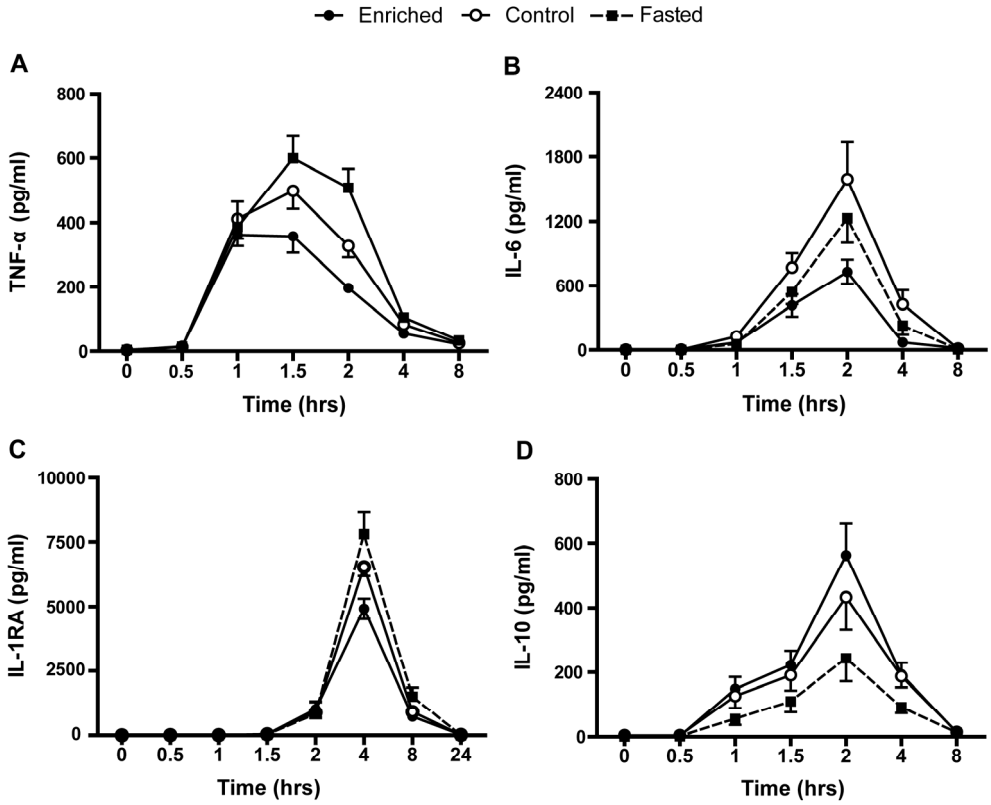

Figure 2: Enriched nutrition modulates the inflammatory response during endotoxemia. Mean plasma concentrations of TNF-α (A), IL-6 (B), IL-1RA (C) and IL-10 (D) following intravenous LPS administration. Enriched nutrition attenuates TNF-α, IL-6 and IL-1RA levels compared with control nutrition ($p < .05$) and fasting ($p < .0001$). Administration of the control product displays a trend towards lower TNF-α levels ($p = .06$). The enriched nutrition enhances IL-10 release ($p < .0001$ vs fasted), while a trend is observed with control nutrition ($p = .07$ vs fasted).

The control nutrition demonstrated a trend towards lower TNF-α plasma levels compared with fasted subjects ($p = 0.06$).

Enriched nutrition significantly reduced IL-6 plasma concentrations during the endotoxemia protocol compared with control nutrition ($p < 0.001$) and fasting ($p < 0.05$. Figure 2B), while the control nutrition did not affect IL-6 compared with fasted subjects ($p = 0.63$). Administration of enriched nutrition attenuated peak levels of IL-6 with 41 ± 9% compared to fasted subjects and 54 ± 7 % compared to control nutrition.

Intravenous injection of LPS is known to trigger a complex compensatory anti-inflammatory response. The specific IL-1 receptor antagonist, IL-1RA is released during inflammation and is thought to control the immune-modulatory effects of IL-1. Enriched nutrition decreased circulating IL-1RA during the experiment compared with control nutrition ($p < 0.0001$) and fasted ($p < 0.0001$; Figure 2C). Peak levels of IL-1RA were 37 ± 8% lower in the enriched nutrition group compared with fasted subjects and 25 ± 6% compared with control nutrition. The control nutrition did not affect IL-1 RA levels compared with fasted.

Continuous postpyloric infusion of enriched nutrition resulted in elevated plasma concentrations of IL-10 over time compared with fasted ($p < 0.0001$), while the control nutrition demonstrated a trend towards higher IL-10 levels ($p = 0.07$; Figure 2D). Enriched nutrition enhanced peak levels of IL-10 with 231 ± 19% compared with fasted subjects and 130 ± 12% with control nutrition.

Enterocyte damage remains unaffected by enteral nutrition

In all subjects, administration of LPS resulted in a gradual increase in i-FABP plasma levels until 4 hours post-LPS, representing the occurrence of enterocyte damage (Figure 3A). From 4 hours post-LPS to 8 hours, levels of i-FABP in all groups returned to baseline. Fasted subjects and subjects receiving control nutrition displayed a more prominent increase in i-FABP levels during the experiment compared with subjects fed with enriched nutrition. Total i-FABP release tended to be lower for the enriched nutrition group compared with control nutrition and fasted subject, although this did not reach statistical significance (Figure 3B).

Increased plasma CCK levels during administration of enteral nutrition

In order to assess the effect of continuous duodenal infusion on CCK release, plasma CCK levels were assessed on indicated time points (Figure 1). CCK levels increased from non-detectable values (< 0.3 pmol/l) before administration of enteral nutrition (t = -1 hrs) to 2.3 ± 0.5 pmol/l at 1 hour after onset of continuous administration of enriched and control nutrition (t= 0; Figure 4A).

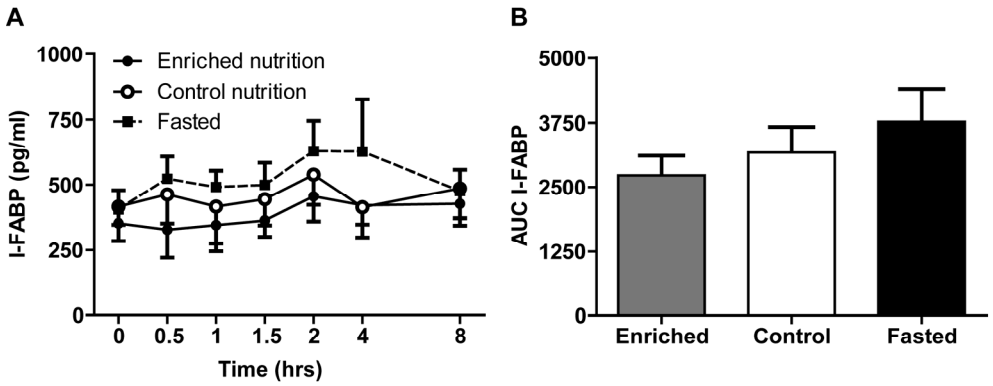

Figure 3: Administration of LPS results in sub-clinical intestinal damage. Intravenous administration of LPS results in a gradual increase of plasma i-FABP levels in all groups (A). Subjects fed enriched nutrition display a smaller increase in circulating i-FABP levels (A-B), although this does not reach statistical significance.

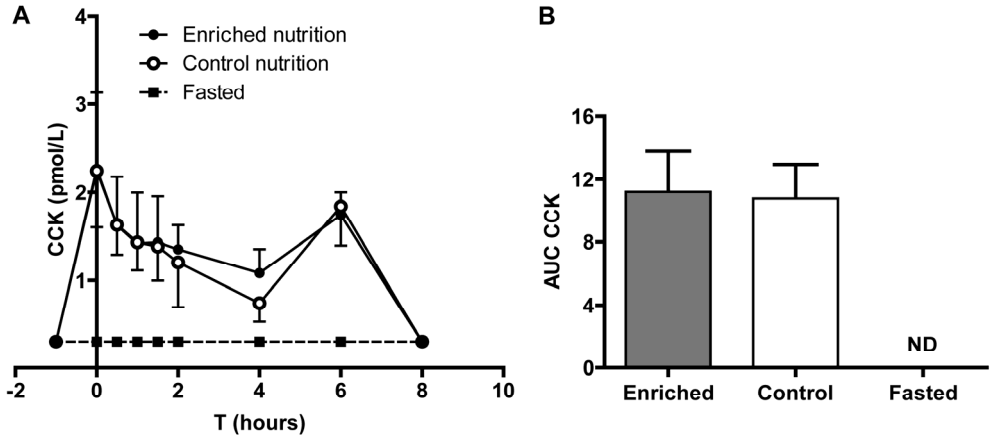

Figure 4: Postpyloric administration of control or enriched nutrition increases plasma levels of CCK. Continuous enteral administration of both the control and enriched nutrition results in an increase in CCK plasma levels compared with fasted subjects. Both nutritional interventions demonstrate a slight decrease in CCK plasma levels at 4 hours following LPS administration. After cessation of nutrient infusion, plasma CCK levels fall below detection level (A). There is no significant difference in total CCK release between control and enriched nutrition (B). ND, not detectable.

Four hours after intravenous LPS injection CCK plasma levels in the control group dropped (0.7 ± 0.2 pmol/l; $p < 0.05$) compared with the levels at 1 hour. The drop in CCK levels tended to be smaller in the enriched group. There were no significant differences in total plasma CCK release between enriched or control nutrition (Figure 4B). CCK levels dropped to none detectable levels at 8 hours after cessation of the nutrient infusion. In fasted subjects, plasma CCK levels were below detection level throughout the protocol.

Discussion

The present study is the first to investigate the immediate immunomodulatory effect of continuous enteral administration of nutrition enriched with lipids and protein in man. Our data reveal that enriched nutrition modulates the innate immune response during human endotoxemia, resulting in attenuated plasma levels of TNF-α, IL-6, IL-1RA and elevated circulating IL-10, indicating a clear shift from a pro-inflammatory to an anti-inflammatory phenotype.

During the last decades, the catabolic state of surgical and critically ill patients increasingly gained interest [23, 24]. The observed negative correlation between catabolism and clinical outcome resulted in more liberal nutritional regimes, such as reduced pre-operative fasting and early administration of enteral nutrition [24.] Implementation of these renewed nutritional support egimes reduced morbidity and length of hospital stay [9, 10]. Although the exact mechanisms behind these beneficial effects are not well known, it is assumed that adequate nutritional support prevents immunodeficiency induced by caloric deficits [25]. In addition to caloric support, prolonged ingestion of nutrition enriched with intrinsic anti-inflammatory compounds, such as long-chain n-3 polyunsaturated fatty acids and glutamine results in immune-modulating effects and improves outcome by influencing specific metabolic processes, including eicosanoid production, glutathione synthesis and generation of heat shock proteins [26, 27]. Our group has revealed a novel pathway in which enteral nutrition modulates the immune response via a fast hard-wired pathway. Enteral lipid-enriched nutrition was shown to limit inflammation and reduce organ damage via a CCK-mediated activation of the cholinergic anti-inflammatory pathway in several rodent models [16-19, 28]. Herein, we present the first evidence that this nutritional anti-inflammatory mechanism is also effective in man.

Virtually every surgical, trauma and ICU patient suffers from systemic inflammation. The complex interplay between pro- and anti-inflammatory mechanisms during such a systemic inflammatory response is still incompletely understood [29]. The human endotoxemia model does not replicate these clinical

conditions, but has been extensively employed to study the acute systemic inflammatory response *in vivo* [21]. Administration of endotoxin affects various systemic physiologic and metabolic processes in a manner similar to the early phase of injury and infection, making it a suitable human model for proof-of-principle studies [21].

Excess release of TNF-α is known to contribute to the development of systemic inflammatory response syndrome, organ damage and mortality in sepsis [30]. Furthermore, circulating levels of TNF-α and IL-6 are correlated with the severity of sepsis in patients [31]. In line with our animal data [16, 17], the current study demonstrates that enriched nutrition limits inflammation during human experimental endotoxemia by attenuating circulating levels of TNF-α and IL-6. Moreover, the intervention with enriched nutrition resulted in decreased IL-1RA plasma levels. These data are conform with previous reports, demonstrating that TNF-α and IL-6 enhance IL-1RA release during endotoxemia, while inhibition of these cytokines lowers circulating IL-1RA [32, 33]. In accordance, attenuation of the inflammatory response using epinephrine or glucocorticoids downregulates IL-1RA and IL-1 plasma levels [34, 35]. In parallel with these reports, our findings that enriched nutrition not only decreases plasma levels of TNF-α and IL6 but also of IL-1RA, reflect an overall reduced pro-inflammatory state. Interestingly, postpyloric administration of enriched nutrition amplified the anti-inflammatory response to endotoxin as evidenced by a pronounced increase in circulating IL-10 compared with fasted subjects. IL-10 is considered to be part of the host-protective mechanism that counterbalances the pro-inflammatory response during infection and inflammation [34]. Furthermore, administration of IL-10 has been shown to reduce endotoxin-induced lethality in mice [36]. The beneficial effect of enriched nutrition on the immune response during human endotoxemia is in line with previous studies using well-known pharmacological agents, such as epinephrine and glucocorticoids which inhibit plasma levels of pro-inflammatory cytokines and augment circulating IL-10 [34, 37]. Together, these data indicate that enriched enteral nutrition is a promising and physiological intervention to control acute inflammation.

Intestinal epithelial cell damage often accompanies sepsis, trauma and major surgery and is related to the degree of gastrointestinal hypoperfusion [38-40]. Additionally, intestinal compromise has been implicated in the development of inflammatory complications following injury [41]. Here, we show that intravenous administration of LPS resulted in increased i-FABP levels. The rise in plasma i-FABP levels tended to be smaller in subjects treated with enriched nutrition compared with control nutrition or fasted subjects, although this did not reach statistical significance. These data are supported by rodents studies demonstrating that lipid-enriched nutrition preserves intestinal integrity [17, 19]. The small increase in i-FABP

plasma levels is likely attributable to the relative low dose of LPS and limited hypoperfusion due to the prehydration protocol. Future studies are therefore needed to establish a gut-protective effect of enriched nutrition in man.

CCK-mediated activation of vagal afferents plays a dominant role in nutrient-induced digestive, metabolic and immunologic feedback [19, 42, 43]. Intestinal release of CCK is predominantly triggered by the luminal presence of lipid and protein [20, 44], while termination of nutrient exposure results in a rapid drop of CCK levels [44]. Taking these considerations into account, we chose to continuously administer nutrition enriched with lipids and proteins to induce a prolonged stimulation of the CCK-mediated anti-inflammatory pathway. Our nutritional intervention resulted in detectable circulating CCK levels during the entire endotoxin-induced inflammatory response. The fact that bolus administration of a lipid-rich milkshake containing 100 g fat prior to endotoxin challenge failed to affect the immune response [45], underlines the importance of continuous nutrient administration. In comparison, continuous infusion of enriched nutrition delivered approximately 60 g fat in total. Although enriched nutrition displayed a more powerful anti-inflammatory effect than control nutrition, significant differences in CCK levels could not be detected in plasma. This might be explained by the fact that circulating CCK levels do not reflect local intestinal concentrations and subsequent activation of afferent vagal fibers in the gut. In this context, it is interesting that plasma concentrations of exogenous CCK have to be at least 10-fold higher compared with postprandial CCK levels to obtain a similar satiety effect [46]. Future studies using specific CCK-1 receptor antagonists, which are currently not available, should specify the role of local CCK levels. In addition, these studies should also focus on the role of other intestinal peptides, including glucagon-like peptide 1 (GLP-1). Recently, our group implicated GLP-1 as co-stimulatory peptide of the nutritional anti-inflammatory pathway in rodents, since administration of GLP-1 receptor antagonists partially reduced the inhibitory effect of lipid-rich nutrition on systemic inflammation [47].

In conclusion, the current proof-of-principle study demonstrates for the first time that: 1) short-term continuous administration of enteral nutrition immediately modulates inflammation in humans, and 2) enrichment of the nutritional composition with lipid and protein enforces this anti-inflammatory potential. Our findings show that the anti-inflammatory effects of enriched nutrition as previously observed in rodents also apply to the human situation. Taken together, the current study implicates continuous and even per-operative administration of enriched nutrition as a promising intervention to modulate inflammatory conditions in the clinical setting.

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Chapter 5

General discussion and summary

The immune system is a complex interrelated construction, comprised of innate and adaptive immune responses that evolved over millions of years to protect the host against injury and infection. The innate immune system orchestrates an immediate but non-specific response, which precedes and activates the slower but highly specific adaptive immune response. These two components of the immune system overlap and supplement each other to initiate a multifaceted response to defend the host to virtually every pathogen [1]. Recently, the nervous system has been recognized as regulatory component of the immune system, fine-tuning the ongoing inflammatory reaction via selective and reversible hard-wired pathways [2].

While an adequate response of the immune system to infection and trauma is critical for survival, an excessive immunological reaction will result in tissue damage, organ failure and eventually even death. In a well-orchestrated immune response, pro- and anti-inflammatory reactions are simultaneously triggered in order to maintain immunological homeostasis. In the early phase, the pro-inflammatory state dominates to be followed by a balanced anti-inflammatory reaction in the later phase. An excessive immune response, however, is characterized by an initial hyperinflammatory reaction followed by an immunosuppressive response. Specifically surgical, trauma and ICU patients are prone to develop such an excessive immune response, which can result in high morbidity and mortality [3,4]. Mortality in the hyperinflammatory phase has been drastically reduced following new developments in intensive care medicine. Mortality rates in the late immunosuppressed phase however remain unaltered high, as patients succumb to opportunistic infections [5]. Current supportive interventions fall short in this phase since modification of the immune system to control the dysregulation of the immune response has appeared to be complicated [5]. Interventions directed at inhibiting single pro-inflammatory mediators demonstrated promising results to improve survival in the experimental setting. Unfortunately, these interventions have failed to improve outcome in the clinical setting.

Over the past years, our group has shown that lipid-enriched enteral nutrition attenuates systemic inflammation and reduces tissue damage in a hemorrhagic shock model in rats. In the current thesis, the mechanisms underlying nutritional activation of this anti-inflammatory pathway were investigated in rodents while the anti-inflammatory actions of a specifically developed enteral nutrition were studied in humans. To this extent, rodent models of hemorrhagic shock, endotoxemia and postoperative ileus were used to investigate activation of the nutritional anti-inflammatory mechanism (**first aim**) and the therapeutic window of enteral lipid-enriched nutrition (**second aim**). Subsequently, these experimental findings were extended to the human setting. Various nutritional compositions were investigated in healthy subjects to identify a suitable nutritional intervention. Finally, the

immune-modulating effects of the chosen enteral lipid- and protein-enriched nutrition were studied using an endotoxemia model in humans (**third aim**).

Previously, our group has reported that enteral lipid-enriched nutrition attenuates inflammation via cholecystokinin (CCK)-receptor mediated stimulation of the autonomic nervous system [6]. In this pathway, physiological activation of the efferent vagus nerve triggers release of acetylcholine that limits inflammation by binding to nicotinic receptors on inflammatory cells. The effector side of this pathway, termed the cholinergic anti-inflammatory pathway, was first described by the group of Dr Tracey, which demonstrated that electrical stimulation of the vagus nerve reduced inflammation via activation of the nicotinic acetylcholine receptor alpha-7 subunit [7,8]. Physiological stimulation of this pathway using enteral nutrition appears an appealing intervention to modulate inflammation in man.

Luminal nutrients can stimulate the autonomic nervous system via the circulation and the afferent vagus nerve [9].Therefore, the **first aim** of this thesis was to gain insight into processes that underlie nutritional activation of the autonomic nervous system leading to modulation of the immune response. In **chapter 2.1** we demonstrate that lipid-enriched nutrition attenuates inflammation in rats via activation of the afferent vagus nerve. To this end, afferent vagal fibers were selectively disrupted using perivagal application of capsaicin, while sparing efferent fibers [10]. Administration of lipid-enriched nutrition in rats with an functional afferent vagus nerve attenuated hemorrhagic shock-induced systemic inflammation and intestinal damage, whereas these beneficial effects of lipid-enriched nutrition were absent in deafferented rats. These findings indicated that the nutritional anti-inflammatory pathway is peripherally activated via afferent vagal fibers. Additionally, applying a peripherally acting CCK-1 receptor antagonist to non-deafferented rats blunted the beneficial effects induced by lipid-enriched nutrition, while bolus administration of PEGylated-CCK9, a compound known to selectively activate peripheral CCK-1 receptors, mimicked the anti-inflammatory actions of lipid-enriched nutrition. Together, these findings substantiate a peripheral CCK-mediated activation of the nutritional anti-inflammatory pathway. In conclusion, **chapter 2.1** reveals a previously unappreciated gut-brain-immune axis triggered by luminal nutrients. Discovery of this CCK/CCK-1 receptor mediated vagovagal anti-inflammatory reflex underlines the versatility of the vagally-mediated gut-brain axis to maintain homeostasis, as luminal nutrients also regulate digestive and metabolic responses via the same pathway [11-13].

Lipid-induced CCK release has been shown to be dependent on the intestinal processing of lipids into chylomicrons [14,15]. In addition, chylomicrons are known to attenuate gastric emptying via a CCK-1 receptor mediated duodenal afferent pathway [14,15]. In line with these data, **chapter 2.2** shows that lipid-enriched

nutrition needs to be processed into chylomicrons within the intestine in order to activate the nutritional anti-inflammatory pathway. Supplementation of lipid-enriched nutrition with Pluronic L-81, a compound that disrupts chylomicron formation without affecting intestinal lipid uptake [16], prevented activation of mesenteric afferents in an *ex vivo* setting and inhibited the immune-modulatory actions of the nutritional vagovagal anti-inflammatory reflex in rodent models of hemorrhagic shock and endotoxemia. These findings are supported by the fact that pluronic L-81 prevents lipid-induced activation of the nucleus of the solitary tract, the primary relay centre for vagovagal information [17].

Next to CCK, several other intestinal peptides, including leptin, glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) are known to activate the vagus nerve in response to luminal nutrients [9,18]. Moreover, it has been suggested that administration of CCK-receptor antagonists lowers afferent vagal sensitivity to other intestinal peptides as well [19,20]. In addition, lipid-induced GLP-1 and PYY release was shown to be dependent on activation of CCK-receptors [21,22]. Therefore, involvement of other intestinal peptides in activation of the nutritional anti-inflammatory pathway was also investigated in **chapter 2.2**. Our data reveal that nutritional stimulation of GLP-1, but not PYY, receptors is involved in the nutrition-induced anti-inflammatory reflex and thus implicate GLP-1 as co-stimulatory peptide. These findings are in line with those of other investigators, who could not demonstrate PYY involvement in the lipid-induced delay of gastric emptying, which is known to be vagally mediated [23,24]. Consequently, provoking the release and receptor activation of not only CCK but also GLP-1 should be kept in mind when developing a nutritional anti-inflammatory intervention in man.

Additionally, by using leptin-deficient Ob/Ob mice we investigated a co-stimulatory role for leptin within the nutritional anti-inflammatory pathway (**chapter 2.2**)**.** Administration of a standard dose of lipid-enriched nutrition failed to attenuate systemic inflammation and intestinal damage in endotoxemic Ob/Ob mice. Interestingly, increasing the nutritional dose resulted in an effective inhibition of the endotoxin-induced inflammation and damage. As it has been shown that simultaneous administration of CCK and leptin synergistically reduces food intake and attenuates inflammation [18,25], our findings suggest a co-stimulatory role for leptin in the nutritional pathway. In order to substantiate these findings, we administered leptin receptor specific nanobodies that selectively antagonize leptin receptors to endotoxemic wild type mice. Interestingly, this pharmacologic inhibition of leptin receptors did not affect the immune-modulating actions of lipid-enriched nutrition. All together, these results suggest that leptin is not directly involved in the nutritional anti-inflammatory pathway but that Ob/Ob mice may have a higher stimulation threshold to activate afferent vagal fibers. This higher threshold can be the result of overfeeding, a situation that applies to Ob/Ob mice,

since prolonged ingestion of a high-caloric diet has been shown to desensitize afferent vagal fibers and lower lipid-induced activation of the nucleus of the solitary tract [11,26,27]. However, future studies are needed to elucidate the effect of long-term high-fat intake on activation of the vagovagal anti-inflammatory reflex.

The **second aim** of this thesis was to explore the therapeutic window of the nutritional anti-inflammatory reflex. In this section, we examined the anti-inflammatory effects of lipid-enriched nutrition in two different animal models that both reflect clinically relevant conditions. In **chapter 3.1**, the nutritional intervention was applied following hemorrhagic shock to mimic the clinical setting of trauma. In trauma patients, inflammatory complications are specifically difficult to treat as interventions can only be started after the inflammatory insult of trauma. In other words, the inflammatory response is already ongoing and tissue damage is already present at time of administering such intervention. The hyperinflammatory response that is frequently observed in this patient group likely results in systemic inflammatory response syndrome and sepsis. Both conditions are associated with a high morbidity and mortality [28,29]. Our study revealed that administration of lipid-enriched nutrition following hemorrhagic shock effectively attenuated inflammation and reduced intestinal damage and bacterial translocation via activation of CCK-receptors. Interestingly, lipid-enriched nutrition displayed an overall lowering effect on the inflammatory response by reducing circulating levels of pro- and anti-inflammatory mediators. The nutrition-induced observed reduction in intestinal damage is suggested to be essential for any anti-inflammatory intervention, as loss of intestinal integrity has been implicated in the development of various inflammatory complications, including respiratory dysfunction, organ damage and sepsis, in surgical, trauma and critically ill patients [30,31]. All together, our results implicate that early nutritional stimulation of the CCK-mediated vagovagal reflex could be a novel therapeutic approach to limit the hyperinflammatory response and tissue damage that unfold following trauma.

The second clinically relevant condition, in which the therapeutic window of the nutritional anti-inflammatory reflex was tested, is post-operative ileus. Postoperative ileus is a major complication in surgical clinics, leading to extended hospital stay and increased morbidity[32]. Abdominal interventions in particular are known to result in a protracted cessation of bowel movement due to the intestinal manipulation that occurs during these events. Indeed, such manipulation induces an inflammatory process in the intestinal muscle layers, thereby interfering with muscle contractibility and activating inhibitory neural reflexes resulting in a panenteric ileus [33-35]. Over the last years, strategies to minimize the occurrence of postoperative ileus have focussed on reducing surgical stress and promoting postoperative mobilization and recovery [36]. Experimental evidence, however, has

implicated anti-inflammatory treatments as a promising novel intervention to reduce postoperative ileus (see also **chapter 3.2** for review). Consequently, we examined the anti-inflammatory effects of lipid-enriched nutrition in **chapter 3.3** using a rat model of intestinal manipulation to induce postoperative ileus. Our intervention with enteral lipid-enriched nutrition effectively reduced the inflammatory phenomena that occur following intestinal manipulation, i.e. mast cell activation, activation of resident macrophages and influx of neutrophils into the intestinal muscular layers. In accordance, the manipulation-induced impairment of gastrointestinal transit was reduced by enteral lipid-enriched nutrition. Moreover, administration of CCK-receptor antagonists abrogated both the immune-modulating actions and the increase in intestinal transit triggered by lipid-enriched nutrition. These findings indicate that nutritional stimulation of the CCK-receptor mediated anti-inflammatory pathway is a promising intervention to prevent and/or treat postoperative ileus. Additionally, our data are in line with other studies showing that electric stimulation of the vagus nerve and pharmacologic stimulation of cholinergic receptors reduce postoperative ileus by inhibiting the intestinal inflammatory response [37,38].

The **third and final aim** of the current thesis was to extend our experimental rodent findings to humans. Crossing the gap between a nutritional intervention that modulates inflammation via activation of an anti-inflammatory vagovagal reflex in rodents and administrating enteral nutrition during surgical interventions to improve clinical outcome is a big leap. To this end, an experimental model of endotoxemia was used as proof-of-principle study to obtain human data regarding the enteral administration of specifically enriched nutrition. The human endotoxemia model is interesting to investigate the immunemodulatory effects of enteral nutrition for several reasons. Firstly, electrical stimulation of the vagus nerve and pharmacological stimulation of nicotinic receptors has been shown to reduce systemic inflammation and improve survival in a murine model of endotoxemia [7,39], indicating that the cholinergic pathway modulates inflammation in this inflammatory model. Secondly, the human endotoxemia model displays clinical relevance due to the fact that intravenous administration of endotoxin affects various systemic physiologic and metabolic processes in a manner similar to the early clinical phase of injury and infection [40]. Lastly, this model is useful as proof-of-principle study as it allows for testing of an experimental intervention in a rather homogenous group of subjects in highly standardized circumstances.

Before the nutritional intervention was applied to humans, the anti-inflammatory potential of lipid-enriched nutrition was verified in a murine endotoxemia model (**chapter 4.1**). In line, nutritional stimulation of the vagovagal anti-inflammatory reflex dose-dependently attenuated systemic inflammation

within this model. As increasing of the nutritional dose results in gastric distension and gastric distension has been shown to activate vagal afferents via CCK-1 receptors [41], involvement of this process in activation of the anti-inflammatory reflex was investigated by administering a non-caloric volume load in an equivalent dose as the nutritional compositions. Enteral administration of this non-caloric volume load prior to endotoxemia did not affect systemic inflammation, suggesting that activation of the vagovagal anti-inflammatory pathway is predominantly nutrient-based. Additionally, lipid-enriched nutrition was shown to reduce endotoxin-induced circulating TNF-α levels more effectively than low-lipid nutrition, indicating that enrichment of the nutritional composition with lipids augmented its anti-inflammatory potential. These results are in accordance with previous reports, describing a superior anti-inflammatory potential of lipid-enriched compared with low-lipid nutrition during hemorrhagic shock and postoperative ileus [42,43](see also **chapter 3.1** and **3.3**). Furthermore, lipid-enriched nutrition attenuated endotoxin-induced damage to intestinal epithelial cells and lowered hepatic and splenic inflammation, thereby substantiating the broad immune-modulating actions of the CCK-mediated anti-inflammatory reflex during an evolving systemic inflammatory response.

The experimental studies performed in this thesis indicate that that the nutritional anti-inflammatory reflex is principally triggered by intestinal release of CCK (**chapter 2 and 3**). In humans, the luminal presence of all macronutrients is known to result in CCK release, with protein and lipid as the most potent CCK-secretagogues [44,45]. Therefore, the nutritional intervention studies in **chapter 4.2** were initiated to select a nutritional composition, to be used as anti-inflammatory intervention in a human proof-of-principle study, based on their capacity to induce CCK release. As local CCK levels cannot be analyzed in the human setting until today, the CCK plasma response following ingestion of various nutritional compositions was determined as surrogate marker for intestinal CCK release. Ingestion of a commercially available lipid-rich nutrition, Diasip© and low-lipid nutrition, Respifor© displayed a similar CCK plasma response. These findings are of interest, as the compositions of these feeds are akin to the feeds used in the rodent studies and here the lipid-enriched nutrition displayed a significantly higher anti-inflammatory potential compared with the low-lipid nutrition [46-50]. Unfortunately, plasma levels of CCK were undetectable in these rodent studies (unpublished data). Remarkably, even ingestion of a custom-made lipid- and protein-enriched nutrition, composed of specific nutrients which are described to maximize intestinal CCK release [51-53], demonstrated similar plasma levels compared with a clinically used standard tube feed. In conclusion, the current study indicated that determination of the plasma CCK response was an inadequate tool to select a nutritional composition to be used as anti-inflammatory intervention. Moreover, it

should be mentioned that systemic levels of CCK do not reflect local intestinal levels and that other intestinal peptides, of which GLP-1 was recently identified, (**chapter 2.1**) are also involved in activation of the nutritional anti-inflammatory reflex.

In **chapter 4.2**, postpyloric administration of nutrition was implicated as an effective intervention to maintain a stable CCK stimulus. The performed studies demonstrated that doubling of the nutritional dose resulted in similar peak CCK levels following ingestion of a higher dose compared with a low dose, while the CCK plasma response was protracted. This can be explained by the phenomenon of gastric emptying. Gastric emptying, which is predominantly mediated via CCK, is the rate limiting step in nutrient transport from the stomach to the duodenum [54-56]. Therefore, increasing the intragastric nutritional volume will not necessarily enhance plasma CCK levels, but will lead to a prolonged CCK response. Postpyloric administration of nutrients bypasses gastric emptying and is known to warrant stable plasma CCK levels [45,57]. Taken together, a continuous postpyloric infusion of custom-made lipid- and protein-enriched nutrition was chosen as nutritional intervention for our human proof-of-principle study, while an isocaloric low-lipid and low-protein composition was assigned as control.

In the last step of extending the rodent data to the human population, the anti-inflammatory actions of postpyloric administration of enriched nutrition were investigated during human endotoxemia (**chapter 4.3**). In this study, the enriched nutrition was continuously administered starting 1 hour prior to endotoxin administration and lasting until 6 hours afterwards. By implementing this nutritional intervention, the CCK-mediated anti-inflammatory pathway is continuously stimulated during the entire inflammatory response evoked by the intravenous endotoxin administration [58]. Additionally, plasma levels of GLP-1 are known to peak at 30 to 60 minutes following nutrient ingestion [21,59]. Therefore, the applied nutritional intervention will also trigger GLP-1 receptors, which contribute to the activation of the nutritional anti-inflammatory reflex, from the time of endotoxin administration onwards (**chapter 2.2**). Our human endotoxemia study showed that continuous administration of the enriched nutrition reduced plasma levels of TNF-α, IL-6 and IL-1 receptor antagonist over the course of the endotoxin-induced inflammatory response compared to the isocaloric control nutrition or the fasted control group. Additionally, enriched nutrition augmented circulating levels of the anti-inflammatory cytokine, IL-10 compared with the fasted subjects. These data signify that the application of an enteral enriched nutrition during human endotoxemia leads to a clear shift in the inflammatory balance from a pro- to a more anti-inflammatory state. Moreover, the enriched nutrition tended to lower endotoxin-induced enterocyte damage. Taken together, our findings indicate that continuous administration of enriched nutrition is a promising intervention to

modulate the immune response in the early course of systemic inflammation in humans.

Implications and future perspectives

For decades or even centuries, food intake and the intestinal processes leading to the absorption of nutrients have simply been regarded as a way to acquire building stones and energy to fuel cellular metabolism. The concept that enteral nutrients are more than simple calories is increasingly recognized. Nowadays, it is known that intestinal nutrients regulate digestive, metabolic and behavioural processes via humoral and neural pathways. Moreover, it has recently been shown that prolonged supplementation of our enteral diet with omega-3 fatty acids or glutamine modulates the inflammatory response via various metabolic processes.

The studies described in this thesis reveal that enteral lipid-enriched nutrition activates a hard-wired gut-brain-immune axis that attenuates inflammation and reduces tissue damage in rodents. We hypothesize that this potent endogenous anti-inflammatory pathway has evolved as a highly effective intestinal feedback mechanism to maintain intestinal barrier function and homeostasis in the constant face of threat. After all, absorption of luminal nutrients inevitably exposes the host to antigenic and possible toxic components that are able to trigger the immune system [60,61]. Even mild activation of the immune response by our diet is an energy consuming process and could result in the formation of immune complexes directed against essential nutrients. The balance between energy uptake and energy expenditure would become unfavorable when ingestion of nutrients results in manifest inflammation, whereas development of an immunological memory to nutrients would result in an inability to absorb nutrients from the intestine. Simultaneous activation of a hard-wired anti-inflammatory pathway together with intestinal nutrient absorption would prevent energy loss while maintaining the capacity of nutrient absorption by reducing such an unwanted inflammatory response. Under physiologic conditions, this nutritional anti-inflammatory reflex most likely represents an important mechanism to regulate low-grade nutrient-induced inflammation. Under non-physiologic conditions, such as surgery, trauma and infection however, well-timed and nutritional stimulation of this pathway seems a promising anti-inflammatory intervention. In these conditions, fasting has been demonstrated to be detrimental for immune competence, while enteral administration of nutrients prevents this inability to respond to inflammation via metabolic processes. Next to nutrient supply, the current thesis describes that enteral administration of specifically enriched nutrition activates a potent endogenous hard-wired pathway to modulate immune responses.

Above all, the current thesis extends the promising experimental data, obtained in animal models, to the human setting. The finding that enteral administration of enriched nutrition effectively attenuated innate immune responses during experimental endotoxemia in man provides justification to study the anti-inflammatory effect of this nutritional intervention in the clinical setting.

From a scientific point of view, additional studies should affirm whether the anti-inflammatory effects of enriched nutrition in the human setting are dependent on the release of CCK and/or other intestinal peptides and their subsequent activation of the vagus nerve. As our nutrition was designed to maximally induce CCK release and our data point towards activation of a rapid acting mechanism, it is tempting to speculate that intestinal release of CCK and subsequent activation of the vagovagal reflex are indeed responsible for the observed effects. However, drawing this conclusion would be moving to far in front of the actual data.

From a clinical point of view, future studies, investigating the effect of the nutritional intervention on clinical outcome, are required before nutritional stimulation of the vagal anti-inflammatory pathway can be successfully added to the armamentarium of clinicians. Based on the experiments described in the current thesis, a nutritional intervention study in surgical patients is proposed. Performing such a clinical trail in surgical patients has several benefits. Firstly, surgical procedures are scheduled in advance and are more or less standardized, thereby minimizing inter-individual differences, aiding experimental logistics and promoting patient inclusion. Additionally, based on the fact that early modulation of an unfolding immune response is preferable over treatment of its advanced counterpart, the nutritional intervention can be started prior to the inflammatory response, being the surgical procedure. Administration of enteral nutrition during surgical procedures also poses some difficulties, however, as surgical patients are deprived of enteral nutrition prior to surgery to prevent aspiration and it would thus require a change of well established clinical practice. Postpyloric administration of nutritional compositions would overcome this problem, as it bypasses the stomach and effectively activates the nutritional anti-inflammatory pathway in the proximal intestine. Although no adverse events were reported during the proof-of-principle study, a well-designed safety study should point out whether enriched nutrition can be administered postpylorically during surgical interventions.

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Nederlandse samenvatting

Het immuunsysteem is een complex mechanisme, dat bestaat uit een aangeboren en een verworven deel. Beide delen werken op ingenieuze wijze samen om het binnendringen van virussen en bacteriën in het lichaam tegen te gaan en binnen gedrongen pathogenen te verwijderen. Tevens ruimen zij beschadigde weefsels op, zodat er een milieu voor weefselherstel gecreëerd wordt. Recent is ontdekt dat het immuunsysteem onder controle van het zenuwstelsel staat.

Een goed functionerende afweerrespons is essentieel voor overleving. Echter, een ontregelde ontstekingsrespons kan de overleving juist negatief beïnvloeden. Een dergelijke reactie kan namelijk weefselschade, orgaanfalen en uiteindelijk de dood tot gevolg hebben. In een gecontroleerde afweerreactie wordt een pro-inflammatoire respons gelijktijdig met een anti-inflammatoire respons geactiveerd. In de vroege fase van een afweerreactie heeft de pro-inflammatoire respons de overhand, terwijl de anti-inflammatoire respons later de overhand krijgt. In het geval van een ontregelde afweerreactie wordt de vroege fase juist gekenmerkt door hyperinflammatie en de late fase door immuunsuppressie. Een dergelijk ontregelde afweerreactie, welke vaak optreedt bij chirurgische, trauma en intensive care patiënten, resulteert in een hoge morbiditeit en mortaliteit. Recente ontwikkelingen in de intensive care hebben ertoe geleid dat de mortaliteit in de vroege fase de laatste jaren sterk is afgenomen. De mortaliteit in de late, immuunsuppressieve, fase is echter onveranderd hoog gebleven. De meeste patiënten overlijden aan opportunistische infecties in deze fase, omdat in deze situatie de afweerreactie erg lastig te beïnvloeden is en de huidige ondersteunende interventies ontoereikend zijn. In de experimentele setting zijn veelbelovende resultaten behaald om de overleving ten tijde van een ontregelde afweerreactie te verbeteren. Deze interventies, welke veelal gericht waren op het selectief blokkeren van pro-inflammatoire mediatoren, hebben echter tot dusver nog geen positief effect gehad in de klinische setting.

In de afgelopen jaren heeft onze groep in proefdieren laten zien dat het enteraal toedienen van vetrijke voeding de systemische inflammatie en het optreden van weefselschade remt via activatie van het autonome zenuwstelsel. In het huidige proefschrift worden de mechanismen, die verantwoordelijk zijn voor de activatie van het anti-inflammatoire mechanisme door voeding onderzocht. Hiertoe werd in proefdiermodellen voor hemorrhagische shock, postoperatieve ileus en endotoxemie het ontstekingsremmende mechanisme (**eerste doel**) en de therapeutische breedte van enterale toediening van vetrijke voeding onderzocht (**tweede doel**). Vervolgens werden deze experimentele bevindingen vertaald naar de humane situatie, waarin we de immuunmodulerende effecten van een enterale vet- en eiwitverrijkte voeding in een humaan endotoxine model hebben bestudeerd (**derde doel**).

In het eerste deel van het proefschrift laten we zien dat enterale toediening van vetrijke voeding de ontstekingsreactie remt via cholecystokinine (CCK)-CCK-1 receptor gemedieerde activatie van de afferente vezels van de nervus vagus (**Hoofdstuk 2.1**). Hiertoe werden de afferente vezels van de nervus vagus in ratten kapot gemaakt middels het stofje capsaicine. Op de zenuwuiteinden van deze vezels zijn CCK-1 receptoren gelokaliseerd. Toediening van vetrijke voeding in deze ratten bleek de ontstekingsrespons niet meer te kunnen remmen en weefselschade kon niet voorkomen worden. Anderzijds bootste toediening van gepegyleerd CCK9, een stof die selectief perifere CCK-1 receptoren activeert, in gevaste ratten juist de beschermende effecten van vetrijke voeding na. Samenvattend onthullen de experimenten in **hoofdstuk 2.1** een voorheen onbekende darm-hersen-immuun as, welke geactiveerd wordt door luminale nutriënten.

Hoofdstuk 2.2 gaat dieper in op de intestinale processen, die leiden tot activatie van het nutritionele anti-inflammatoire mechanisme. Hierbij laten we zien dat chylomicron formatie een belangrijke rol speelt. De rol van chylomicron formatie is getest door de formatie ervan te remmen middels toevoeging van Pluronic L-81 aan de vetrijke voeding. Deze toevoeging voorkwam activatie van mesenteriale afferente zenuwen en verhinderde de ontstekingsremmende werking van vetrijke voeding ten tijde van hemorrhagische shock in ratten en endotoxemie in muizen.

Naast CCK zijn er ook andere darmhormonen, zoals leptine, glucagon-like peptide-1 (GLP-1) en peptide YY (PYY), die in staat zijn om vagale afferenten te activeren na voedselinname. Onze data laten zien dat het blokkeren van de GLP-1 receptoren, maar niet van PYY of leptine de ontstekingsremmende effecten van vetrijke voeding gedeeltelijk remmen. Deze resultaten impliceren dat afgifte van GLP-1 een co-stimulatoire rol in de anti-inflammatoire reflex speelt. Bij de ontwikkeling van een nutritionele interventie in de mens moet er dan ook aan gedacht worden dat niet alleen de afgifte van CCK, maar ook de afgifte van GLP-1 gestimuleerd wordt.

Een interessante bevinding van deze studie was dat in leptine-deficiënte muizen toediening van een standaard dosis vetrijke voeding niet in staat was om de endotoxine-geïnduceerde ontstekingsreactie en darmschade te remmen. Echter, het verhogen van de toegediende dosis resulteerde in een effectieve remming van zowel de ontsteking als de darmschade in deze dieren. Deze gegevens laten zien dat leptine niet direct in het mechanisme betrokken is, maar impliceren mogelijk dat de nervus vagus in Ob/Ob muizen een hogere stimulatie drempel heeft. Deze hogere stimulatie drempel zou veroorzaakt kunnen worden door overvoeding, een bekend fenomeen in Ob/Ob muizen, waarvan bekend is dat het resulteert in een gedesensitizeerde vagus. Toekomstige studies zullen het effect van een langdurige

vetinname op de activatie van de vagovagale anti-inflammatoire reflex moeten uitwijzen.

In het **tweede deel** van het proefschrift werd de therapeutische breedte van het nutritionele anti-inflammatoire mechanisme onderzocht. In dit gedeelte bestudeerden we de ontstekingsremmende werking van vetrijke voeding in twee verschillende proefdiermodellen, die overeenkomsten vertonen met klinisch relevante ziektebeelden. In **hoofdstuk 3.1** werd de vetrijke voeding toegediend na hemorrhagische shock om de complexe immunologische situatie van trauma patiënten na te bootsen. Bij dergelijke patiënten kan een behandeling pas na het trauma gestart worden. Onze studie heeft laten zien dat het toedienen van een vetrijke voeding na een verbloedingsshock de ontstane ontstekingsreactie en darmschade reduceert middels activatie van CCK-receptoren. De bevindingen van hoofdstuk 3.1 geven aan dat stimulatie van de CCK-gemedieerde vagvagale reflex met voeding in een trauma setting een nieuwe interventie kan zijn om de reeds geactiveerde ontstekingsrespons te remmen en daarmee geassocieerde weefselschade te voorkomen.

De werkzaamheid van vetrijke voeding werd ook getest in een setting van postoperatieve ileus, een veelvoorkomende chirurgische complicatie. Gedurende de laatste jaren is er veel onderzoek gedaan naar ontstekingsremmende middelen om postoperatieve ileus en de daarmee gepaard gaande morbiditeit en mortaliteit te verminderen (zie ook **hoofdstuk 3.2** voor een review). In **hoofdstuk 3.3** wordt aangetoond dat toediening van vetrijke voeding de activatie van mestcellen en residente macrofagen remt. Tevens voorkomt de voeding het binnendringen van neutrofielen in de spierlagen van de darmen. De toediening van voeding remde niet alleen de ontstekingsreactie in de darm, maar zorgde er ook voor dat de motiliteit van de darm gestimuleerd werd. Deze positieve resultaten werden teniet gedaan door gelijktijdige toediening van een CCK-receptor antagonist. Samenvattend laten deze data zien dat vetrijke voeding via een CCK-receptor gemedieerd mechanisme postoperatieve ileus remt.

Het **derde en laatste deel** van dit proefschrift is erop gericht om de vertaalslag van dierexperimenteel onderzoek naar de mens te maken. Hiertoe werd de ontstekingsremmende werking van specifiek verrijkte voeding in een humaan model van experimentele endotoxemie onderzocht. Aangezien endotoxemie tot een ander type ontstekingsrespons leidt dan een verbloedingsshock of postoperatieve ileus, werd eerst in een muis endotoxine model onderzocht of enterale vetrijke voeding ontsteking remt en orgaanschade kan verminderen. In **hoofdstuk 4.1** wordt duidelijk dat vetrijke voeding in endotoxemische muizen systemische inflammatie remt op een dosis-afhankelijke manier. Tevens laat dit

hoofdstuk zien dat het oprekken van de maag door voeding, wat zorgt voor activatie van afferente vezels van de nervus vagus, niet betrokken is bij het geobserveerde ontstekingsremmende effect. Deze resultaten onderstrepen onze eerdere bevindingen dat stimulatie van de ontstekingsremmende vagovagale reflex voedingsgemedieerd is. Ook in dit model is de werking van vetrijke voeding aanzienlijk krachtiger dan de werking van vetarme voeding. Toediening van vetrijke voeding remde tegelijkertijd de endotoxine-geinduceerde schade aan darmepitheel en de ontstekingsreactie in de lever en milt. Met deze studie werd aangetoond dat de CCK-gemedieerde anti-inflammatoire reflex een belangrijke immuunmodulerende werking heeft tijdens een ontwikkelende systemische inflammatoire respons. Tevens impliceren deze resultaten dat er een positief effect te verwachten is van enterale voeding in een humane endotoxine studie.

In **hoofdstuk 4.2** wordt een voedingsstudie beschreven die tot doel had om een optimale voedingscompositie te identificeren voor het gebruik als anti-inflammatoire interventie in een humane studie. Omdat lokale intestinale afgifte van CCK nog niet meetbaar is in de mens, werd de CCK plasma concentratie gebruikt om een inschatting te maken van de mate waarin een specifieke voeding de anti-inflammatoire reflex stimuleert. De inname van een commercieel beschikbare hoog-vet voeding (Diasip©) en een laag-vet voeding (Respifor©) resulteerde in een vergelijkbare plasma CCK respons. Deze bevinding is opvallend aangezien de macronutriënten samenstelling van beide voedingen vergelijkbaar is met de eerder geteste proefdiervoedingen, waarbij de vetrijke voeding een significant sterker anti-inflammatoir effect liet zien dan vet-arme voeding. Deze humane studie toonde verder aan dat een experimentele voeding, specifiek samengesteld om zoveel mogelijk CCK afgifte te bewerkstellingen, een vergelijkbare CCK plasma respons liet zien ten opzichte van een standaard sondevoeding. Concluderend kan gezegd worden dat het systemisch meten van CCK geen juiste benadering is om een voeding te selecteren voor een humane anti-inflammatoire interventie. Hieraan dient toegevoegd te worden dat plasma CCK waarden geen accurate weerspiegeling zijn van de lokale CCK waarden in de darm en rondom de afferente vagus. Tevens spelen er mogelijk meerdere darmhormoneneen rol in de activatie van de vagovagale reflex, zoals recent aangetoond werd voor GLP-1 (**hoofdstuk 2.1**).

Het toedienen van een bolus voeding via de maag, zoals tijdens deze studie resulteerde in een duidelijke piek in de plasma CCK waarden gevolgd door een graduele afname. Deze karakteristieke respons kan worden toegeschreven aan de maaglediging. Om een continue en stabiele afgifte van CCK te krijgen zou derhalve continue en postpylorisch gevoed moeten worden. Samenvattend werd op basis van deze studie gekozen voor een lipide- en eiwit-verrijkte voeding, die op

theoretische gronden de meeste CCK afgifte in de mens geeft. Daarnaast is gekozen voor een isocalorische laag-lipide en –eiwit als controle voeding.

Hoofdstuk 4.3 laat zien dat het continue postpylorisch toedienen van een lipide- en eiwitverrijkte voeding aan proefpersonen de endotoxine-geïnduceerde plasma waarden van TNF-alfa, IL-6 en IL-1 receptor antagonist verlaagd ten opzichte van de gevaste groep en de groep, die de controle voeding kreeg. Daarnaast toont deze studie dat de compensatoire afgifte van IL-10 zeer sterk gestimuleerd werd door de lipide- en eiwit-verrijkte voeding in vergelijking met de gevaste groep en de groep met de controle voeding. Ook was de darmschade, gemeten als plasma waarden intestinal fatty acid binding protein, lager in de lipide- en eiwit-verrijkte voeding groep. Deze data geven impliceren dat het enteraal toedienen van een lipide- en eiwit-verrijkte voeding ten tijde van endotoxemie in de mens een duidelijke verandering veroorzaakt in de ontstekingsrespons. Deze verandering wordt gekenmerkt door een vermindering van de pro-inflammatoire reactie, een bevordering van de anti-inflammatoire reactie en een remming van darmschade tot gevolg. Concluderend kan gesteld worden dat het enteraal toedienen van specifiek verrijkte voeding een veelbelovende interventie is om de ontstekingsreactie in de vroege fase van systemische inflammatie in de mens te beïnvloeden.

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Na vier jaar onderzoek met pieken afgewisseld met een dal hier en daar, komt er "ineens" dat moment dat je de laatste hand aan je proefschrift legt. Een fantastisch gevoel en tevens een mooi moment om terug te blikken op een bijzondere periode van persoonlijke groei en de fijne samenwerking met veel gemotiveerde, inspirerende en bevlogen personen door wie dit proefschrift mede gevormd is tot wat hier nu ligt.

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Vanaf 1 januari 2011 begon mijn opleiding tot algemeen chirurg in het Maxima Medisch Centrum te Veldhoven onder leiding van Dr. W.J. Prakken en Dr. R.M.H.

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Full Papers:

T Lubbers, J de Haan, Y Zhang, D Grundy, JW Greve, WA Buurman. Activation of the CCK-mediated nutritional anti-inflammatory pathway: involvement of chylomicron formation and intestinal peptides. Journal of Nutritional Biochemistry. Epub 2011

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EM Lemmens, T Lubbers, EA Beuls, G Hoogland. Gender differences in febrile seizure-induced proliferation and survival in the rat dentate gyrus. Eplilepsia 2005;46:1603–1612

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Submitted papers or papers in preparation:

T Lubbers, M Kox, J de Haan, JW Greve, JC Pompe, BP Ramakers, P Pickkers, WA Buurman. Lipid- and Protein-Enriched Enteral Nutrition Limits Inflammation in a Human Endotoxemia Model. Submitted

J de Haan, M Hadfoune, T Lubbers, B Winkens, I Verbaeys, M Luyer, W Buurman, J Greve. Lipid-Enriched Enteral Nutrition Controls Mast Cell Activation via the Vagal Anti-Inflammatory Pathway. Submitted

E Pastille, J de Haan, F Wirsdoerfer, T Lubbers, J Greve, U Schade, W Buurman, S Flohe. Lipid-enriched enteral nutrition reduces immunosuppression in polymicrobial sepsis. Submitted

S Hanssen, M Oostendorp, T Lubbers, E Villamor, W Backes, C Peutz-Kootstra, W Buurman, M Jacobs. Inhaled NO Attenuates the Deleterious Effects of Cell-Free Hemoglobin on Microcirculatory Blood Flow and Injury in the Rat Kidney. Submitted

J de Haan, I Vermeulen-Windsant, T Lubbers, M Hadfoune, J Greve, M Jacobs, W Buurman. Lipid-enriched nutrition attenuates hemolysis-induced vasoconstriction and associated tissue damage. Submitted

T Lubbers, S Achterfeldt, J de Haan, I Verbaeys, J Greve, W Buurman. PEGylated-CCK9 dose-dependently reduces inflammation in gram-negative hyperinflammation. In preparation

Patents

WO2009099316: T Lubbers, H Bouritius, M Luyer, W Buurman, J Greve, Z Hofman. Use of lipid-enriched nutrition for the treatment of post-operative ileus. Based on chapter 3.2.

Filed patent: Z Hofman, M Klebach, T Lubbers, J de Haan, W Buurman, J Greve, A Vriesema. Food composition for intra-operative tube feeding. Based on chapter 4.3.

Grants and prizes

2011 Dutch Society for Parenteral and Enteral Nutrition; Best abstract award
2011 Travel grant; Nederlandse vereniging voor gastroenterologie
2009 Travel grant; Nederlandse vereniging voor gastroenterologie
2009 Travel grant; United European Gastroenterology Week, London
2008 Clinical research traineeship from the Netherlands Organisation of Health Research and Development
2008 Young investigator award; International Federation of Shock Societies
2008 Travel grant; United European Gastroenterology Week, Vienna

Curriculum Vitae

Tim Lubbers was born on the 22th of May 1979 in Heteren, the Netherlands. From 1991 to 1997, he attended high school (gymnasium) at the Lorentz College in Arnhem. After graduation, he moved to Maastricht in 1997 to study Health Sciences at the University of Maastricht with the specialization Biological Health Sciences. His MSc thesis entitled "Gender differences in febrile seizure-induced proliferation and survival of rat dentate gyrus neurons" was based on research conducted at the department of neurosurgery of the University Hospital Maastricht under the supervision of Dr. G. Hoogland. Starting in 1999, he commenced his medical education at the same university where he obtained his medical degree in 2005. Following medical school, he started as a PhD-student at the Department of General surgery under the supervision of Prof. Dr. W.A. Buurman and Prof. Dr. J.W.M. Greve. In 2008, he obtained a clinical research traineeship stipendium from the Netherlands Organisation of Health Research and Development (ZonMw). From July 2010 to January 2011, he worked as a surgical resident at the Atrium Medical Centre in Heerlen (Dr. R.J.T.J. Welten, Dr. M.N. Sosef). He started his surgical training in January 2011 at the Maxima Medical Centre in Veldhoven (Dr. W.J. Prakken, Dr. R.M. Roumen), which is part of the educational region of the Academic Hospital Maastricht (Dr. L.P.S. Stassen, Prof. Dr. C.H.C. Dejong).