Nutrient-mucosal interactions in the human small intestine : the role of iron and lactoferrin

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Nutrient-mucosal interactions in the human small intestine

the role of iron and lactoferrin

The Graduate School

The studies presented in this thesis were performed at the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which participates in the graduate school VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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Ter verkrijging van de graad van doctor
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CHAPTER 1

GENERAL INTRODUCTION

Introduction

Iron and lactoferrin as model ingredients to study small intestinal functions

Structure and function of the small intestine

Small intestinal permeability

Intestinal oxidative stress

Iron-mediated gene expression

Iron, zinc and copper absorption routes

Lactoferrin

Aims of this thesis

Outline of the thesis

Introduction

The gastrointestinal (GI) tract is an important organ for many homeostatic processes in the body. It contains numerous pathogenic compounds from exogenous, dietary sources as well as those from endogenous origin, such as the intestinal microflora, which are potentially dangerous to the host. A healthy gut protects against detrimental effects of pathogenic compounds present in the intestinal lumen, and it allows digestion and absorption of micro- and macronutrients, which are essential for proper functioning and development of the host. The GI tract is composed of several functionally and anatomically distinctive sections. The mouth, oesophagus and stomach are important for food grinding and digestion. Furthermore, the low gastric pH provides a defence mechanism against (micro)organisms, and can stimulate or inhibit several pH-dependent processes. In addition to the upper GI tract, food digestion continues in the small intestine. Most energy, water and nutrient absorption takes place in this part of the GI tract. The colon is responsible for additional water absorption. Moreover, it contains large bacterial colonies, the intestinal microflora, which contribute to final food digestion.

Although the role of the small intestine in the pathogenesis of gastrointestinal diseases has drawn much attention over the years, the exact mechanism of action of many of the ongoing processes in the small intestine and their functional role in the aetiology of diseases remain to be elucidated. The literature provides surprisingly little information about physiological processes in the human *in vivo* situation. The vast majority of studies of small intestinal processes has been performed in cell lines, developed and cultured as a model for the human epithelial barrier. Despite the obvious advantage of convenient accessibility of cell lines to study processes, the *in vitro* situation often does not accurately resemble the human *in vivo* situation. The same problem arises when studying different animal species as a model for the human *in vivo* situation. The development of validated methodologies to study small intestinal physiology and biochemistry has been hampered by its anatomical position in the body. This often requires invasive techniques to study site-specific processes. The need to develop human models to study nutrient-mucosal interactions in the small intestine has initiated the variety of studies described in this thesis.

Iron and lactoferrin as model ingredients to study small intestinal functions

The study of the *in vivo* consequences of iron administration to humans is a central theme in this thesis. Iron is an essential micronutrient for many processes. Iron deficiency is one of the most abundant nutrient deficiencies, affecting approximately 30 % of all people worldwide (WHO, 1999). It can result from any condition in which dietary iron does not meet the body's demand. This is often observed in children and adolescents, and in people with increased iron losses, such as people suffering from various gastrointestinal diseases, wound bleeding or menstruating women (Andrews, 1999). Inadequate absorption of iron may result in iron deficiency. This is the case in people consuming iron deficient diets or diets with a low iron bioavailability. Also,

absorption interactions with other micronutrients or gastrointestinal disorders may inhibit iron bioavailability. Iron deficiency is the principal cause of iron deficiency anaemia. This disorder is associated with a decreased maximal workload, a decreased immune function and cognitive and physical impairment. It is commonly treated with oral ferrous iron containing supplements, generally prescribed because of their higher bioavailability compared to ferric iron supplements (Frewin, Henson & Provan, 1997). High intra-luminal amounts of ferrous iron may, however, induce damage to the intestinal wall, owing to its high toxicity. Hence, enough iron needs to be consumed to maintain or restore sufficient iron levels, but the potential iron toxicity limits the prescription of high dosages of iron.

In the studies described in this thesis, iron was administered to study small intestinal processes, function, stress and defence mechanisms *in vivo*, applying recently developed and novel models. Furthermore, the application of the milk protein lactoferrin to prevent damage to the small intestinal mucosa was investigated. Lactoferrin has been proposed as a functional food ingredient due to its antioxidative, antiviral, antimicrobial and anti-inflammatory properties.

Structure and function of the small intestine

The small intestine has an important function in many processes. It provides a physical barrier against luminal pathogens, mediates immune and endocrine processes, and is essential for proper food digestion and energy, water and nutrient absorption. Furthermore, several nutrients are transformed into biologically active or -inactive forms during small intestinal transit.

The small intestine comprises different compartments. The lumen, the mucosa including the brush border, the submucosa and a muscle layer, surrounded by the serosa, can be distinguished (Figure 1A). The lumen of the small intestine contains intestinal fluid. This intestinal fluid is a mixture of, among other things, electrolytes, mucins, enzymes, (bile) acids, glycoproteins, microorganisms and immunoglobulins (Lentner, 1981). The small intestinal mucosa comprises the brush border, covered with intestinal lining fluid, the epithelium and the lamina propria. The intestinal lining fluid is an unstirred layer of fluid. The brush border contains a number of digestive enzymes located on microvilli. These microvilli increase the luminal surface or absorptive area of the small intestine by a factor of about 20, and hence serve to optimize small intestinal absorption (Figure 1B). The epithelium is a single cell layer, linked together through tight junctions. It functions as a selectively permeable barrier capable of both secretion and absorption as required for absorption (Figure 2). The epithelium rests upon the lamina propria. This is a compact stroma, composed mainly of reticular- and elastin fibres, and provides support to the structure of the intestinal wall.

The underlying submucosa comprises a matrix of collagenous and elastic fibres in which larger blood vessels, lymph vessels and nerves ramify. The muscle layer, which covers the submucosa, contains a circular and a longitudinal muscle layer. Its combined actions are responsible for the propulsion of luminal contents (peristalsis). It is surrounded by the serosa, which permits the intestine to slide over adjacent organs during peristaltic movements (Magee & Dalley, 1986).

Figure 1. Anatomy of the small intestine. **A** Morphology of the intestinal wall. **B** Intestinal mucosa and submucosa. Adapted from Moog (1981).

Figure 2. Schematic representation of tight junctions, connecting two adjacent enterocytes. The microvilli are located on the apical side of the intestinal mucosa, directed towards the intestinal lumen. Actin filaments are represented as the small circles, myosin by the lines, and structural tight junction proteins by the large bold circles.

The intestine is innervated by the extrinsic and the intrinsic or enteric nervous system (ENS). Nerves from the extrinsic nervous system synapse with so-called ENS cells. The ENS has a complex structure and organization. The most prominent anatomically distinct networks are situated in the myenteric and submucosal plexuses. These plexuses consist of nerve cell bodies, axons, dendrites, and nerve endings, and can function autonomously to regulate a wide variety of intestinal processes (Johnson, 1991).

Luminal contents are potentially harmful to the intestinal mucosa. Damage to the small intestinal mucosa will impair the mucosal barrier, and may initiate considerable health problems, such as local intestinal inflammation, but also in systemic activation of inflammation or sepsis. The integrity of the mucosal barrier is reflected by small intestinal permeability. Since the intestinal integrity is an important end-point in intestinal stress studies, the mechanisms responsible for an impaired mucosal barrier function, with special emphasis on the detrimental effects of intestinal oxidative stress, will be explained in more detail in the next paragraph.

Small intestinal permeability

The physical construction of small intestinal mucosa creates the first line of defence against luminal pathogens. The epithelial layer of the small intestine mediates nutrient absorption, whereas it only allows passage of minute amounts of pathogenic and toxic molecules. Pathogens that do permeate the intestinal epithelium are potentially dangerous to the host. They may initiate immune activation, which aims to attack and eliminate the toxic compound. In healthy individuals, a low rate of intrusion of pathogens into the epithelium and a subsequent initiation of an immune response is a continuous process and does not harm the epithelial barrier. Damage to the intestinal mucosa however may disrupt the integrity of tight junctions. Tight junctions connect adjacent enterocytes, and normally allow permeation of molecules in a charge and size dependent manner (Figure 2). These tight junctions are formed by a multiprotein complex including occludin, claudin, several zona occludens proteins (ZO1, ZO2 and ZO3), actin, myosin, cingulin and 7H6 (Mitic & Anderson, 1998; Mitic, Van Itallie & Anderson, 2000; Nusrat, Turner & Madara, 2000). The transmembrane proteins occludin and claudin form fibrils that interact with fibrils from an adjacent cell to close the paracellular space between two enterocytes. These fibrils are linked to an intracellular cytoskeleton in the enterocytes. Tight junctions can be affected by several mechanisms. Structural tight junction proteins can be cleaved, the actin cytoskeleton or the peri-junctional actomyosin ring can be modified or even disrupted, and pathogen intrusion can trigger transmigration of polymorphonuclear leukocytes (PMNs) that may directly affect tight junctions. Furthermore, synthesis of tight junction-associated proteins may be modified, resulting in deformation of the tight junctions or the intracellular cytoskeleton (Berkes *et al.*, 2003; Nusrat *et al.*, 2001; Sears, 2000), and intracellular myosin light chain kinase (MLCK) may be phosphorylated by luminal contents. This stimulates cytoskeletal contraction of the actin cytoskeleton as well as of the peri-junctional actomyosin ring, and result in tension on the tight junctions and, therefore, increased permeability (Atisook, Carlson & Madara, 1990; Turner *et al.*, 1997), and is an important feature of the 'compromised gut'.

Invasion of pathogens triggers a complex activation of the immune system that is not yet fully elucidated. Initially, proinflammatory interleukins trigger interleukin-8 secretion on the basolateral membrane. This attracts circulating neutrophils to the apical surface of epithelial cells. Other specific signals are responsible for further neutrophil migration towards the pathogens, such as Pathogen Elicited Epithelial derived Chemoattractant (PEEC) (Berkes *et al.*, 2003; McCormick *et al.*, 1998). The resulting inflammatory response may cause disruption of the tight junctions and, consequently, induce an increase in intestinal permeability. This may result in further intrusion of enteric pathogens, such as lipopolysaccharide (LPS) and microbes, which initiates an additional response of the local intestinal immune system. This response involves PMNs translocation, activation of intraepithelial lymphocytes (IELs), which release, among others, the cytokines interleukin 4 and 10 (IL-4 and IL-10) and interferon- γ (IFN- γ), which were shown to be the major barrier-disruptive cytokines (Nusrat *et al.*, 2000). Synchronously, nuclear factor κ B (NF κ B) is released. NF κ B mediates most proinflammatory cytokines, chemokines, immune receptors, and cell surface adhesion molecules, and is considered to be a major regulator of intestinal inflammation (Berkes *et al.*, 2003). Although such inflammatory responses are necessary for a proper immune function, it also induces additional damage to the mucosal barrier. The cytokines may alter tight junctions through modulating the cytoskeleton activity (Perez, Barber & Ponz, 1997), which will aggravate the mucosal damage and progressively increase permeability. Progressive damage to the mucosa of the small intestine may result in bacterial translocation. This passage of gastrointestinal microflora across the lamina propria to local mesenteric nodes, and then to extranodal sites, initiates an additional cytokine response, a further invasion of pathogens and may eventually initiate a systemic inflammatory response or sepsis (Thomson *et al.*, 2000).

Damage to tight junctions in the small intestinal epithelium causes disruption of the integrity of the intestinal mucosa. This causes an increase in small intestinal permeability, which can be determined non-invasively by urinary sugar recovery tests. These tests are based on the comparison of intestinal permeation of molecules with different sizes. A typical measurement of small intestinal permeability comprises administration of inert sugar probes, such as the monosaccharide L-rhamnose and the disaccharide lactulose, and subsequent determination of the 5-h urinary lactulose/rhamnose excretion. This method has been validated previously (van Nieuwenhoven *et al.*, 1999) and is commonly applied in healthy volunteers, as well as in patients. It is regarded as a reliable parameter of the integrity of the small intestinal mucosa.

Increased permeability has been associated with several gastrointestinal disorders, such as Crohn's disease (Meddings, 1997), coeliac disease (Smecuol *et al.*, 1997), viral gastroenteritis (Isolauri *et al.*, 1989) and food allergy (Crowe & Perdue, 1992). The maintenance of the intestinal barrier function is of pivotal importance for a proper gut function and to avoid health problems.

Intestinal oxidative stress

Nutrients, drugs and other intra-luminal contents can induce several adverse effects in the small intestine. Dietary oxidants or substrates that cause production of reactive oxygen species (ROS) may induce oxidative damage to any component of the small

intestine, such as to the brush border, epithelial cells, enzymes or tight junctions. This implies a serious health threat, because intestinal oxidative stress is associated with the development of impaired barrier function, intestinal inflammation and even carcinogenesis. However, despite the common concern about possible detrimental effects of oxidative stress to the human small intestine, its causes and consequences remain to be elucidated.

A potential pro-oxidant is iron, which if ingested in large amounts, as occurs during iron supplementation, may result in oxidative stress in the small intestine. Iron supplements are commonly prescribed to treat iron deficiency anaemia. Typical oral iron repletion therapies consist of three daily dosages of iron supplements containing 65 mg iron each. In healthy people, approximately 15 % of all dietary iron is absorbed in the gastrointestinal tract, whereas about 1 % of the unabsorbed iron fraction remains in the intestine as free iron ions (Lund *et al.*, 1999). Free ferrous iron ions readily react with hydrogen peroxide, which is formed as a result of superoxide radical (O_2^-) production, a by-product of normal metabolism, to form ferric iron and highly reactive hydroxyl radicals (equation 1), a process denoted as Fenton chemistry. In the presence of O_2^- , ferric iron ions are reduced to ferrous iron with concomitant production of molecular oxygen (equation 2). The resultant of these reactions (equation 3) is known as the Haber-Weiss reaction (adapted from (Crichton, 2001)).

Fenton-mediated production of hydroxyl radicals is a continuous process in most living organisms. Hydroxyl radicals are extremely reactive, as is indicated by their very short half life (10^{-9} sec) (Sies, 1991), and can inflict damage to any biological molecule in its direct environment. The free electron is able to transfer from one molecule to another, thereby initiating a cascade of damaging reactions. ROS, such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, may inflict damage to lipids, proteins, carbohydrates and DNA, and hence may initiate or propagate carcinogenesis (Blakeborough, Owen & Bilton, 1989; Ray *et al.*, 2000; Simic, 1994). In homeostasis, continuous ROS production participates in several metabolic processes, such as apoptosis and immune responses. They do not induce damage to healthy tissue because ROS production is balanced by the antioxidant network. Antioxidants are able to scavenge or sequester free radicals, thereby inhibiting their harmful effects.

Excessive ROS production induces a disturbance in the pro- and antioxidant balance, and results in oxidative damage. This may occur in the presence of large amounts of free iron, which is the case during iron supplementation. Oral iron treatment is associated with considerable gastrointestinal side effects, such as nausea, vomiting, heart-burn, abdominal pain and constipation, whereas intravenous iron treatment does not induce GI discomfort (al-Momen *et al.*, 1996). Furthermore, previous investigations showed that oral iron supplements induced intestinal oxidative stress in rats (Srigiridhar & Nair, 1998; Srigiridhar *et al.*, 2001), aggravated lactic acid-induced damage to the barrier function of the small intestine epithelium (Gonzalez *et al.*, 1997), promoted colon carcinogenesis in mice (Siegers *et al.*, 1992) and induced inflammatory proc-

esses in rats with colitis (Aghdassi *et al.*, 2001; Reifen *et al.*, 2000). Epidemiological studies showed a role of dietary iron in the development of colon cancer (Wurzelmann *et al.*, 1996). Iron-induced oxidative stress in the intestine after oral ingestion of iron supplements may in part be responsible for these adverse effects.

Iron-mediated gene expression

As discussed above, iron administration has several functional consequences in the human small intestine. Iron-induced oxidative stress and its metabolic consequences mediate gene expression in epithelial cells. Recent studies showed that gene expression could be modified by dietary interventions (Davis & Johnson, 2001; Massimino *et al.*, 1998). Prior to the development of cDNA microarrays, gene expressions were determined by direct quantification of the amount of mRNA representing a specific gene in a sample. The application of genome-wide cDNA microarrays, developed in 1995 (Schena *et al.*, 1995), enables simultaneous monitoring of the expression of thousands of genes. This technique, commonly referred to as transcriptomics, provides unique insights into the many processes that, in a continuous interplay and under control of local gene expression, result in homeostasis or in a response to disturbances in the homeostatic equilibrium. Its greatest merit lies in explorative studies with no obvious empirical based hypothesis. In such studies, cDNA microarrays may be applied to obtain information about many processes and to design more specific studies. However, the large number of genes studied using genome wide microarrays is also the most critical drawback of the technique. The likelihood of false-positive results increases with the number of genes studied. This problem can partially be overcome by mathematical calculations, thereby limiting the sensitivity but increasing the specificity of the assays.

In combination with human intervention techniques, the application of genome wide cDNA microarrays in gastrointestinal research will help to unravel the complex and largely unidentified processes and mechanisms, which are involved in proper functioning of the gut. This information will also be useful in the design of studies to the pathogenesis, prevention and treatment of gastrointestinal diseases.

Iron, zinc and copper absorption routes

In addition to the capacity to catalyze ROS production, iron supplementation may exert other adverse effects in the small intestine. Iron administration is associated with a decreased absorption of other divalent metals, such as zinc and copper. After oral administration, intestinal iron is reduced by a ferric reductase and transported across the apical membrane of the small intestine by divalent metal transporter 1 (DMT1), a protein that also transports zinc and copper (Gunshin *et al.*, 1997). Competitive binding to DMT1 in the presence of iron may decrease zinc and copper absorption. Furthermore, dietary iron mediates DMT1 expression (Yeh *et al.*, 2000) through signalling iron levels in enterocytes (Frazer *et al.*, 2003). Hence, iron repletion may reduce zinc and copper transfer by down-regulating DMT1.

Studies to date are not conclusive about iron-zinc and iron-copper interactions. Some investigators showed competitive absorption between iron and zinc (Dawson, Albers & McGanity, 1989; Simmer & Thompson, 1985) and iron and copper (Burns &

Paterson, 1993; Yu *et al.*, 1995) respectively, whereas others did not observe such interactions (Davidsson *et al.*, 1995; Donangelo *et al.*, 2002; Sheldon *et al.*, 1985). Zinc deficiency is associated with impaired immune function, growth, appetite and wound healing and with mucosal dystrophy, whereas copper deficiency may cause microcytic hypochromic anaemia, neutropenia, impaired growth of neonates, arthritis, myocardial disease, low serum ceruloplasmin, low red blood cell superoxide dismutase and platelet cytochrome-c oxidase (Bonham *et al.*, 2002; Castillo-Duran & Uauy, 1988; Danks, 1988; Williams, 1983). For a well-balanced prescription of micronutrient supplements, it is important to fully elucidate the interactions between them.

Lactoferrin

Lactoferrin is a glycoprotein of the transferrin family. It is present in milk, saliva, tears, bile, blood plasma, mucosal and genital secretions (Britigan, Serody & Cohen, 1994; Levay & Viljoen, 1995) and released by neutrophil specific granules (Davidson & Lönnnerdal, 1989). It was suggested that lactoferrin increases iron absorption (Rosa & Trugo, 1994; Sanchez *et al.*, 1996), because it binds iron with high affinity (Anderson *et al.*, 1989; Lönnnerdal & Iyer, 1995), and specific lactoferrin receptors are present in the intestinal brush border (Davidson & Lönnnerdal, 1988; Kawakami & Lönnnerdal, 1991). However, others were not able to confirm this hypothesis (Davidsson *et al.*, 1994; Fairweather-Tait *et al.*, 1987). Several other biological effects, such as bactericidal (Bellamy *et al.*, 1992; Zagulski *et al.*, 1998), anti-inflammatory (Nuijens, van Berkel & Schanbacher, 1996), antiviral (Harmsen *et al.*, 1995; Ikeda *et al.*, 2000) and antioxidative (Baldwin, Jenny & Aisen, 1984; Britigan *et al.*, 1994) activities, have been attributed to lactoferrin. Its mechanisms of action are largely unknown, despite extensive research in this field. Human lactoferrin is slightly different from that of other species, especially with regard to the composition of the glycans. Bovine lactoferrin does not bind to the human lactoferrin receptor (Davidson & Lönnnerdal, 1986; Davidson & Lönnnerdal, 1988). The observation that lactoferrin is present in large quantities in colostrum and in smaller concentrations in normal milk suggests an important function in newborns. Furthermore, it was shown that oral lactoferrin administration suppresses the release of the pro-inflammatory cytokines interleukin-1, interleukin-6 and TNF α by activated monocytes and macrophages (Crouch, Slater & Fletcher, 1992; Mattisby-Baltzer *et al.*, 1996; Zimecki *et al.*, 1999; Zimecki *et al.*, 1998). Hence, lactoferrin is able to mediate immunological responses. Lactoferrin is a well-recognized antioxidant, owing to its strong iron chelating capacity. The iron ions bound to lactoferrin are not able to participate in Fenton chemistry. This diminishes the production of hydroxyl radicals and consequently attenuates oxidative stress (Britigan *et al.*, 1994). The capacity of lactoferrin to participate as an antioxidant in the presence of iron may provide a functional application of lactoferrin. It may prevent iron-induced oxidative damage in the small intestine, especially if large amounts of iron are present, as occurs during iron repletion therapies.

Aims of this thesis

This thesis presents and discusses seven studies related to the interactions between iron, as a specific micronutrient, in relation to the small intestinal mucosa *in vivo* in

humans as well as in relation to other micronutrients or bioactive compounds. The specific aims of these studies were:

- To investigate the effects of iron application on
 - small intestinal oxidative stress and mucosal integrity
 - bioavailability of the micronutrients zinc and copper
- To investigate whether synchronous lactoferrin administration as a bioactive compound attenuates acute gastroenteropathy, induced by indomethacin.

Outline of the thesis

Iron administration may cause intestinal oxidative damage. This may in turn increase small intestinal permeability. Chapter 2 describes the effects of a one-month iron supplementation study on small intestinal permeability in iron-deficient premenopausal women. In this study, after the four weeks of iron supplementation, indomethacin was administered to investigate the effects of iron supplementation in a situation of acute intestinal damage.

Due to the unavailability of a model to directly investigate oxidative stress in the human small intestine, an intestinal perfusion model was developed for this purpose. Chapter 3 describes the first study applying this newly developed model to quantify intestinal oxidative stress. In this experiment, the effects of intraduodenal iron administration on lipid peroxidation and on the antioxidant network were investigated. The results from this study were used to expand the model and design the study described in chapter 4. An intervention study in healthy volunteers was performed, applying the perfusion model in combination with flexible duodenoscopy to obtain mucosal samples. In this study, iron-induced oxidative stress by different dosages of iron was investigated. Furthermore, the chapter describes the effects of these iron interventions on the expression of all functionally mapped genes. Gene expression was measured with genome-wide cDNA microarrays.

In addition to affecting ROS production, iron may have other adverse effects in the small intestine. It may affect the bioavailability of other micronutrients. Chapter 5 describes a study of the interactions between iron, zinc and copper absorption in the human small intestine. Zinc absorption was calculated from urinary excretion of orally administered stable zinc isotopes in ileostomy patients. Copper absorption was determined by calculating the balance of stable copper isotopes administered orally and their excretion in ileostomy effluent.

Lactoferrin, a glycoprotein with strong iron-binding properties, may be applied as a functional ingredient of food supplements. Before studying its biological effects after oral administration, its metabolic fate needs to be investigated to obtain detailed information about its bioavailability. Chapter 6 describes a study to investigate lactoferrin digestion in the stomach in healthy volunteers. The data from this study justified the design of a study to the survival of lactoferrin after passage of the stomach and small intestine, which is described in chapter 7. This information about the bioavailability of orally administered lactoferrin in the colon is pivotal for the design of future studies to investigate the role of lactoferrin in that tissue.

The non-steroidal anti-inflammatory drug indomethacin causes a reproducible, acute enteropathy and a subsequent increase in intestinal permeability in the small intestine in healthy subjects, corresponding to that observed in gastrointestinal mucosal dis-

eases. The effects of lactoferrin administration in the stomach and small intestine after indomethacin administration were investigated applying the lactulose/rhamnose/sucrose gut permeability test. This method provides a commonly accepted model to determine integrity of the gastric and small intestinal mucosa. This study is described in chapter 8.

All studies are discussed separately and in relation to each other in chapter 9. The consequences of the findings of the different studies in relation to small intestinal health and future research are discussed.

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CHAPTER 2

MUCOSAL INTEGRITY OF THE SMALL INTESTINE IN WOMEN USING IRON SUPPLEMENTS

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Submitted

Abstract

Background Iron supplementation causes Fenton driven free radical production in the intestine. This may induce oxidative damage to the mucosa and consequently impair the integrity of the intestinal wall. This study aimed to investigate the effects of iron supplementation on the integrity of the small intestine in iron deficient women.

Design Thirty-one iron deficient but otherwise healthy women were randomly allocated to receive placebo (n=16) or 300 mg iron/d as ferrous fumarate (n=15) for 1 month. At baseline and after 28 days of supplementation, small intestinal permeability was determined applying the lactulose/rhamnose (L/R) gut permeability test. After 31 days of supplementation, 75 mg and 50 mg of indomethacin was administered 9 and 1 h prior to a third permeability experiment to investigate the effects of acute intestinal stress on small intestinal permeability after one month iron application.

Results Serum ferritin was significantly increased after 4 weeks of iron supplementation (24 ± 12 $\mu\text{g/L}$) compared to placebo (10 ± 7 $\mu\text{g/L}$). Urinary L/R ratios, as parameter of small intestinal permeability, did not differ between the groups receiving placebo or iron supplements in any of the experiments.

Conclusion Iron supplementation in iron deficient but otherwise healthy women does not impair the mucosal integrity of the small intestine, which suggests that the human small intestine has a powerful antioxidant defence mechanism against luminal oxidative stress.

Introduction

Iron deficiency is one of the most abundant nutrient deficiencies worldwide. As many as 14 % of all adult females suffer from iron deficiency in Europe and North America, and approximately 30% of all people worldwide (DeMaeyer & Adiels-Tegman, 1985). It causes iron deficiency anaemia owing to decreased erythropoiesis and microcytosis. Symptoms of iron deficiency anaemia are pallor, fatigue, poor exercise tolerance, and decreased exercise tolerance (Andrews, 1999). It is commonly treated with prescribed or over-the-counter oral iron supplements, which often contain divalent ferrous salts because of their higher bioavailability compared to the trivalent ferric salts. Iron supplementation may comprise up to 195 mg of elemental iron/d divided over three single dosages as ferrous sulfate, fumarate or gluconate respectively (Frewin, Henson & Provan, 1997). However, free ferrous iron participates in Fenton chemistry, which results in instantaneous production of highly reactive hydroxyl radicals (Fodor & Marx, 1988; Srigiridhar & Nair, 1998). These hydroxyl radicals can induce damage to biological molecules, such as lipids, proteins, carbohydrates and DNA. In normal homeostasis, hydroxyl radicals and other reactive oxygen species (ROS) are balanced by the antioxidant network. Increased ROS production may, however, exceed the capacity of the antioxidant network to trap or neutralize ROS and, hence, result in oxidative damage. Previous studies showed that increased ROS production, caused by iron repletion in iron deficient rats, promoted oxidative stress at the site of iron absorption (Srigiridhar & Nair, 1998). Iron repletion with synchronous supplementation of antioxidants, however, protected the gastrointestinal tract of iron deficient rats to oxidative stress during oral iron repletion (Srigiridhar & Nair, 2000). This supports the hypothesis that oxidative damage mainly occurs when the maximal capacity of the antioxidant network is exceeded.

The clinical observation that oral iron therapy regularly induces gastrointestinal (GI) side effects, such as nausea, vomiting, heartburn, abdominal pain and constipation in some patients, in contrast to intravenous iron treatment (al-Momen *et al.*, 1996), may well be associated with iron-induced oxidative stress in the gastrointestinal tract. Especially in patients with an already compromised gut or an impairment of the mucosal or systemic antioxidant capacity, such as in Inflammatory Bowel Disease or celiac disease (Geerling *et al.*, 1998; Lih-Brody *et al.*, 1996; Marx & van Asbeck, 1996; Odetti *et al.*, 1998; Yamada & Grisham, 1991), oral iron therapy may induce intestinal oxidative stress and aggravate gastroenteropathy in these patients.

This study aimed to investigate the effects of one-month oral iron supplementation on the integrity of the small intestinal mucosa in iron deficient but otherwise healthy premenopausal women, reflected by small intestinal permeability. Secondly, this study aimed to investigate the detrimental effects of oral iron therapy on small intestinal permeability in subjects with acute mucosal damage, applying administration of the non-steroidal anti-inflammatory drug indomethacin as a model for gastrointestinal disorders-associated enteropathy.

Methods

Subjects

Thirty-one iron deficient, premenopausal women (age 21 ± 2 y) were recruited. All subjects received detailed information and gave their written informed consent before participation. Volunteers with a history of gastrointestinal complaints and those who had used oral iron-containing supplements at any time during the 6 months prior to testing were excluded from participation. The study design was approved by the Ethics Committee of the University Hospital Maastricht, Maastricht, The Netherlands.

Study design

Iron deficiency criteria for inclusion in this study were determined by assessing blood parameters (serum ferritin < 15 $\mu\text{g/L}$, blood haemoglobin < 7.5 mmol/L, blood haematocrit < 0.36 L/L) in a screening blood sample drawn from an antecubital vein in the forearm. After inclusion, subjects were randomly allocated to either the iron- or the placebo group.

On the day prior to the first experiment, a venous blood sample was obtained to assess baseline iron status. The next morning, after an overnight fast and after voiding, small intestinal permeability was measured using the lactulose/rhamnose gut permeability test as described below. For the 31 days following the baseline measurement, subjects ingested a masked, sealed gelatine capsule containing 100 mg iron as ferrous fumarate or placebo three times a day. Subjects were instructed to take the supplement one hour before breakfast, one hour before lunch and one hour before dinner to ensure maximal bioavailability of the iron.

Twenty-eight days after the start of supplementation, iron status and small intestinal permeability was assessed using the lactulose/rhamnose gut permeability test. Thirty days after the start of the supplementation, 75 mg indomethacin (Genfarma BV., Maarssen, The Netherlands) was administered at 1100 PM. Subsequently, after an overnight fast, 50 mg indomethacin was administered at 0700 AM, and a third small intestinal permeability test was performed one hour later.

Small intestinal permeability

Small intestinal permeability was assessed using the lactulose/rhamnose gut permeability test. This test is based on the comparison of intestinal permeation of molecules of different sizes. After voiding, a hyperosmolar drink containing 20 g sucrose, 5 g lactulose (Genfarma bv., Maarssen, The Netherlands) and 0.5 g L-rhamnose (ICN Biomedicals Inc., Aurora, OH, USA) in 100 mL water was administered. Subsequently, urine was collected for five hours. Subjects refrained from eating during the time of urine collection. Consumption of water was allowed *ad libitum* during the last two hours of urine collection. The urine volume was determined, and urine aliquots were taken and stored at -80°C until analysis.

The fractional 5-h urinary recovery of the sugar probes was determined. The urinary lactulose/rhamnose excretion ratio provides an accurate parameter of small intestinal permeability (van Nieuwenhoven *et al.*, 1999). Sucrose was added to obtain a hyperosmolar solution, which improves the sensitivity of the test compared to isosmolar solutions (Sigthorsson *et al.*, 1998). Lactulose and rhamnose were determined by fluorescent detection high-pressure liquid chromatography (HPLC). All samples were spiked with lactose as an external standard that does not interfere with determination of lactulose and rhamnose. Samples were incubated overnight with derivatization reagent (9-fluorenylmethyl chloroformate hydrazine) at 65°C. After cooling to room temperature, the reaction mixture was injected into a dedicated HPLC-system. The fluorescence was detected with a fluorescence detector (B&L systems, Zoetermeer, The Netherlands). The protocol for lactulose and rhamnose analysis was well validated and described in detail previously (Rooyakkers, van Eijk & Deutz, 1996).

Statistics

Lactulose/rhamnose ratios of the placebo- and the iron group at baseline, after 4 weeks of iron supplementation and after the indomethacin challenge were compared applying ANOVA, entering placebo and iron as factors. Haematological parameters of the placebo- and the iron group at baseline and after 4 weeks of iron supplementation were compared with paired Students t-tests. Differences were regarded as significantly different at a significance level of $P < 0.05$. All statistical comparisons were performed using the Statview 5.0 software package for the Macintosh.

Results

None of the subjects suffered from adverse effects of the iron supplements, besides occasional black colorization of the stool, which is a common phenomenon during ingestion of supplemental iron.

Baseline serum ferritin did not significantly differ between the group receiving placebo (11 ± 6 µg/L) or iron supplements (9 ± 7 µg/L). After 4 weeks, serum ferritin was significantly higher in the group receiving iron compared to the placebo group (24 ± 12 and 10 ± 7 µg/L, respectively; $P < 0.05$). None of the other haematological parameters differed between the two groups at baseline or after 4 weeks of supplementation (Table 1).

Urinary lactulose/rhamnose ratios were 0.013 ± 0.009 and 0.016 ± 0.007 at baseline, 0.016 ± 0.006 and 0.017 ± 0.009 after four weeks of iron administration, and 0.031 ± 0.020 and 0.032 ± 0.013 after the indomethacin challenges in the groups receiving placebo or iron supplements, respectively, and did not differ at any time (Figure 1). Urinary lactulose- and rhamnose recoveries did not significantly differ between the groups receiving placebo or iron supplements either at baseline, after four weeks of iron supplementation or after the indomethacin challenges (Table 2).

Table 1. Haematological parameters at baseline and after 4 weeks of supplementation with either placebo or 300 mg iron/day

		baseline	4 weeks
Ferritin (µg/L)	placebo	11 ± 6	10 ± 7
	iron	9 ± 7	24 ± 12*
Haemoglobin (mmol/L)	placebo	8.2 ± 0.5	8.1 ± 0.5
	iron	7.8 ± 0.5	8.1 ± 0.6
Haematocrit (%)	placebo	38.9 ± 2.5	36.9 ± 2.60
	iron	37.5 ± 2.6	37.1 ± 2.7

Data are expressed as mean ± sd. * Statistically different from placebo, *P*<0.05

Figure 1. Urinary lactulose/rhamnose ratios

Five-h urinary lactulose/rhamnose ratio after ingestion of a hyperosmolar test drink containing 5 g lactulose 0.5 g rhamnose and 20 g sucrose at baseline, after 4 weeks of placebo or iron supplementation (300 mg iron/d as ferrous gluconate; black bars) and, subsequently, after ingestion of 75 and 50 mg indomethacin at 9 and 1 h before ingestion of the lactulose/rhamnose test drink (average+sd). No significant differences between the treatments were observed at any time; *P*<0.05.

Table 2. Urinary lactulose- and rhamnose recoveries

	baseline		4 weeks		indomethacin	
	placebo	iron	placebo	iron	placebo	iron
% lac recovery	0.13 ± 0.08	0.18 ± 0.09	0.2 ± 0.1	0.19 ± 0.09	0.3 ± 0.18	0.39 ± 0.27
% rham recovery	11.7 ± 6.05	13.2 ± 6.3	14.4 ± 8.5	13.1 ± 7.2	11.3 ± 6.1	12 ± 5.4

Five-h urinary lactulose (lac) and rhamnose (rham) recoveries (mean ± sd) after administration of a lactulose/rhamnose/sucrose solution at baseline, after 4 weeks of supplementation with either placebo (n=16) or 300 mg iron as ferrous gluconate/d (n=15) and, subsequently, after administration of 75 mg and 50 mg of indomethacin at 9 and 1 h before ingestion of the sugar drink, respectively.

Discussion

The present study shows that iron supplementation with large daily dosages of ferrous iron supplements does not increase small intestinal permeability in premenopausal healthy women, despite the previous observation that iron administration causes free radical production in the intestine (Srigiridhar *et al.*, 2001) (Lund *et al.*, 1999). Also, iron supplementation did not cause an increased susceptibility of the small intestine to acute enteropathy, obtained by an indomethacin challenge. However, small intestinal permeability data should be interpreted with caution. Epithelial cells, tight junctions, intracellular cytoskeletons, intestinal microvasculature and other components that are involved in the control and maintenance of mucosal integrity were not damaged by the iron intervention to such an extent that permeability of the small intestine increased. This does not exclude oxidative damage to other intestinal compounds, such as brush border microvilli, enzymes etc, which may induce adverse effects *in vivo* in humans. Although not the scope of this study, supplementation with 300 mg iron/d for 28 d increased serum ferritin levels in these subjects. After the supplementation period, 12 out of 15 subjects were no longer considered as iron deficient according to the criteria, whereas in the group receiving placebo, 14 out of 16 subjects still were iron deficient after the supplementation period.

The mucosa of the small intestine provides a tightly controlled barrier against exogenous, orally ingested pathogens. Oxidative damage in the intestine may disrupt the integrity of tight junctions, decrease villus heights and induce apoptosis (Baker, Baker & LaRosa, 1995; Madesh *et al.*, 1999; Srigiridhar *et al.*, 2001). This disrupted integrity is reflected by an increased intestinal permeability (Baker *et al.*, 1995). An increase in permeability may result in intrusion of enteric pathogens, such as lipopolysaccharide (LPS) and microbes, which initiates a response of the local intestinal immune system. This response involves polymorphonuclear leukocytes (PMNs) translocation, a cytokine response and activation of intraepithelial lymphocytes (IELs), all of which induce further damage to the mucosal wall (Nusrat, Turner & Madara, 2000; Perez, Barber & Ponz, 1997). Progressing damage to the mucosa of the small intestine may result in bacterial translocation and, owing to an additional cytokine response, a further invasion of pathogens and may eventually initiate a systemic inflammatory response or sepsis (Thomson *et al.*, 2000). Furthermore, increased intestinal permeability is associated with a large array of gastrointestinal diseases, such as Crohn's disease (Andre *et*

et al., 1988; Meddings, 1997), celiac disease (Smecuol *et al.*, 1997), viral gastroenteritis (Isolauri *et al.*, 1989) and food allergy (Crowe & Perdue, 1992). It is, therefore, important to maintain the integrity of the intestinal wall and to limit any changes in permeability.

During iron therapy, relatively large amounts of iron remain in the intestine in a form that is likely to participate in Fenton reactions. During Fenton chemistry, free ferrous iron ions catalyze the formation of hydroxyl radicals in the presence of superoxide anion radicals and hydrogen peroxide, both produced as a result of normal metabolism. Hydroxyl radicals are highly reactive and can inflict chemical or structural damage to any biologic molecule in its direct environment. The radical can instantaneously transfer to another molecule and thus initiate a cascade of reactions. The catalyzing role of iron in the production of ROS *in vivo* has been established previously in animal studies. Micromolar concentrations of ferrous and ferric iron were shown to induce lipid peroxidation to microvillus membranes of rabbits (Fodor & Marx, 1988). Additionally, oral iron supplements aggravated lactic acid-induced damage to the barrier function of the small intestine epithelium (Gonzalez *et al.*, 1997), promoted colon tumorigenesis in mice (Siegers *et al.*, 1992) and inflammatory processes in rats with colitis (Aghdassi *et al.*, 2001; Reifen *et al.*, 2000). EPR spectroscopy in rats *in vivo* showed that oral iron therapy with ferrous sulfate resulted in iron mediated oxidative stress through hydroxyl radicals in the small intestine. This stress resulted in a decrease in cell turnover, shortening of micro-villus height and partial or complete erosion of the microvilli in the duodenum (Srigiridhar *et al.*, 2001). The free radical generating capacity from faeces in healthy volunteers was markedly increased after moderate iron supplementation (Lund *et al.*, 1999). However, this was determined *ex vivo*. The true radical production and its consequences in the intestine *in vivo* remain to be elucidated.

The findings of the present study do not confirm the data from animal studies. Iron ingestion did not induce oxidative damage to the permeation routes in the healthy human small intestine. Considering the well-established capacity of iron to catalyze free radical formation in the intestine, the human small intestine must have a powerful defence system against oxidative challenges. This supports the findings of a recent study from our group. Ingestion of a single, acute dosage of 80 mg iron as ferrous sulfate directly into the human duodenum *in vivo* induced significant lipid peroxidation in the lumen of the small intestine. This lipid peroxidation was accompanied by an instantaneous, massive increase in the total antioxidant capacity of the intestinal secretions (Troost *et al.*, 2003). From a physiological point of view, there is an obvious need for a powerful defence network against dietary oxidants in the small intestine, considering the high amount of oxidants and other potentially hazardous compounds available in food.

Furthermore, iron supplementation did not cause the small intestine to be more susceptible to indomethacin administration. The indomethacin challenge, which was shown previously to induce enteropathy and consequently a rise in small intestinal permeability by a factor 2-3 (Bjarnason *et al.*, 1993; Bjarnason *et al.*, 1989; Bjarnason *et al.*, 1986), did not cause a larger increase in small intestinal permeability in the

group receiving iron supplements compared to placebo. This supports the conclusion that iron supplementation does not induce extensive oxidative damage in the small intestine in these healthy subjects. However, the observation that the small intestinal permeability did not differ between the groups receiving placebo or iron may also be explained by the damage induced by indomethacin itself. Theoretically, the observed rise in permeability following indomethacin administration, which was approximately 2-fold, may reflect the maximal extent of enteropathy that can be detected by the lactulose/rhamnose gut permeability test and, hence, additional damage cannot be detected. Although this sugar recovery test is well validated and widely used, no data are available regarding the maximal permeability changes that can be detected. Previous studies applying the sugar test however do show permeability changes up to approximately 3-fold (Daenen *et al.*, 1991; Lambert *et al.*, 2001), which implies that any synergistic damage of indomethacin administration and iron supplementation would have been detected in the present study.

It was previously shown that IBD patients have an imbalance between ROS production and antioxidant status (Geerling *et al.*, 1998; Lih-Brody *et al.*, 1996), which puts them at higher risk for developing oxidative stress-associated enteropathy than the iron-replete, healthy subjects tested in the present study. Hence, the results of the present study cannot directly be extrapolated to pathological situations, in which iron supplementation may aggravate enteropathy.

The findings of the present study show that one month of high-dose iron supplementation does not impair the mucosal integrity of the small intestine in healthy females, either in the presence or absence of an indomethacin challenge as a model to stress the intestinal mucosa, compared to placebo. This may be explained by the presence of a powerful antioxidant network in the human small intestine *in vivo*. Future research will have to elucidate the capacity and mechanism of action of the antioxidant network in the human small intestine, both in the healthy and in the compromised gut.

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CHAPTER 3

A NEW METHOD TO STUDY OXIDATIVE DAMAGE AND ANTIOXIDANTS IN THE HUMAN SMALL BOWEL: EFFECTS OF IRON APPLICATION

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Abstract

Background Iron may induce oxidative damage to the intestinal mucosa by its catalyzing role in the formation of highly reactive hydroxyl radicals. This study aimed to determine iron-induced oxidative damage provoked by a single clinical dosage of ferrous sulfate and to elucidate the antioxidant defence mechanisms in the human small intestine *in vivo*.

Design A double lumen perfusion tube was positioned orogastrically into a 40-cm segment of the proximal small intestine in six healthy volunteers (25 ± 5 y). The segment was perfused with saline and subsequently with saline containing 80 mg iron as ferrous sulfate at a rate of 10 mL/min. Intestinal fluid samples were collected at 15-min intervals.

Results Thiobarbituric acid reactive substances (TBARS) concentrations as indicator of lipid peroxidation increased significantly from 0.07(0-0.33) μM during saline perfusion to 3.35(1.19-7.27) μM during iron perfusion ($P < 0.05$). Non-protein antioxidant capacity increased significantly from 474(162-748) μM to 1314(674-1542) μM ($P < 0.05$).

Conclusion These data show that a single dosage of ferrous sulfate induces oxidative damage and the subsequent release of an antioxidant in the small intestine *in vivo* in healthy volunteers.

Introduction

The prevalence of iron deficiency anaemia in Europe and North America is 1 % in adult males and 14 % in adult females (DeMaeyer & Adiels-Tegman, 1985). The usual treatment of iron deficiency anaemia involves oral iron supplementation with ferrous salts. Ferrous salts, prescribed in amounts up to 195 mg of elemental iron/d divided over three single dosages as ferrous sulfate, fumarate or gluconate respectively (Frewin, Henson & Provan, 1997), are commonly preferred over ferric salts because of their higher bioavailability. Oral ferrous iron therapy may, however, induce oxidative stress due to its catalyzing role in Fenton chemistry, resulting in the production of highly reactive hydroxyl radicals (Fodor & Marx, 1988; Srigiridhar & Nair, 1998). They can react with any molecule in their direct environment, resulting in a cascade of reactions in which lipids, proteins and DNA may get damaged. The production of reactive oxygen species (ROS), such as superoxide anion radicals, hydroxyl radicals and hydrogen peroxide, is normally balanced by the antioxidant system. Excessive ROS production, however, induces a disturbance in the pro- and antioxidant balance, resulting in oxidative damage.

Oral iron therapy, commonly indicated in people with an elevated risk for developing iron deficiency, such as children, adolescents, the elderly, (pregnant) women, vegetarians and athletes, may induce considerable gastrointestinal (GI) side effects, such as nausea, vomiting, heartburn, abdominal pain and constipation, in contrast to intravenous iron treatment (al-Momen *et al.*, 1996). Iron-induced oxidative damage in the intestine after oral ingestion of iron supplements may in part be responsible for these GI side effects. Additionally, oral iron therapy in patients suffering from gastrointestinal disorders associated with an impaired mucosal or systemic antioxidant capacity, such as Inflammatory Bowel Disease or celiac disease, may attribute to the development of major oxidative damage (Geerling *et al.*, 1998; Lih-Brody *et al.*, 1996; Marx & van Asbeck, 1996; Odetti *et al.*, 1998; Yamada & Grisham, 1991).

Oral iron supplements were shown to aggravate lactic acid-induced damage to the barrier function of the small intestine epithelium (Gonzalez *et al.*, 1997), to promote colon tumorigenesis in mice (Siegers *et al.*, 1992) and inflammatory processes in rats with colitis (Aghdassi *et al.*, 2001) (Reifen *et al.*, 2000). Moreover, EPR spectroscopy in rats *in vivo* showed that oral iron therapy with ferrous sulfate resulted in iron mediated oxidative stress through hydroxyl radicals in the small intestine. This stress resulted in a decrease in cell turnover, shortening of microvillus height and partial or complete erosion of the microvilli in the duodenum (Srigiridhar *et al.*, 2001). Epidemiological studies showed a role of dietary iron in the development of colon cancer (Wurzelmann *et al.*, 1996). Additionally, ROS may induce development of several forms of cancer (Blakeborough, Owen & Bilton, 1989; Nelson *et al.*, 1989).

Because of the complexity of the direct measurement of oxidative damage in the lumen of the human small intestine *in vivo*, no data are available regarding the effects of clinically prescribed amounts of iron on oxidative damage in the human intestine *in vivo*. Also, little is known about the antioxidant defence mechanisms against ROS production in the intestine. For this purpose, we developed a perfusion technique to

directly quantify oxidative damage and antioxidant capacity *in vivo* in the small intestine in healthy adults. This study aimed to determine iron-induced oxidative damage provoked by a single clinical dosage of ferrous sulfate in the small intestine in humans and to obtain more information about the antioxidant defence mechanisms in the human small intestine *in vivo*.

Methods

Subjects

Six healthy non-smoking volunteers (25 ± 5 y, 3 males, 3 females) without a history of a gastrointestinal disorder participated in the present study. Volunteers were in good physical health at the time of participation as checked by medical interview. All subjects gave their written informed consent before participation. The study protocol was approved by the Ethics Committee of the University Hospital Maastricht, Maastricht, The Netherlands.

Study design

After an overnight fast, a perfusion catheter was ingested orogastrically by each volunteer. The catheter consisted of three silicon tubes connected to a rubber balloon on the distal end of the catheter. One of the tubes, called the sample port, had three small openings at 5 cm proximal of the distal end of the catheter to enable fluid sampling from the small intestine at the level of the distal end of the catheter. The other two tubes were connected in an open loop with the balloon to enable fluid and air transport in and out of the balloon. A fourth lumen had a single opening at 45 cm from the distal end. This port, referred to as the perfusion port, served to administer a test solution directly into the duodenum.

After installation of the catheter tip into the stomach, the rubber balloon was filled with 1.5 mL of mercury. Subjects were instructed to remain in the supine position on the right hand side with the upper body lifted 45° and the feet raised 20 cm until the balloon entered the duodenum. Positioning of the catheter was checked with continuous pH-registration at the tube tip. When the pH shifted from low (approx. 1-2.5) to approx. 4-6, the mercury was aspirated and the balloon was subsequently inflated with 15 mL of air. Subjects were instructed to stay in the supine position on the back with the upper body lifted 45° . The catheter propagated subsequently by the peristaltic movements of the GI tract. Catheter positioning was successful when the perfusion port was placed about 5-10 cm distally from the pylorus. This was checked by a second pH-registration channel, which was located at 5 cm proximal from the perfusion port. After positioning, the balloon was deflated to stop it from progressing in the GI tract. Throughout the experiment, the pH of the second pH channel was continuously registered to ensure proper positioning of the perfusion port. After the perfusion experiment, subjects removed the catheter by pulling it gently.

Through the perfusion port, a saline solution was injected at 10 ml/min during 195 min. This period was necessary to reach steady state conditions as was observed in pilot experiments (data not shown). Subsequently, a 300-mL saline solution containing 80 mg elemental iron as ferrous sulfate (Merck, Darmstadt, Germany) was perfused at 10 ml/min during 30 min. Finally, a saline solution was perfused again for 60 minutes to rinse out the iron solution and intestinal secretions.

Intestinal fluid samples were obtained by using the lever properties of the catheter. After an initial adaptation period of 195 min, intestinal fluid content was levered

through the sample lumen into a beaker placed in melting ice. Intestinal fluid excretions were pooled over 15-min intervals and stored at -80°C until analysis.

Biochemical analysis of intestinal fluid samples

Thiobarbituric acid reactive substances (TBARS) assay

The determination of TBARS was based on the formation of a coloured adduct of malondialdehyde (MDA) with 2-thiobarbituric acid (TBA). One hundred μL sample was added to 900 μL of a reagent (containing 0.12 mol/l TBA, 0.32 mol/l o-phosphoric acid, 0.68 mmol/l butylated hydroxytoluene (BHT) and 0.01 % (m/v) EDTA). The mixture was incubated for 1 hour at 100°C in a water bath. After cooling the MDA-products were extracted with 500 μL butanol. Thirty μL of the butanol layer was injected on a HPLC system (Agilent, Palo Alto, CA, USA) equipped with a fluorescence detector, set on excitation wavelength of 532 nm and emission wavelength of 553 nm, and a Nucleosil C18 column, 150 x 3.2 mm, particle size 5 μm (Supelco Inc. PA, USA). Samples were eluted with 65 % (v/v) 25 mmol/l phosphate buffer pH 4.8, 35 % (v/v) methanol. A calibration curve was constructed using malonaldehyde bis(diethylacetal) as standard.

Antioxidant capacity assay

The antioxidant capacity assay was carried out as described previously (Berg van den *et al.*, 1999) with some modifications. The ABTS^{*+} radical was produced by incubating a solution of 0.23 mmol/l ABTS and 2.3 mmol/l ABAP in 100 mM sodium phosphate buffer pH 7.4 at 70°C until the absorption of the solution at 734 nm was between 0.680 and 0.720. Deproteination was done by mixing the sample with an equal volume of a solution of 10 % (w/v) trichloro-acetic acid. In the reaction of antioxidants with the blue/green ABTS radical, the blue green colour disappears. This decolourisation after 5 minutes is determined spectrophotometrically at 734 nm. The reduction in absorbance is related to that of trolox, a synthetic, hydrophilic vitamin E analogue, which gives the trolox equivalent antioxidant capacity (TEAC) value. The TEAC value is calculated as molar trolox equivalents of the sample.

Glutathione assay

Total glutathione was determined using the recycling method (Vandeputte *et al.*, 1994). To 50 μL of a sample 100 μL of a 0.4 mmol/l NADPH/ 0.3 mmol/l DTNB solution in 143 mmol/l phosphate buffer pH 7.4, containing 6.3 mmol/l EDTA, is added in a 96- well plate. Then the reaction is started by adding 50 μL of a solution containing 4 U/ml glutathione reductase. The increase of the absorption ($\Delta\text{A}/\text{min}$) is followed for 2 minutes at 405 nm. The $\Delta\text{A}/\text{min}$ of the samples is compared to that of the calibrators and the concentration of total glutathione is calculated.

Uric acid assay

Uric acid was determined in ultra filtrates using a HPLC method described previously (Lux, Naidoo & Salonikas, 1992) with minor modifications. The sample was filtered over a 3 kD filter unit (Centrex UF 0.5, Schleicher & Schuell, Dassel, Germany) by

centrifugation for 45 minutes at $14,000 \times g$ and 4°C . $25 \mu\text{L}$ of the filtrates was analyzed on the HPLC. The system (Agilent, Palo Alto, CA, USA) consisted of an Hypersil BDS C-18 end-capped column, $125 \times 4 \text{ mm}$, particle size $5 \mu\text{m}$ (Agilent, Palo Alto, CA, USA) with a mobile phase of 5 mmol/L sodium phosphate buffer $\text{pH} = 3.3$. Detection was performed using a variable wavelength detector set on a wavelength of 292 nm . Concentrations of uric acid were calculated using a calibration curve of uric acid.

Statistics

Differences were assessed by Univariate Analyses of Variance using SPSS 10.0 software. Differences were regarded as significant when $p < 0.05$. Multiple comparisons, used to localize time differences, were adjusted with the Least Significant Difference.

Results

One to two hours after ingestion of the catheter, the distal end of the catheter entered the duodenum. Catheter positioning was completed within 5 to 8 h in all volunteers. The TBARS levels were significantly higher at 15, 30, 45 and 60 min after the start of the ferrous sulfate perfusion compared to the baseline level measured at $t = 0$ ($P < 0.05$) and returned to baseline levels after 90 min (Figure 1). The TEAC of the intestinal fluid was significantly higher at 30 and 45 min after the start of the ferrous sulfate perfusion compared to the baseline level measured at $t = 0$ ($P < 0.05$) (Figure 1).

Figure 1. Lipid peroxidation and antioxidant capacity

*Thiobarbituric Acid Reactive Substances (TBARS) concentrations, an indicator of lipid peroxidation, and Trolox Equivalent Antioxidant Capacity (TEAC), an indicator of total antioxidant capacity, in intestinal perfusion samples, collected over 15-min intervals during perfusion of a 40 cm-segment in the proximal small intestine in 6 subjects. Baseline values at $t=0$ were obtained during saline perfusion. Subsequently saline containing 80 mg elemental iron as ferrous sulfate was perfused for 30 min, and finally saline was perfused for 60 min at 10 mL/min (mean $\pm$ SEM; differences vs $t=0$ tested with Analysis of Variance, * $P < 0.05$).*

Table 1. Uric acid concentrations in intestinal perfusion samples pooled over 15-min periods

time (min)	Uric acid (μM)
0	37.9 ± 15.6
15	34.4 ± 15.7
30	32.5 ± 12.6
45	27.1 ± 8.2
60	25.2 ± 4.8
75	28.7 ± 8.7
90	26.9 ± 10.2

(mean ± sd)

Median alkaline phosphatase concentrations of the perfusate samples did not differ significantly between t=0 and t=30 min (502 ± 377 and 510 ± 363 U/L respectively). Uric acid concentrations of the perfusate samples were not significantly different from baseline during the first 75 min after the start of the ferrous sulfate perfusion (Table 1). At t = 90, uric acid was statistically lower than baseline values (P<0.05). Glutathione concentrations in the perfusate samples were under the detection limit of the equipment (< 0.10 μmol/L).

Discussion

The present study clearly shows that iron ingestion *in vivo* induces intestinal oxidative damage as indicated by the occurrence of lipid peroxidation. The magnitude of the TBARS concentration in the intestinal fluid samples as a result of iron ingestion was remarkably high. The TBARS assay was applied to estimate malondialdehyde (MDA) concentration, which is an end product of lipid peroxidation. MDA is a well-accepted parameter of oxidative damage (Hageman, Bast & Vermeulen, 1992). However, it is not specifically produced as a result of free radicals attack, but may be formed during the *ex vivo* sample incubation stage. In order to prevent formation of MDA *ex vivo*, the chain-breaking antioxidant BHT was added. The ability of BHT to prevent TBARS formation during the analytical assay was checked in *ex vivo* experiments with intestinal fluid samples. In these experiments, we observed an instantaneous rise in TBARS formation upon iron addition, but BHT was effective in preventing TBARS formation during the assay. Other possible analytical shortcomings of the conventional TBARS-assay are resolved by the use of the applied HPLC technique (Halliwell & Chirico, 1993). Therefore, in the present study TBARS provides an accurate indicator of oxidative damage *in vivo*. The finding that antioxidant capacity increases in the lumen of the small intestine following an oxidative challenge has not been described previously. The rapid and massive increase in antioxidant capacity of the intestinal fluid samples indicates a powerful defence mechanism of the small intestine against exogenous oxidants.

The TBARS concentrations in the intestinal fluid samples after the iron challenge are roughly 3–5 times higher than those observed in plasma in healthy adults. This is a highly interesting finding in the view of the dilution of the intestinal fluid samples. In pilot experiments we measured the dilution of the intestinal fluid with the saline or saline/iron solution in the intestinal segment by addition of an inert marker, lactulose, to the ingoing solution and subsequent measurement of the lactulose concentration in the perfusate samples. A dilution of approximately 100-fold was found in all samples. This dilution was constant throughout the experiment (data not shown). In view of the fact that sample dilution did not change during the experiment, independently of the presence or absence of iron, intestinal fluid dilution did not interfere with the results of the study and hence was not measured in the present study.

On average, approximately 15 mg of iron is present in the daily diet. Approximately 2.5 mg iron entering the intestine will be absorbed. About 1 % of the dietary iron remains in the lumen in a form that is likely to participate in free radical generation. During oral iron therapy in adults using dosages of 60 to 195 mg iron/d this proportion remains the same, resulting in a much larger amount of iron available for Fenton reactions. During Fenton chemistry, ferrous iron catalyzes the formation of hydroxyl radicals in the presence of superoxide anion radicals and hydrogen peroxide, both produced as a result of normal metabolism. Hydroxyl radicals are highly reactive and can inflict damage to any biologic molecule in its direct environment. The free electron can transfer to another molecule and hence initiate a cascade of damaging reactions. Previously it was shown that the free radical generating capacity from faeces in healthy volunteers was markedly increased after iron supplementation (19 mg/d iron as ferrous sulfate for 14 d) (Lund *et al.*, 1999). However, this was measured in faecal samples *ex vivo* in an *in vitro* assay. This does not necessarily reflect the conditions from the present study in which the true effects of iron ingestion in the small intestine *in vivo* were investigated. The results of the present study confirm the finding that iron ingestion leads to an increase in free radical production in the gut. The model used in the present study mimics ingestion of an oral iron supplement, which dissolves in the gastric juice and is subsequently released gradually into the small intestine.

From the data (Figure 1) it is clear that oxidative damage occurs immediately following iron administration into the small intestine. This damage continues during saline perfusion after the ferrous sulfate perfusion. This is likely due to the low perfusion flow in the experimental setup. After discontinuation of the iron perfusion, it takes some time to rinse out all of the iron and the intestinal juices secreted during the iron perfusion from the small intestine. After 60 min of saline perfusion, no iron-induced rise in TBARS is observed in the intestinal fluid samples anymore. These data are in line with those of a rat study where it was shown that high dosages of iron supplements induce lipid peroxidation in the colon (Rimbach *et al.*, 1997). Any effects of iron supplements in the small intestine were not investigated in that study.

The lipid peroxidation observed in the present study preceded a marked rise in TEAC (Figure 2). From this it may be concluded that iron-induced oxidative stress induces the release of an antioxidative component in the small intestine. As for the TBARS

concentrations, the TEAC values are also remarkably high, especially in view of the previously mentioned dilution of the intestinal fluid samples with the ingoing solution. The data of the present study indicate that a powerful physiologic mechanism in the small intestine protects against iron-induced oxidative damage. In view of the fast release of the antioxidant(s), it is unlikely that the iron-induced oxidative damage directly induced production of an antioxidative agent, which is subsequently released into the lumen. It is more likely that an antioxidant already present in a storage compartment in the small intestine was released upon oxidative damage. In the present study, all samples were completely deproteinized during the TEAC assay. Sample deproteinization is routine practice during the TEAC assay to reduce the analysis of variance. In pilot experiments, we observed that sample deproteinization did not affect the average TEAC values compared to duplicate non-deproteinized samples. Hence, the high antioxidant capacity was exclusively caused by a non-protein species. This excludes a role for enzymatic antioxidants or metallothionines in the observed rise in antioxidant capacity.

The rise in TEAC might be explained by lysis of epithelial or mucosal cells. If the membranes of intact cells were disrupted, its cellular content was excreted into the lumen. As a result, the antioxidant capacity would predominantly originate from the cytosolic antioxidant capacity of the epithelial cells. However, this would have been accompanied by a rise in total alkaline phosphatase, which we did not observe. Also, removal of intact epithelial cells present in the intestinal fluid samples by centrifugation did not significantly affect the antioxidant capacity of the samples (data not shown). This suggests that the strong rise in TEAC following iron-induced oxidative stress was not due to cell lysis but was brought about by a specific anti-oxidant mechanism.

Data regarding the non-protein antioxidant capacity of the small intestine are scarce. It was shown in a rat study that the small intestine has a powerful reducing capacity, which is protective against triglyceride hydroperoxides (Mohr *et al.*, 1999). The underlying mechanism or the site of action (luminal, mucosal or epithelial) was not identified. In the present study, glutathione and uric acid were determined in the intestinal fluid samples in an attempt to identify the origin of the rise in antioxidant capacity following iron perfusion. Due to the intestinal fluid dilution by the perfusing solution, glutathione concentrations were below the detection threshold of the equipment used. Uric acid, a powerful antioxidant present in lung- and intestine epithelial lining fluid (Karasawa, Ishi & Kubota, 1991), was present in low amounts that were not affected by the iron intervention. Vitamin C, present in low amounts in most samples, did not show a correlation with antioxidant capacity (data not shown). Hence, neither of these antioxidants was responsible for the rise in antioxidant capacity following the iron challenge in the present study.

The perfusion model to investigate oxidative stress and antioxidant capacity in the human small intestine enabled us to determine the effects of a pro-oxidative agent over a standardized segment of the small intestine. A major advantage of the technique is the rather non-invasive protocol of the catheter positioning, as this occurred

using normal peristaltic movements. The technique is applicable to healthy volunteers as well as patients. The perfusion technique can only be used to study a limited segment of the proximal small intestine. Hence, results may not be directly extrapolated to the entire small intestine. However, due to a decreasing nutrient concentration along the small intestine, nutrient-associated oxidative damage probably takes place predominantly in the proximal small intestine. The rate of perfusion needs to be as low as possible to avoid effects of the perfusion experiment on normal functioning of the small intestine. Because of the low perfusion flow, it is not possible to monitor acute, short-term responses in the small intestine. Additionally, sampling occurs at 15-min intervals to ensure sufficient sample collection during each collection interval. The perfusion technique provides the unique feature to study oxidative damage and antioxidant capacity in secretions of the small intestine *in vivo* in humans under strictly standardized conditions. This model is applicable to a variety of intestinal disorders associated with a disturbance in the equilibrium between oxidative stress and the antioxidant defence network. Increased oxidative stress occurs in acute and chronic inflammation, certain drugs and nutrients. Decreased antioxidant capacity may be pre-sent in disorders of mucosal integrity and chronic malabsorption disorders associated with antioxidant deficiencies (Geerling *et al.*, 1998). A disturbance in the equilibrium between oxidative stress and the antioxidant defence network in the small intestine may be an important factor in the pathophysiology of these disorders. Additionally, the perfusion technique can be used to evaluate the efficiency of nutritional antioxidant interventions in restoring a sufficient antioxidant network against oxidative attack in the small intestine.

Iron ingestion induced Fenton-driven oxidative stress in the human small intestine, followed by a massive rise in total antioxidant capacity in the lumen. This rise may reflect the release of a powerful, yet unidentified, non-protein antioxidant. Future work should focus on identification of this antioxidant and on the possibilities to increase the antioxidant capacity against oxidative stress with nutritional interventions in disorders related to a high intestinal oxidative stress or a low intestinal antioxidant defence network.

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CHAPTER 4

***IN VIVO* REGULATION OF GENE EXPRESSION IN THE SMALL INTESTINAL MUCOSA BY IRON-INDUCED OXIDATIVE STRESS**

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Submitted

Abstract

Background Iron-induced oxidative stress in the small intestine may alter gene expression in the intestinal mucosa. The present study aimed to determine which genes are mediated by an iron-induced oxidative challenge in the human small intestine.

Design Ten healthy volunteers (22 $\pm$ 2 y) were tested on two separate occasions, in a randomized cross-over design. After duodenal tissue sampling by gastroduodenoscopy, a perfusion catheter was inserted orogastrically to perfuse a 40-cm segment of the proximal small intestine with saline and, subsequently, with either 80 or 400 mg iron as ferrous gluconate. After the intestinal perfusion, a second duodenal tissue sample was obtained.

Results Thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation, in intestinal fluid samples increased significantly and dose-dependently at 30 min after the start of perfusion with 80 or 400 mg iron, respectively ($P<0.001$). During the perfusion with 400 mg iron, the increase in TBARS was accompanied by a significant, momentary rise in trolox equivalent antioxidant capacity (TEAC), an indicator of total antioxidant capacity ($P<0.05$). The expression of eighty-nine gene reporters was significantly altered by both iron interventions. Functional mapping showed that six distinct processes were importantly mediated by both iron dosages. Three of those processes involved G protein receptor coupled pathways. The other processes were associated with cell cycle, complement activation and calcium channels.

Conclusion Iron administration in the small intestine induced, dose-dependent, lipid peroxidation and a momentary antioxidant response in the lumen, mediated the expression of at least 89 individual genes, and affected at least six biological processes.

Introduction

It is generally accepted that oxidative stress in the intestine is an aetiological factor in the pathophysiology of several inflammation-associated disorders, such as Inflammatory Bowel Disease (IBD) and celiac disease (Geerling *et al.*, 1998; Lih-Brody *et al.*, 1996; Marx & van Asbeck, 1996; Odetti *et al.*, 1998; Yamada & Grisham, 1991). Diet-related components may be important mediators of oxidative damage. Previous studies showed that iron supplements induce oxidative damage to the small intestine, owing to its catalyzing role in Fenton chemistry, which results in the production of highly reactive hydroxyl radicals (Fodor & Marx, 1988; Srigiridhar & Nair, 1998). Furthermore, iron intake was associated with impairment of the barrier function of the small intestine epithelium (Gonzalez *et al.*, 1997), the development of inflammation in ulcerative colitis (Aghdassi *et al.*, 2001) (Reifen *et al.*, 2000), a decrease in cell turnover, shortening of microvillus height and erosions of the microvilli in the duodenum (Srigiridhar *et al.*, 2001) and the development of colon cancer (Siegers *et al.*, 1992) (Wurzelmann *et al.*, 1996). We recently showed that a single iron dose administered directly into the small intestine induces lipid peroxidation and the release of, yet unidentified, antioxidants in the lumen of the small intestine (Troost *et al.*, 2003). The cellular mechanisms involved in the intestinal oxidative stress and defence responses are not clear. The rapidly evolving cDNA microarray technology provides a sophisticated method to synchronously monitor the expression of thousands of genes in order to obtain essential information about the regulation of numerous biochemical processes in the intestinal mucosa on the transcriptional level. This technique has the potential to elucidate complex biochemical or physiological pathways, such as intestinal oxidative stress and its consequences.

The present study aimed to assess iron-induced oxidative damage and the antioxidant response in the human small intestine *in vivo* after administration of commonly used iron substitution dosages, and to investigate the effects of intestinal oxidative stress on genome-wide gene expression in the small intestine, in order to obtain detailed information about intestinal transcriptomics *in vivo*.

Methods

Subjects

Eight healthy non-smoking volunteers (24 ± 4 y (average $\pm$ sd), 8 males, 2 females) without a history of gastrointestinal disorders participated in the study. Volunteers were free of medication and in good physical health. All subjects gave their written informed consent before participation. The study protocol was approved by the Ethics Committee of the University Hospital Maastricht, Maastricht, The Netherlands.

Study design

This study was designed to conform to a randomized cross-over design. Each subject was tested on two separate days. Baseline duodenal biopsies, taken by standard forceps in the proximal horizontal part of the duodenum (15 cm distally from the pylorus), were obtained by flexible gastroduodenoscopy. Biopsies were imbedded in tissue-tek (Electron Microscopy Sciences, Washington, PA, USA), snap-frozen in melting isopentane and liquid nitrogen respectively, and stored at -80°C until analysis. After obtaining the mucosal samples, a guide wire was inserted with the distal tip positioned about 60 cm distally from the pylorus, facilitated by the duodenoscopy. After retraction of the endoscope, a double lumen perfusion catheter (home made from PVC tubing, inner diameter 2.5 mm, outer diameter 4 mm) was inserted over the guide wire into the proximal small intestine with the tip of one lumen, which served as an injection port, located in the proximal descending duodenum and the tip of the second lumen, with three small holes in the last 3 cm to serve as a sample port, 40 cm distally from the injection port. After positioning, the guide wire was removed. The whole procedure was performed under short interval fluoroscopic control. To check positioning of the injection port in the duodenum throughout the experiment, intraluminal pH was continuously measured at 5 cm proximal of the injection port by a pH electrode (Single Crystal Antimony Multi-Use pH Catheter, Synectics Medical, Queluz, Portugal). The 40-cm segment of the proximal small intestine was perfused with saline at 10 ml/min using a peristaltic pump for 195 min to be sure to have obtained steady state conditions (Troost *et al.*, 2003). After reaching steady state, either 80 or 400 mg iron as ferrous gluconate dissolved in 300 mL saline was perfused. The iron solutions were perfused over a 30-min period. Finally, saline was perfused for another 60 min. All ingoing solutions were kept at 37°C in a water bath. Throughout the perfusion experiment, intestinal fluid samples were obtained continuously using the lever properties of the catheter. After the initial 180 min adaptation period, intestinal fluid was levered through the sample lumen into a beaker placed in melting ice. Intestinal fluids were pooled over 15-min intervals and stored at -80°C until analysis.

Fifteen minutes after the perfusion experiment, the perfusion catheter was removed and a second gastroduodenoscopy was performed to obtain tissue samples from the same intestinal region after the iron challenge. The entire protocol was repeated on another day, approximately two weeks after the first experiment, to investigate the effects of the other iron dosage. The administration of dosages was in random order.

Gene expression analysis

Total RNA was isolated from each tissue sample and RNA quality was assessed by SDS-page gel electrophoresis. After quality check, at least one tissue sample from 3 out of 8 subjects provided low quality RNA and hence, all tissue samples from these subjects were excluded from the microarray analyses. The remaining 5 subjects were pooled into two groups (samples from subjects 1 and 2 were pooled in pool 1, samples from subjects 3, 4 and 5 were pooled in pool 2). Microarrays were carried out on both pools at baseline, after the low and after the high dosage intervention respectively.

cRNA preparation and *in vitro* transcription

10 µg total RNA was used as starting material for the cDNA preparation. The first and second strand cDNA synthesis was performed using the SuperScript II System (Invitrogen, Taastrup, DK) according to the manufacturers instructions except using an oligo-dT primer containing a T7 RNA polymerase promoter site. Labelled cRNA was prepared using the BioArray High Yield RNA Transcript Labelling Kit (Enzo, Farmingdale, NY, USA). Biotin labelled CTP and UTP (Enzo) were used in the reaction together with unlabeled NTP's. Following the IVT reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen, Hilden, GE).

Array hybridisation and scanning

Fifteen µg of cRNA was fragmented at 94°C for 35 min in a fragmentation buffer containing 40 mM Tris-acetate pH 8.1, 100 mM KOAc, 30 mM MgOAc. Prior to hybridisation, the fragmented cRNA in a 6xSSPE-T hybridisation buffer (1 M NaCl, 10 mM Tris pH 7.6, 0.005% Triton), was heated to 94°C for 5 min and subsequently to 45°C for 5 min before loading onto the Affymetrix HG U133A probe array cartridge. The probe array was then incubated for 16 h at 45°C at constant rotation (60 rpm). The *washing and staining procedure* was performed in the Affymetrix Fluidics Station. The probe array was exposed to 10 washes in 6xSSPE-T at 25°C followed by 4 washes in 0.5xSSPE-T at 50°C. The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate, final concentration 2 mg/ml (Molecular Probes, Eugene, OR) in 6xSSPE-T for 30 min at 25°C, followed by 10 washes in 6xSSPE-T at 25°C. Subsequently, an antibody amplification step was performed, using normal goat IgG as blocking reagent, final concentration 0.1 mg/ml (Sigma, Copenhagen, DK) and biotinylated anti-streptavidin antibody (goat), to obtain a final concentration of 3 mg/ml (Vector Laboratories, Burlingame, CA, USA). This was followed by a staining step with a streptavidin-phycoerythrin conjugate, final concentration 2 mg/ml (Molecular Probes, Eugene, OR) in 6xSSPE-T for 30 min at 25°C, and 10 washes in 6xSSPE-T at 25°C. The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope (Hewlett Packard GeneArray Scanner G2500A). The readings from the quantitative scanning were analysed with the Affymetrix Gene Expression Analysis Software.

Each gene on the U133A chip was represented by 11 pairs of 25-mer oligodeoxyribonucleotide probes. Each probe pair consists of a perfect match oligonucleotide that

precisely matches the gene sequence, and a mismatch oligonucleotide that differs only in one single nucleotide mismatch in the centre position. The data from the different sets of probe pairs were used to perform the statistical analyses. For comparison from array to array, all signal data from the quantitative readings were scaled to a global intensity of 150, as previously published (Birkenkamp-Demtroder *et al.*, 2002; Thykjaer *et al.*, 2001).

The signal intensities from the microarrays were scaled and further analyzed using the Affymetrix Microarray Suite version 5.0 software (Affymetrix, Santa Clara, CA). Relative mRNA changes were expressed as differences in gene expressions between the 80- or the 400 mg iron challenge compared to baseline samples, respectively. The definition of increase, decrease or no change of expression for individual genes was based on p-values of the comparisons of the normalized signal intensities, as calculated by the software. Genes that were either up- or down regulated in the same direction in both pools were further investigated. If the expression of a gene was increased in one pool while it did not change or decreased in the other pool, it was assumed that the gene expression was not mediated by the iron interventions. Likewise, only those genes that were mediated in the same direction by both the low and the high iron dosage interventions were further investigated.

To allow functional mapping of expression profiles, the annotation of individual probes with the Swissprot (<http://us.expasy.org/sprot/sprot-top.html> (Boeckmann *et al.*, 2003)) database ID for the corresponding protein was used. Using these IDs, expression changes for each of the four, pooled, samples were studied using functional MAPPs (MicroArray Pathway Profiles) available from the GenMapp initiative (<http://www.genmapp.org/>) with GenMapp v1.0 beta software (Dahlquist *et al.*, 2002). MAPPs generated from the Gene Ontology database (<http://www.geneontology.org/> (Consortium, 2001)), the G-Protein Coupled Receptor Database (<http://www.gpcr.org/> (Horn, Vriend & Cohen, 2001)), plus a small number of MAPPs based on the KEGG database (<http://www.genome.ad.jp/kegg/> (Kanehisa *et al.*, 2002)) and MAPPs specifically designed for GenMapp (so called local MAPPs) were used. MappFinder software was used to select the MAPPs with relatively high numbers of differentially expressed genes with a fold change of at least 2.3, as was indicated by the individual Z-scores. The Z-score increases when higher numbers of changing genes are found, taking into account the number of genes present in the MAPP that are represented on the array, and the total number of genes involved in the concerning MAPP. MAPPs were selected for further study if all four samples reached an arbitrary Z-score of at least 1 on that MAPP.

Real-time polymerase chain reaction (RT-PCR)

In order to validate the observed mRNA changes from the microarray analyses, RT-PCR was performed on seven randomly selected genes. cDNA was synthesized from single samples previously analyzed on GeneChips. The microarray gene expression profiles of the 7 genes, with Unigene cluster IDs nm_005410, nm_001924, nm_002306, nm_005118, nm_00075, nm_003355 and nm_003056, were compared with RT-PCR. Reverse transcription was performed using Superscript II RT (Invitrogen). 1 µg total RNA and 3 mL 150 pmol/mL random nonamer-primer in a total volume of

12 ml was incubated 4 min. at 70°C and chilled on ice. After adding 4 ml 1st Strand Buffer (from supplier), 2 ml DTT (0,1M), 1 ml dNTP mix (10mM), and 1 ml SuperScript RT II (200U/ml) the reaction was incubated 1 hour at 45°C, and finally 15 min. at 70°C. The cDNA was diluted 1:20 for use in Real Time PCR. Real-time PCR analysis was performed on the selected genes using primers, designed with PrimerExpress software from Applied Biosystems (Foster City, CA). Triple determinations were performed on ABI PRISM® 7000 Sequence Detection System using the SYBR® Green PCR Master Mix (Applied Biosystems). The PCR reaction consisted of 12.5 ml SYBR Green PCR Master Mix, 300 nM of forward and reverse primers, and 2.0 ml 1:20 diluted template cDNA in a total volume of 25 ml. The reaction was thermocycled using the default settings of "ABI Prism 7000 SDS Software 1.0": 2 min. at 50°C, 10 min. at 95°C, followed by 40 rounds of 15 sec. 95°C and 1 min at 60°C. A dissociation protocol was added after thermocycling, determining dissociation of the PCR products from 65°C to 95°C. All samples were normalized to GAPDH, as was applied previously (Birkenkamp-Demtroder *et al.*, 2002).

Biochemical analysis of intestinal fluid samples

TBARS assay

The determination of thiobarbituric acid reactive substances (TBARS) was based on the formation of a coloured adduct of malondialdehyde (MDA) with 2-thiobarbituric acid (TBA). 100 µl sample was added to 900 µL of a reagent (containing 0.12 mol/L TBA, 0.32 mol/L o-phosphoric acid, 0.68 mmol/L butylated hydroxytoluene (BHT) and 0.01 % (m/v) EDTA). The mixture was incubated for 1 hour at 100 °C in a water bath. After cooling, the TBARS-products were extracted with 500 µl butanol. Thirty µL of the butanol layer was injected on a HPLC system (Agilent, Palo Alto, CA, USA), equipped with a fluorescence detector, set on excitation wavelength of 532 nm and emission wavelength of 553 nm, and a Nucleosil C18 column, 150 x 3.2 mm, particle size 5 µm (Supelco Inc. PA, USA). Samples were eluted with 65 % (v/v) 25 mmol/l phosphate buffer pH 4.8, 35 % (v/v) methanol. A calibration curve was constructed using malonaldehyde bis(diethylacetal) as standard.

Antioxidant capacity assay

The antioxidant capacity assay was carried out as described previously (Berg van den *et al.*, 1999) with some modifications. The ABTS+• radical was produced by incubating a solution of 0.23 mmol/L ABTS and 2.3 mmol/L ABAP in 100 mmol/L sodium phosphate buffer pH 7.4 at 70°C until the absorption of the solution at 734 nm was between 0.680 and 0.720. Deproteination was obtained by mixing the sample with an equal volume of a solution of 10 % (w/v) trichloro-acetic acid. In the reaction of antioxidants with the blue/green ABTS radical, the blue green colour disappears. This decolourisation after 5 minutes was determined spectrophotometrically at 734 nm. The reduction in absorbance is related to that of trolox, a synthetic, hydrophilic vitamin E analogue, which gives the trolox equivalent antioxidant capacity (TEAC) value. The TEAC value was calculated as molar trolox equivalents of the sample.

Statistics

Statistical analyses of the scaled microarray data were performed with the Affymetrix Microarray Suite version 5.0 software package, applying Wilcoxon's signed rank tests to detect differences between arrays. Time effects of MDA and TEAC within each iron intervention, compared to baseline values obtained at $t=0$, were investigated with ANOVA Repeated Measures. Significant time differences were located with Scheffé post hoc analyses, including Bonferroni correction for multiple comparisons. Univariate Analyses of Variance were used to assess differences between the two iron dosages. Differences were regarded as significant if $P < 0.05$.

Results

Gene expression

After normalization of the microarray signals, 8542 gene reporters showed a significant expression in both pools at baseline. Another 476 gene reporters were marginally expressed according to the Affymetrix default cut-off values, whereas the remaining 13271 reporters were not expressed in the duodenal tissue samples.

The microarray analyses showed that the expressions of 89 reporters were consistently increased or decreased compared to baseline by both iron challenges in both pools; 61 genes were decreased (Table 1a), whereas 28 genes were increased (Table 1b).

Mapping the individual genes to shared biological pathways using MAPPFinder software revealed that, considering the biological processes observed in both pools, 6 biologically processes were mediated by both iron dosages. Three out of those six processes involved G protein coupled receptors, whereas the other three processes were associated with complement activation, calcium channels and cell cycle. The processes, which were mediated by the two interventions in both sample pools, are listed in Table 2. The table shows the smallest number of gene reporters, mediated by any pool in any intervention. Furthermore, it shows the number of gene reporters involved in each process, which were represented on the microarray chip, and the total number of genes involved in each process, as was defined by the databases described in the Methods section.

RT-PCR analyses of 7 selected genes showed good similarity with microarray analyses of the same genes, considering the direction of changes in gene expression profiles following the iron interventions (Table 3).

TBARS and TEAC

The TBARS concentration in intestinal fluid samples after administration of 80 mg iron increased significantly from $0.2 \mu\text{mol/L} \pm 0.16$ at baseline to $2.9 \mu\text{mol/L} \pm 1.2$, $2.3 \mu\text{mol/L} \pm 1.5$ and $2.4 \mu\text{mol/L} \pm 1.4$ at 30, 45 and 60 min respectively after the start of the iron perfusion ($P < 0.0001$). The TBARS concentration after administration of 400 mg iron increased significantly from $0.4 \mu\text{mol/L} \pm 0.5$ at baseline to $3.8 \mu\text{mol/L} \pm 1.1$, $4.1 \mu\text{mol/L} \pm 1.4$ and $3.8 \mu\text{mol/L} \pm 1.5$ at 30, 45 and 60 min after the start of the iron perfusion ($P < 0.001$). Univariate analysis of variance showed that TBARS production after administration of 400 mg iron was significantly higher compared to 80 mg iron ($P < 0.001$; Figure 1).

The TEAC concentration after administration of 80 mg iron increased from 597 $\mu\text{mol/L} \pm 187$ at baseline to 1032 $\mu\text{mol/L} \pm 609$ at 30 min after the start of the iron perfusion. Statistical analysis did not detect an overall time effect. The TEAC concentration after administration of 400 mg iron intervention increased significantly from 806 $\mu\text{mol/L} \pm 293$ at baseline to 1889 $\mu\text{mol/L} \pm 635$ and 1490 $\mu\text{mol/L} \pm 413$ at 30 and 45 min respectively after the start of the iron perfusion ($P < 0.05$). Univariate analysis of variance showed that TEAC after administration of 400 mg iron was significantly higher than after 80 mg iron ($P < 0.001$; Figure 1).

Figure 1. Lipid peroxidation and antioxidant capacity

Thiobarbituric Acid Reactive Substances (TBARS) concentrations, an indicator of lipid peroxidation, and Trolox Equivalent Antioxidant Capacity (TEAC), an indicator of total antioxidant capacity, in intestinal perfusion samples, collected over 15-min intervals during perfusion of a 40 cm-segment in the proximal small intestine in 8 subjects. Baseline values at $t=0$ were obtained after 180 min saline perfusion. Saline containing 80 (grey lines) or 400 mg (black lines) elemental iron as ferrous gluconate was perfused for 30 min, followed by saline perfusion for 60 min at 10 mL/min (mean $\pm$ SEM; * differences compared to baseline, $P < 0.001$).

Table 1a. Genes consistently downregulated by perfusion of a small intestinal segment with 80 and 400 mg iron, on two separate test days. Probe identification numbers (Probe IDs) refer to the Affymetrix reference number of the measured probe pairs

Probe ID	Gene title	Probe ID	Gene title
200617_at	IAA0152 gene product	209777_s_at	solute carrier family 19 (folate transporter), 1
201009_s_at	thioredoxin interacting protein	209782_s_at	D site of albumin promoter binding protein
201275_at	farnesyl diphosphate synthase	210095_s_at	insulin-like growth factor binding protein 3
201790_s_at	7-dehydrocholesterol reductase	210830_s_at	
201876_at	paraoxonase 2	211576_s_at	
202017_at	epoxide hydrolase 1, microsomal (xenobiotic)	211618_s_at	
202025_x_at	acetyl-Coenzyme A acyltransferase 1	211699_x_at	hemoglobin, alpha 1
202122_s_at	mannose 6 phosphate receptor binding protein	211745_x_at	hemoglobin, alpha 2
202433_at	UDP-galactose transporter related	211922_s_at	
202793_at		213706_at	
203414_at	monocyte to macrophage differentiation-associated	213787_s_at	
203633_at	carnitine palmitoyltransferase I, liver	214274_s_at	
203943_at	kinesin family member 3B	216474_x_at	
204039_at	CCAAT/enhancer binding protein (C/EBP), alpha	216894_x_at	cyclin-dependent kinase inhib. 1C (p57, Kip2)
204233_s_at	choline kinase	217414_x_at	hemoglobin, alpha 2
204266_s_at	choline kinase	217478_s_at	
204429_s_at	solute carrier family 2 (facilitated glucose/fructose transporter), 5	218094_s_at	chromosome 20 open reading frame 35
204544_at	alpha integrin binding protein 63	218421_at	hypothetical protein FLJ23239
204607_at	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	218688_at	DKFZP586B1621 protein
204760_s_at	nuclear receptor subfamily 1, group D, member 1	219398_at	protein FLJ20871 similar to FSP27
204997_at	glycerol-3-phosphate dehydrogenase 1 (soluble)	219534_x_at	cyclin-dependent kinase inhib. 1C (p57, Kip2)
205071_at	cytochrome P450 subfamily III (arachidonic acid epoxidase) pp 2	220070_at	hypothetical protein FLJ13798
205749_at	cytochrome P450, subfamily I (aromatic compound-inducible), pp 1	221529_s_at	plasmalemma vesicle associated protein
208108_s_at	glucose phosphate isomerase	221552_at	lipase protein
208684_at	coatomer protein complex, subunit alpha	221679_s_at	lipase protein
208998_at	uncoupling protein 2 (mitochondrial, proton carrier)	221951_at	
209122_at	adipose differentiation-related protein	31637_s_at	nuclear receptor subfamily 1, group D, 1
209123_at	quinoid dihydropteridine reductase	38158_at	extra spindle poles, <i>S. cerevisiae</i> , homolog of
209355_s_at	phosphatidic acid phosphatase type 2B	45288_at	lipase protein
209775_x_at	solute carrier family 19 (folate transporter), member 1	65630_at	Similar to RIKEN cDNA 5530601119 gene,
209776_s_at	solute carrier family 19 (folate transporter), member 1	209777_s_at	solute carrier family 19 (folate transporter), 1

Table 1b. Genes consistently upregulated by perfusion of a small intestinal segment with 80 and 400 mg iron, on two separate test days. Probe identification numbers (Probe IDs) refer to the Affymetrix reference number of the measured probe pairs.

Probe ID	Gene title	Probe ID	Gene title
201362_at	NS1-binding protein	208921_s_at	Sorcin
201368_at		208949_s_at	galectin 3
201427_s_at	selenoprotein P, plasma, 1	209301_at	carbonic anhydrase II
201695_s_at	nucleoside phosphorylase	210177_at	Tripartite motif-containing 15
201925_s_at	decay accelerating factor for complement	211548_s_at	Hydroxyprostaglandin dehydrogenase 15NAD
203287_at	ladinin 1	211962_s_at	
203574_at	nuclear factor, interleukin 3 regulated	212130_x_at	
203725_at	growth arrest and DNA-damage-inducible, alpha	213372_at	
203824_at	transmembrane 4 superfamily member 3	215363_x_at	
203914_x_at	hydroxyprostaglandin dehydrogenase 15-(NAD)	217761_at	SIPL protein
205769_at	fatty-acid-Coenzyme A ligase, very long-chain 1	219508_at	glucosaminyl (N-acetyl) transferase 3
205771_s_at	A kinase (PRKA) anchor protein 7	220639_at	hypothetical protein FLJ22800
205860_x_at	folate hydrolase (prostate-specific membrane antigen) 1	222303_at	
205969_at	arylacetamide deacetylase (esterase)	40016_g_at	KIAA0303 protein

Table 2. Processes in small intestinal mucosa, affected by iron administration.

Process	MAPP	Gene reporters changed / measured / total
Cell cycle	KEGG	6 / 9 / 104
GPCRs, Class C	GPCRDB Mapp	3 / 12 / 18
Calcium channels	Local Mapps	7 / 22 / 28
Complement activation, classical pathway	Local Mapps	4 / 12 / 16
Peptide GPCRs	Local Mapps	9 / 45 / 69
GPCRs, class A	GPCRDB Mapp	22 / 112 / 289

Processes most strongly mediated by the two iron interventions in both pools, identified by Mappfinder as described in the Methods section. The second column shows which database was used for the identification of the biological pathway. The number of mediated gene reporters, changed more than 2.3-fold by both iron interventions in both pools, the total number of gene reporters involved in each process and represented on the microarray chips (HG U133A), and the total number of gene reporters involved in each process as specified by the consecutive databases, are listed in the right hand column.

Table 3. Comparison of RT-PCR- and microarray results

GenBank		1 L	1 H	2 L	2 H
nm_005410	RT-PCR	1.30	1.90	2.04	1.56
	microarray	1.28	1.68	1.39	1.24
nm_001924	RT-PCR	1.68	2.92	2.56	2.44
	microarray	1.62	1.76	1.69	1.98
nm_002306	RT-PCR	1.56	1.96	2.26	3.20
	microarray	1.46	1.66	1.32	1.39
nm_005118	RT-PCR	7.06	13.53	8.32	11.80
	microarray	2.12	2.05	2.29	1.95
nm_000775	RT-PCR	-1.31	-1.29	-1.21	-1.36
	microarray	-1.38	-1.37	-1.12	-1.35
nm_003355	RT-PCR	-1.29	-1.17	-1.41	-1.44
	microarray	-1.28	-1.18	-1.36	-1.62
nm_003056	RT-PCR	-1.76	-1.75	-1.57	-1.62
	microarray	-1.71	-1.76	-1.44	-1.77

RT-PCR analysis of selected genes (top rows) and microarray data from the same genes (bottom rows). Data are expressed as fold-changes compared to baseline. The first column (1 L) shows the gene expression data from pool 1 after administration of the low dosage iron compared to baseline, column 1 H shows data from pool 1 after administration of the high iron dosage. The third and fourth column (2 L and 2 H)) show the data from pool 2 after administration of the low- and high iron dosage respectively.

Discussion

In the present study, state of the art-methods to study nutrient-gut interactions and nutrient-gene interactions in the human small intestine were combined to synchronously investigate several intestinal responses to luminal oxidative challenges, provoked by different iron dosages. Acute exposure of the proximal small intestine to either a low or a high dosage of ferrous iron induced dose dependent lipid peroxidation, as was indicated by TBARS concentrations, in the intestinal lumen and a synchronous increase in luminal antioxidant capacity. The acute, single-dose, iron challenge mediated gene expression of a number of genes. Both iron interventions strongly affected at least six distinct biological pathways on the transcriptional level.

We recently showed that perfusion of a 40 cm duodenum segment with 80 mg iron as ferrous sulfate induced lipid peroxidation and the release of an unknown antioxidant (chapter 3). The rise in antioxidant capacity following iron ingestion was not caused by ex vivo TBARS formation during the analytical assay, cell lysis of epithelial cells, glutathione, uric acid or vitamin C and was of non-protein origin (Troost *et al.*, 2003). The present study, applying ferrous gluconate, confirmed the catalyzing effects of ferrous iron administration in the small intestine to induce oxidative damage and the release of antioxidants into the intestinal lumen. At baseline, during the initial saline perfusion, TBARS were only marginally present. After ingestion of either the low or the high iron dosage, TBARS concentrations increased in a dose-dependent manner. This

increase was accompanied by a similar dose-dependent increase in TEAC. The TBARS and TEAC concentrations were very high in view of the highly diluted intestinal fluid with the saline solution used for perfusion of the intestinal segment. All samples consisted mainly of the ingoing saline solution, mixed with small amounts of intestinal secretions. This suggests that the iron administration resulted in powerful antioxidant responses. In an attempt to identify the origin of the antioxidant response, NMR spectra from samples with high and low anti-oxidant capacity were taken. Although differences between spectra were observed, it has not been possible to identify the compound(s) responsible for these differences (data not shown). Further analyses are required.

The cDNA chip used enabled simultaneous monitoring of the expression *in vivo* of more than 20,000 gene reporters. Among numerous processes that were slightly mediated by the iron interventions, six distinct processes were strongly regulated, as listed in Table 2. Three out of these six processes involved G protein coupled receptors (GPCRs). However, two of these three processes were identified using the GPCRDB database, whereas one was identified by local MAPPS, as was described in the Methods section. We cannot exclude that some gene reporters were attributed to one process in the GPCRDB database, and at the same time to the GPCR process identified by local MAPPS, hence causing an overestimation of the genes involved in GPCRs-associated processes. However, from the results it is clear that GPCRs were strongly mediated by the iron interventions. GPCRs comprise the largest superfamily of proteins in the body. Ligands for these receptors are biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions and proteases. All GPCRs are able to recruit and regulate the activity of intracellular G proteins, which regulate a large array of intracellular processes (Gether, 2000). The observation that GPCRs were regulated by iron induced oxidative stress may indicate a, yet unknown, function of G protein associated processes in response to cellular stress. The observation that complement activation is regulated by the iron administrations suggests that the iron-induced oxidative damage triggered immune activation in the duodenal mucosa. Complement activation has previously been associated with mucosal damage in ulcerative colitis (Ueki *et al.*, 1996), whereas it was also established that mucosal damage owing to autologous complement activation can be prevented by endogenous regulatory mechanisms (Andoh *et al.*, 1993). Furthermore, it was shown that intestinal epithelial cells are able to produce components of complement activation, and that this production can be mediated by cytokines in the intestinal epithelium (Andoh *et al.*, 1993). Increased complement activation, as observed in the iron perfusion experiments, may induce complement-induced damage. Future studies will need to elucidate the consequences of complement activation in duodenal mucosa. The functional role of the cell cycle- and calcium channels related processes, which were affected by the iron interventions, is not yet described to our knowledge, and needs to be elucidated. Interestingly, it was shown that mice fed a low selenium-diet, which is a trace mineral with antioxidant properties, appeared to have an increased activation of genes involved in cell cycle control (Rao, Puschner & Prolla, 2001). This suggested that selenium mediates cell cycle control, which may account for its anti-tumorigenic properties. The present observation that cell cycle processes were mediated by iron-induced oxidative

stress, in addition to the previous data about the effects of the antioxidant selenium on cell cycle control, suggests that this process may mediate the initiation of carcinogenesis if the oxidant/antioxidant balance is disturbed.

Genes involved in the regulation of iron absorption mechanisms have drawn the attention of many investigators, especially in the view of their causal role in the pathogenesis of hereditary haemochromatosis. Several proteins are involved in the direct regulation of iron uptake from the duodenal lumen. Divalent metal transporter 1 (DMT1, a transmembrane transporter for divalent metals including ferrous iron, plays an important role in the absorption of iron from the gut lumen into enterocytes, whereas ferroportin1 (FP1) is a transmembrane iron exporter molecule, involved in the basolateral iron transport from enterocytes into the circulation. In a recent study in mice, iron supplementation for two weeks downregulated the levels of transcripts of DMT1, ferroportin1 (FP1) and transferrin receptor 1 (TfR1), whereas feeding an iron-deficient diet resulted in an increase of these transcripts (Dupic *et al.*, 2002). Furthermore, duodenal gene expression of an iron responsive element-splice variant of DMT1 was significantly downregulated one hour after intragastric iron administration in rats (Frazer *et al.*, 2003). In the present study we showed that the gene expression of DMT1 and TfR1 tended to decrease by approximately 25% in both sample pools following administration of the low and the high iron dosage into the duodenum, although these genes were not regarded as significantly changed after statistical analysis (data not shown). Unfortunately, FP1 was not represented on the gene chip. Our findings contribute to the ongoing debate of the precise regulation of intestinal iron absorption. It is commonly acknowledged that high iron levels in the enterocytes downregulate DMT1 expression (Frazer *et al.*, 2003). The finding of the present study that acute iron ingestion may downregulate DMT1- and TfR1 expression suggests that intestinal iron concentrations directly mediate transcription of these proteins, as was shown previously only for DMT1 expression (Zoller *et al.*, 2001) (Frazer *et al.*, 2003).

The genome-wide microarrays used yielded information about the expression of a myriad of genes. The major advantage of this technique is the ability to synchronously monitor many biological processes. However, the present study design has some limitations. The duodenal mucosal tissue samples comprised a variety of mucosal and submucosal cell types. Changes in gene expression that only occur in one cell type were, therefore, diluted by the presence of other cell types and, hence, may not have reached significance. In addition, gene expression levels differ between individuals. Pooling samples from different volunteers and the inclusion of only those genes that were affected by both the low and the high iron dosage in both pools for further analysis, as was applied in the present investigation, reduced the effects of inter- and intra-individual variations in gene expression. We cannot exclude that, using this approach, some relevant genes that were mediated by the iron interventions were erroneously excluded from further analyses. On the other hand, the procedure followed to identify iron-mediated biological pathways minimized the risk of false positive results. The study results reveal the most important, strongest mediated processes. Finally, the microarray assay itself could have induced false-positive signals due to cross-hybridization or non-specific binding to the probe pairs. This seems highly un-

likely in view of previously published studies, in which the data of Affymetrix microarray chips were successfully compared to RT-PCR analyses (Gerhold *et al.*, 2001; Xiao *et al.*, 2001). In the present study, we also confirmed the microarray data of seven genes that were mediated by the iron interventions with RT-PCR analyses. Table 3 shows that the changes in gene expression profiles in both pools after both iron interventions were roughly the same, either applying RT-PCR or microarray analyses. Some differences between the two methods were present, as can be observed from the results of gene nm_005118. RT-PCR analysis showed a 13.53 fold change in the expression of this gene reporter, whereas microarray analysis showed only a 2.05 fold change in expression. This difference may be attributed to the low baseline level of this gene, which is a common concern in the interpretation of microarray results (Gerhold *et al.*, 2001), especially if results are expressed as fold change. Because all RT-PCR data show the same direction in changes in gene expression profiles, we assume that the microarray data provide valid gene expression profiles for all gene reporters. It was not possible to establish the functional consequences of the regulation of the processes, identified in Table 2. The impact of the iron interventions on gene expression profiles differed between the two pools, owing to biological, inter-individual variation. Future studies of the functional consequences of changes in the gene expression profiles, as identified in this study, need to be designed to determine the biological impact of the present investigation.

This study showed that iron administration in the human small intestine induced lipid peroxidation, and a strong rise in antioxidant capacity of the small intestinal secretions, in a dose-dependent fashion. Both iron dosages mediated the expression of many genes. Besides considerable inter- and intra-individual differences in gene expression profiles, we identified six processes, which appeared to be strongly and consistently regulated by iron administration or iron induced oxidative stress in the small intestine. Further studies are needed to establish the functional consequences of acute and chronic iron administration and oxidative stress in the human small intestine.

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CHAPTER 5

IRON SUPPLEMENTS INHIBIT ZINC- BUT NOT COPPER ABSORPTION *IN VIVO* IN ILEOSTOMY SUBJECTS

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Abstract

Background Iron supplements may inhibit intestinal zinc and copper uptake because these elements may compete for binding to a transporter molecule (DMT1), located at the apical side of the small intestine epithelium. This study aimed to quantify the interaction between different levels of iron administration and zinc and copper absorption in humans.

Design Eleven subjects with an ileostomy (55 ± 9 y) ingested a stable isotope-labelled zinc/copper solution containing 12 mg zinc (^{66}Zn and ^{67}Zn) and 3 mg copper (^{65}Cu) in the presence of 0, 100 or 400 mg iron as ferrous gluconate respectively on three different test days. Subsequently, 1 mg of ^{70}Zn was injected intravenously. Subjects collected ileostomy effluent for 24h and urine for 7d respectively. Zinc status and true zinc absorption were calculated from the urinary excretion of the zinc isotopes. Apparent copper absorption was calculated from ileostomy effluent excretion of the orally administered copper isotopes.

Results Zinc status did not differ between the experiments. Zinc absorption was significantly higher in the absence of iron compared with the concomitant ingestion of 100 or 400 mg iron (44 ± 22 , 26 ± 14 and 23 ± 6 % respectively, $P < 0.05$), whereas zinc absorption did not significantly differ between 100 or 400 mg iron. Apparent copper absorption was 48 ± 14 , 54 ± 26 and 53 ± 7 % in the presence of 0, 100 or 400 mg iron respectively, and did not differ between any of the experiments.

Conclusions Oral iron therapy may impair zinc absorption and thus zinc status in a dose-independent fashion, but does not affect copper absorption.

Introduction

Approximately 2 billion people worldwide suffer from iron deficiency (WHO, 1999). Iron deficiency is often accompanied by other nutrient deficiencies such as zinc and copper deficiencies, especially when the iron deficiency was caused by insufficient dietary intake of micronutrients as is the case in developing countries (Allen, 1994; Solomons & Ruz, 1997). As a result, supplements containing iron, multiple trace elements and minerals are used by millions of people worldwide. It is important to provide detailed information regarding safe upper limits of supplements to minimise adverse effects on mineral absorption caused by mineral-mineral interactions. It has long been recognized that iron metabolism interacts with the metabolism of several other micronutrients, such as zinc and copper. The discovery of Divalent Metal Transporter 1 (DMT1) (Gunshin *et al.*, 1997), provided an explanation of previous indications of the competitive absorption of iron, zinc and copper from the small intestine. Iron was shown to decrease zinc absorption in humans in a dose-dependent way when given in a water solution but not when given with a meal (Valberg, Flanagan & Chamberlain, 1984) (Sandström *et al.*, 1985). Iron supplements have been reported to depress zinc absorption in pregnant women (Simmer & Thompson, 1985), whereas lower serum zinc concentrations are observed in teenage pregnant women taking iron supplements (Dawson, Albers & McGanity, 1989). However, other human studies showed that iron supplementation did not alter markers of zinc status or zinc absorption (Donangelo *et al.*, 2002) (Davidsson *et al.*, 1995) (Sheldon *et al.*, 1985).

The iron-copper interaction may pose a health risk because marginal copper deficiency greatly increases the effect of iron deficiency (Snedeker & Greger, 1983) (Whittaker, 1998). Animal experiments have shown that iron supplementation diminishes copper status in rats and sheep (Yu *et al.*, 1995).

In the present study, the interactions between iron, zinc and copper, iron and isotopically enriched zinc and copper were administered orally in an aqueous solution after an overnight fast, as is generally advised to ensure maximal micronutrient absorption. Zinc and copper absorption were calculated from the appearance of the isotopically enriched stable isotopes in ileostomy effluent and urine. Subjects with an ileostomy were recruited to ensure complete recovery of test drinks. This study aimed to investigate the acute effects of moderate (100mg) and high (400mg) doses of iron routinely used in a clinical setting on the absorption of copper and zinc in adults.

Methods

Subjects

Eleven subjects (9 male, 2 female; $55 \text{ y} \pm 9$) with an ileostomy were recruited for participation. When the indication for proctocolectomy was Crohn's disease, subjects were permitted to the study only if they had been in remission for at least three years and less than 10 cm of the terminal ileum was removed. All participants were free of medication affecting normal functioning of the upper gastrointestinal tract, none had symptoms of an absorptive disorder or nutrient deficiencies. Additionally, all subjects were free of inflammation associated acute phase response of the immune system, as determined by measurement of blood serum C-reactive protein. Detailed information about the study was given orally and in writing. All subjects signed a written informed consent before participation. The Ethics Committee of the University Hospital Maastricht, Maastricht, The Netherlands approved the study.

Preparation of isotopes

The zinc isotopes ^{66}Zn (enriched to 99% abundance), ^{67}Zn (enriched to 90.6% abundance), ^{70}Zn (enriched to 95% abundance) were obtained as zinc metal (ChemGas, Boulogne, France). Both oral and intravenous zinc doses were prepared in the Dose Preparation Unit (DPU) at the Institute of Food Research. Elemental zinc was dissolved in concentrated HCl and evaporated to dryness. The precipitate was dissolved in trisodium citrate and the pH adjusted to 7 using sodium bicarbonate. The final solution was filtered through a $0.2 \mu\text{m}$ filter to remove pyrogens. The oral doses were aliquoted in the DPU, whilst the individual iv doses were sub-sampled, autoclaved and subjected to sterility testing at the Ipswich Hospital Pharmacy, Ipswich, UK.

The copper isotope (^{65}Cu , enriched to 99.8% abundance) was obtained as elemental copper foil (ChemGas, Boulogne, France) and individual oral doses were prepared in the DPU. Elemental copper was dissolved in conc. nitric acid (AristaR) on a hot plate at 150°C . The solution was evaporated to virtual dryness and then redissolved in concentrated HCl (AristaR). The solution was sub-boiled to dryness and the residue redissolved in 6M hydrochloric acid and removed from the heat. Sterile water was added and the pH adjusted to pH 5 with 1M NaOH. The solution was made up to the appropriate volume with sterile water and filtered through a $0.22\mu\text{m}$ filter. However, filtering proved to be progressively difficult and HCl was added to adjust the pH to 3.5 allowing the remaining solution to be filtered. The final solution was sub-sampled into individual doses in the DPU and stored at -20°C until required.

Study design

For a period of three days prior to the first test day and on the day of testing, subjects kept a weighed food intake diary to record their habitual diet. The food diary was used to ensure that volunteers consumed an identical diet prior to all three experimental days. The study was a randomised crossover design.

At 0800 h, following an overnight fast, ileostomy effluent and urine were collected in acid-washed containers at wake-up as baseline samples. A venous blood sample was taken from an antecubital vein in the forearm for measurement of serum ferritin and blood haemoglobin and haematocrit as indicators of iron status, and serum C-reactive protein to confirm the absence of inflammation associated acute phase response. After blood sampling, a test drink, as described below, was consumed, and subsequently all ileostomy effluent was collected for the following 24 h. Effluent samples were immediately frozen on dry ice and stored in plastic air-tight boxes at -20°C prior to analysis. Complete 24h urine collections were made for 7 days post-dosing and 100 mL fractions were also stored at -20°C until analysis. All sample containers were acid-washed prior to sample collection.

Test solutions

The test beverages contained 300 mL of an isotonic maltodextrin solution. To this test beverage, isotope solutions containing 7 mg ^{67}Zn , 5 mg ^{66}Zn and 3 mg ^{65}Cu were added. The test beverage was ingested on three occasions. Once without addition of an iron supplement, one with synchronous ingestion of a pill containing 100 mg iron as ferrous gluconate (Numico, Wageningen, The Netherlands) and once with synchronous ingestion of 400 mg iron as ferrous gluconate.

Zinc was abundantly present as ^{67}Zn and ^{66}Zn . Ingestion of 7 mg of ^{67}Zn was needed to ensure sufficient urinary ^{67}Zn enrichment. The, less expensive, ^{66}Zn was added to the test beverages to increase the amount of zinc to a total amount of 12 mg.

Immediately after oral administration of the test beverage, 1 mg ^{70}Zn was administered intravenously into an antecubital vein in the forearm. The exact amounts of oral and intravenous isotopes were determined for each individual. Subjects refrained from eating for 4 h but were allowed water '*ad libitum*' after one hour post-dosing. After 4 h post-dosing, volunteers consumed their habitual diet as described above.

Sample processing

All glassware, crucibles and other equipment or containers used during sample processing were acid-washed before use.

Ileostomy effluent was freeze-dried, autoclaved and ground using a grinder with no metal parts in the grinding basket. All 24-h effluent samples were pooled and homogenized and subsamples were ashed at 450°C for 48 h. Aliquots and an internal standard of ^{68}Zn were accurately weighed into crucibles and dissolved in 4 mol/L HCl while heating for 2 h on hot plates.

Urine aliquots were accurately weighed into 150 mL beakers, concentrated on hot plates, transferred to silica crucibles, heated to dryness and ashed at 450°C for 48 h. Subsequently the ash was dissolved in 0.5 mol/L HCl while heating for 2 h on hot plates.

Subsequently, zinc was extracted from urine samples using anion exchange chromatography. An anion exchange resin (Type AG1X-8, BioRad Laboratories, Hercules, CA) was soaked in deionised water for at least 24 h before use. Two mL of pre-soaked resin was packed into acid washed glass columns (1 mL pipette tips, Sarstedt, Nüm-

brecht, DE). The columns were connected to a peristaltic pump (Watson Marlow, Falmouth, UK) with a flow rate of 1 mL/min and washed with 2 mol/L HNO_3 for 1 h to remove any minerals. The resin was reconstituted to the chloride form using 0.5 mol/L HCl, which was pumped through for 60 min. The sample solutions were loaded onto the columns, columns were washed with 0.5 mol/L HCl during 30 min and finally zinc was eluted with 1 mol/L HNO_3 . This fraction was collected and the total zinc concentration was adjusted to 1 ppm before for zinc isotopic enrichment determination using Micromass "Isoprobe" Multi Collector Inductively Coupled Plasma Mass Spectrometer (MC-ICP-MS) as described below. For copper extraction from the ileostomy fluid samples, pre-soaked resins were washed with 2 mol/L HNO_3 for 1 h and regenerated into the chloride form by pumping 6 mol/L HCl for 1 h. Subsequently samples were loaded and the columns were washed with 6 mol/L HCl for 30 min. Copper was eluted with 2.5 mol/L HCl, dried down under a hot lamp and made up for MS-ICP-MS analysis in 2% ultra pure HNO_3 .

Stable copper- and zinc isotope ratios were determined using a MC-ICP-MS combined with a Cetac "Aridus" desolvator at the Institute of Food Research (Norwich, UK). The samples were run with bracketing standards and instrument blanks. For correction of instrumental mass bias a simple linear correction between sample and bracketing standards was applied. For reference copper NIST 976 (certified for isotopic composition) was used in the Cu analysis. For zinc, Spectrosol (BDH, Poole, UK) was used for reference in absence of a certified isotopic standard and the Zn isotopic composition was assumed to be the average natural composition as defined elsewhere (Rosman & Taylor, 1998).

The external reproducibility of the calculated Zn isotope enrichment, based on the isotopic variation in the baseline urine samples, was 0.6, 4.9 and 10.1 % for the 66/64, 67/64 and 70/64 isotope ratios respectively. The external reproducibility of the calculated copper isotope enrichment, based on the baseline ileostomy effluent samples, was 4.0 %.

Calculations

Zinc absorption

The mass spectrometric ratios of the urine samples, measured by the MC-ICP-MS, were converted into mole fractions of recovered dose (oral and IV) using the equations set out in Lowe et al (Lowe et al., 1997). It was assumed that 48 hours after dose administration, the zinc from the oral and IV doses would have equilibrated and therefore any 24 hour pooled sample would be adequate to calculate the true zinc absorption of the oral dose. Thus, for the second 24-hour sample ($t=48\text{h}$) this is given by:

$$\text{True Absorption (t=48h)} = \frac{\text{mole fraction}_{\text{oral}}}{\text{mole fraction}_{\text{IV}}} \cdot \frac{\text{dose}_{\text{IV}}}{\text{dose}_{\text{oral}}}$$

Where, "mole fraction_{oral}" and "mole fraction_{IV}" are the mole fractions of the oral and IV doses (respectively) from a single 24-hour pooled urine sample collected at least 48 hours post dose. Also, "dose_{oral}" and "dose_{IV}" are the quantities of labelled zinc given as oral and IV doses.

This can obviously be repeated for all subsequent 24-hour samples (t=72h, 96h, 120h, 144h, 168h). The final true absorption value can then be calculated as the mean of these six values.

Copper absorption

Since copper only has two stable isotopes, faecal monitoring is the method used to calculate the apparent absorption. The details of this are contained in a separate publication (Harvey *et al.*, 2002). Briefly, the following formula was used:

$$\text{Apparent Absorption} = \frac{\text{dose}_{\text{oral}} - \text{recovered}_{\text{oral}}}{\text{dose}_{\text{oral}}}$$

Where, “recovered_{oral}” is the quantity of copper from the oral dose found in the faeces and “dose_{oral}” is the quantity of copper found in the oral dose.

Exchangeable Zinc Pool (EZIP)

This method is based on the technique developed by Miller *et al* (1994). It is assumed that the endogenous zinc contained in the pool exchanges with any newly absorbed zinc in the plasma within 2 days. As mentioned previously, it is possible to use the mass spectrometer ratios to calculate the mole fractions of the individual sources of zinc in the study. These are the oral and IV tracers and the naturally abundant (NA) tracee. Enrichment in any particular urine sample is defined as the ratio of the mole fraction of zinc from the IV source (mole fraction_{IV}) to the mole fraction of zinc from the NA source (mole fraction_{NA}).

$$\text{Enrichment} = \frac{\text{mole fraction}_{\text{IV}}}{\text{mole fraction}_{\text{NA}}}$$

By plotting the natural logarithm of this ratio against time (for t>48hours), fitting a straight line through the data and extrapolating back to t=0, the size of the EZIP can be obtained. This assumes that the entire IV dose was equilibrated in the EZIP instantaneously and that extrapolation back to t=0 compensates for any loss of IV dose from the EZIP.

Statistics

Exchangeable zinc pool, zinc and copper absorption during the three different interventions were compared with ANOVA for repeated measures. Differences were regarded as statistical significant if P<0.05. Statistically significant results were further analysed using Scheffé post hoc tests (P<0.05). Statistics were computed using the Statview 5.0 software package for the Macintosh.

Results

All volunteers had normal iron status; the average serum ferritin was 117.1 ± 85.4 µg/L, blood haemoglobin was 9.5 ± 0.7 mmol/L and blood haematocrit was 44.2 ± 3.3 L/L, whereas C-reactive protein was below 9 mg/L in all volunteers.

The size of the total exchangeable zinc pool was 126 ± 28 , 120 ± 27 and 119 ± 35 mg on the day of ingestion of 0, 100 or 400 mg iron respectively. No statistical significance in EZP was observed between any of the experimental days.

All subjects completed the intervention study. The data from one volunteer for one experimental day were withdrawn from further analyses due to contamination of the samples.

True zinc absorption, calculated from urinary zinc isotope excretion, was significantly lower in the presence of 100 and 400 mg iron compared to the zinc absorption in the absence of iron (average $\pm$ sd zinc absorption was $26.4\% \pm 14.4$, $22.9\% \pm 6.4$ and $44.5\% \pm 22.5$ of the administered dose respectively; $P < 0.05$). Zinc absorption did not differ between the 100- and 400 mg iron intervention (Figure 1).

Apparent copper absorption was $47.8\% \pm 13.6$, $54.1\% \pm 26.1$ and $52.8\% \pm 7.3$ of the administered dose (average $\pm$ sd) in the presence of 0, 100 or 400 mg iron as ferrous gluconate respectively. No significant differences were observed between any of the interventions (Figure 2).

Figures 1 and 2. Zinc and copper absorption

True zinc absorption and apparent copper absorption from a water solution containing 12 mg zinc and 0, 100 or 400 mg iron as ferrous gluconate (mean $\pm$ sem, $n = 11$, ANOVA (Scheffé); * $p < 0.05$ vs 0).

Discussion

The present study clearly shows that iron supplements decrease zinc absorption when given in an aqueous solution. The marked drop in zinc absorption in the presence of 100 and 400 mg iron as ferrous gluconate shows that chronic oral iron supplementation increases the risk of developing or maintaining zinc deficiency. Previous studies have shown that iron, zinc and copper compete for absorption if given as an aqueous solution (Sandström *et al.*, 1985; Valberg *et al.*, 1984). However, this was not the case when iron and zinc were given with a meal, although data from different studies are equivocal (Davidsson *et al.*, 1995; Sandström *et al.*, 1985; Sheldon *et al.*, 1985; Valberg *et al.*, 1984).

The earlier supplementation studies warrant further investigation of iron-, zinc- and copper absorption interactions *in vivo* in humans (Sandström, 2001). In the present study we used a validated and accurate *in vivo* model to assess zinc and copper absorption and at the same time measured the exchangeable zinc pool as a meaningful indicator of zinc status.

During the 24 hours ileostomy effluent collection period, zinc isotopes were excreted into the ileostomy effluent. This zinc was not only the unabsorbed fraction of stable isotope-labelled zinc that was administered via the test meal but also absorbed zinc isotopes, which were rapidly re-excreted in the intestinal lumen. Calculation of zinc absorption from zinc isotopes enrichment in ileostomy effluent would therefore have caused underestimation of zinc absorption. To avoid this problem, zinc absorption was determined using urinary excretion of stable zinc isotopes.

The observed effect of iron on zinc absorption may deteriorate zinc status. Zinc deficiency is associated with an impaired immune function, growth, appetite and wound healing and mucosal dystrophy. The absence of a dose-response relationship between the two iron dosages indicates that the mechanism by which iron and zinc compete for absorption seems to be saturated by ingestion of 100 mg of ferrous iron.

The low, 100 mg iron dosage is medically used as an oral supplement in the treatment of iron deficiency anaemia, whereas the amount of zinc present in the test drink equals the average habitual daily intake of zinc and is also present in comparable quantities in food supplements. The high, 400 mg iron dosage is occasionally prescribed for rapid oral iron replenishment therapy.

The interaction between iron and zinc absorption may be explained by competitive binding to the transporter protein Divalent Metal Transporter 1 (DMT1, formerly called DCT1 or Nramp2) located at the apical membrane in the small intestine, or by an iron-induced decrease in expression of DMT1. Although the exact mechanism of iron absorption is still under investigation, it is clear that DMT1-mediated iron transfer accounts for most iron absorption from the intestine (Andrews, 1999; Gunshin *et al.*, 1997). After reduction by a ferric reductase, ferrous iron crosses the apical membrane of the gut by binding to DMT1. Furthermore, DMT1 also transports other divalent cations such as zinc and copper (Andrews, 1999; Gunshin *et al.*, 1997). Its expression is regulated by the iron concentration in epithelial cells of the small intestine (Frazer *et al.*, 2003; Moos, Trinder & Morgan, 2002). A decrease in DMT1 expression may have caused the observed decrease in zinc absorption. It is, however, not likely that a single dose of iron significantly affects DMT1 expression in the proximal small intestine where most iron absorption takes place, especially considering the relatively short transit period of this segment of the intestine. Furthermore, recent data suggest that the shared absorption pathway for iron and zinc is distinct from binding to DMT1, although the actual absorption mechanism remains to be elucidated (Yamaji *et al.*, 2001). However, in that study, using Caco-2 cells, the effects of 50 and 100 μ M iron respectively on zinc absorption were investigated, in contrast to the millimolar iron concentrations, which were applied in the present study. Because different absorption routes may be involved in the presence of higher iron concentrations, the data from the *in vitro* study may not fully apply to the present *in vivo* investigation.

We showed competitive absorption of iron and zinc. The data show that zinc uptake in the human small intestine is not solely dependent on an absorption route that competes with iron. This confirms data from previous *in vitro* studies that identified several specific zinc transporters, which may serve an important role in mediating zinc absorption from the gut lumen (Cragg *et al.*, 2002; Lioumi *et al.*, 1999). Future research is needed to identify functional zinc transporters in the brush border membranes in humans.

The absence of a dose-response relation between the two iron dosages suggests that the mechanism by which iron and zinc compete for absorption is saturated by ingestion of 100 mg of ferrous iron.

The presence of a feedback mechanism of zinc status mediating zinc absorption, as is the case for iron, is speculative but may indeed be present. Zinc status may change within days as a result of the diet consumed. Therefore, in the present study all subjects were instructed to maintain and record their habitual diet during the three days prior to and on the first test day and to repeat the same diet during the following two test periods to minimize confounding effects of zinc status on zinc absorption. Zinc status was assessed by measuring EZP which provides an accurate, validated indicator of zinc status (Miller *et al.*, 1994). This indicator of zinc status was the same at each test period. Hence, the results of the present study were not affected by changes in either zinc status. Iron status was assessed at the start of the first experiment. It was assumed that iron status does not change within a few weeks provided the same habitual diet is consumed.

It may be concluded that zinc absorption is impaired by both the low and the high dosage of iron. The absorbed zinc fraction in the iron intervention experiments was transported from the gut lumen by mechanisms, which do not compete with iron.

Previous human and animal studies have shown that increased iron intake is associated with decreased serum copper levels and the corresponding activity of cuproenzymes. Animal studies provide indications of competitive absorptive mechanisms for iron and copper. Moderate iron supplementation results in decreased copper levels and copper absorption in rats (Yu *et al.*, 1995). In pregnant rabbits with marginal Cu intake, high iron intake resulted in a very severe Cu deficiency, which causes serious problems for the neonate. In humans, iron-folate supplements decreased copper status (Burns & Paterson, 1993), whereas zinc supplementation lowered iron and copper status in adult females (Yadrick, Kenney & Winterfeldt, 1989). These studies do not provide direct proof for nutrient absorption interactions in the intestine. In the present study, we showed that iron administration does not inhibit copper absorption. This finding is supported by a previous study in Caco-2 cells. It was shown that copper uptake is not merely mediated by DMT1, but that another mechanism must be involved in copper uptake (Arredondo, Uauy & Gonzalez, 2000). Recently, a newly discovered human copper transporter (hCtr1) was identified and characterized (Lee *et al.*, 2002). This protein serves an important role in cellular copper uptake from the plasma, and may also prove to be important in copper absorption from the intestine. However, the exact mechanism of copper absorption in humans remains to be elucidated.

Copper absorption was determined in the present study by the assessment of the intake/output balance of copper isotopes. It was not possible to apply the technique of urinary isotope excretion to measure copper absorption as was applied for calculation of zinc absorption because copper only has two stable isotopes forms, which disables the intravenous administration of a third stable copper isotope. Furthermore, a relation between urinary copper excretion and copper absorption or copper status has not been established. The results of the present study show that, despite previous findings, the DMT1 route is not a rate limiting factor in copper absorption *in vivo* in humans. This confirms an important role for other copper trans-porters such as hCtr1 in the small intestine.

The ileostomy patients recruited for participation had a normal functioning small intestine, as shown by normal serum C-reactive protein concentrations and medical interviews. Iron-, zinc- and copper absorption mainly takes place in the small intestine. The ileostomy model provides an accurate model to determine true absorption of nutrients (Konings *et al.*, 2002; Langkilde *et al.*, 1990). It guarantees full test meal recovery, whereas complete faecal collection is often problematic in healthy volunteers. Unfortunately, it cannot be applied to estimate zinc absorption, owing to an unknown rate of re-excretion of absorbed stable zinc isotopes, as mentioned above.

We conclude that therapeutic amounts of iron inhibit zinc- but not copper absorption in the human small intestine. Iron supplements may impair zinc status, especially in populations with inadequate dietary micronutrients intake. This should be taken into careful consideration when micronutrient supplements are indicated.

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CHAPTER 6

GASTRIC DIGESTION OF BOVINE LACTOFERRIN *IN VIVO* IN ADULTS

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Abstract

Background Lactoferrin, an iron-binding glycoprotein present in milk and other endocrine and exocrine secretions, may exert a number of physiologic effects in the intestine. To study the effects of oral lactoferrin supplementation *in vivo* in the gastrointestinal tract, information about the gastric survival of lactoferrin *in vivo* is of importance.

Design We tested twelve healthy volunteers (age 21 ± 0.3 y) on three separate days according to a randomized cross-over design. A test drink containing 4.5 g of bovine lactoferrin (20 % iron saturated; apoLF) in the presence of a gastric pH buffer (0.1 mol/L sodiumcitrate / citric acid) (apoLFbuf), apoLF without the buffer (apoLF) or iron-saturated lactoferrin (holoLF) was administered into the stomach using nasogastric intubation.

Results Gastric emptying rate, determined by a marker dilution technique, did not differ between any of these drinks. Substantial gastric survival of lactoferrin, analyzed by gelpermeation chromatography under denaturing conditions, was observed after ingestion of all test drinks (64 %, 62 % and 79 % of the lactoferrin in the apoLFbuf, apoLF and holoLF test drink respectively survived gastric transit). Addition of the gastric pH buffer resulted initially in a lower intragastric pH because of its hydroxide buffering effect. However it failed to elevate intragastric pH during a prolonged period of time in order to inhibit intragastric lactoferrin breakdown.

Conclusion We conclude that, after oral administration, a substantial amount of apoLF and holoLF survives gastric transit.

Introduction

Lactoferrin is a protein of the transferrin family. It is present in milk, saliva, tears, bile, blood plasma, mucosal and genital secretions (Britigan, Serody & Cohen, 1994; Levay & Viljoen, 1995). Lactoferrin is able to bind ferric iron (Fe^{3+}) with a high affinity, even in an acidic environment (Anderson *et al.*, 1989; Lönnerdal & Iyer, 1995). Because of this strong iron binding capacity, a number of physiologic effects have been proposed, as reviewed extensively by several investigators (Baker *et al.*, 1991; Iyer & Lönnerdal, 1993; Levay & Viljoen, 1995; Lönnerdal & Iyer, 1995).

Lactoferrin is proposed to act as an antioxidative (Baldwin, Jenny & Aisen, 1984; Davidson & Lönnerdal, 1989; Gutteridge *et al.*, 1981). Iron bound to lactoferrin may not be able to catalyze Fenton chemistry. This however has not yet been investigated *in vivo*. Lactoferrin is also involved in the immune system of the body. It is released by neutrophil specific granules (Davidson & Lönnerdal, 1989). Other cells of the immune system, macrophages and monocytes, can bind lactoferrin by specific lactoferrin receptors on their cell surfaces which enables the presence of lactoferrin at sites where the immune system is active. Several studies were able to show a regulatory function of lactoferrin on interleukin-6 and tumor necrosis factor alfa *in vivo* in mice (Machnicki, Zimecki & Zagulski, 1993; Zimecki *et al.*, 1998). Also, lactoferrin was shown to exert antibacterial activity *in vivo* (Zagulski *et al.*, 1998a; Zagulski *et al.*, 1998b) in mice and recently lactoferrin ingestion with food was shown to facilitate dermatophytosis cure in guinea pigs (Wakabayashi *et al.*, 2000). Lactoferrin is proposed to serve a role in facilitating iron absorption from the gut by a mechanism, which is still poorly understood. Previously, both a facilitating effect (McMillan, Landaw & Oski, 1976) and an inhibitory effect (Vet & Gool, 1974) of lactoferrin on iron absorption were described. These findings indicate that lactoferrin supplementation may be of value in patients prone to intestinal damage, such as people undergoing oral iron therapy, patients with Inflammatory Bowel Disease or patients suffering from intestinal stress in general.

The effects of oral lactoferrin supplementation could be potentially diminished to a certain extent by gastrointestinal breakdown of the lactoferrin molecule. It is not known to what extent lactoferrin is digested in the stomach or in the intestine *in vivo*. *In vitro* incubation of lactoferrin with proteolytic enzymes results in the formation of several lactoferrin fragments. It was shown that lactoferrin is less rapidly digested in gastric juice than casein and transferrin in an acidic environment (Britton & Koldovsky, 1989). An important factor interfering this degradation process is the degree of iron saturation of the lactoferrin. Apolactoferrin, with an iron saturation of approximately 20 %, is more easily digested than hololactoferrin (100 % iron saturated) (Brines & Brock, 1983; Brock *et al.*, 1976). As the biological activity of lactoferrin is located in the intestine it is necessary to look at survival of the molecule during passage through the stomach.

The present study was designed to study the extent of gastric degradation of lactoferrin *in vivo*. Also, the effect of buffering gastric pH on possible breakdown of lactoferrin was studied.

Methods

Subjects

Twelve healthy volunteers (5 males, 7 females, age 21.3 ± 0.3) without a history of gastrointestinal complaints were recruited for this study. All subjects received full information about the protocol and gave their written informed consent before participation. This study was approved by the Ethics Committee of Maastricht University, Maastricht, The Netherlands.

Study design

Subjects were tested according to a randomized cross-over design with a washout period of at least 7 days. On the evening prior to testing, all subjects were instructed to consume a standardized meal *ad libitum* (rice and sweet/sour sauce, Uncle Ben, The Netherlands). After an overnight 12 hours fasting period, subjects arrived at the laboratory. Subjects remained seated until the end of the experiment.

After arrival, a nasogastric tube was inserted. Via the nasogastric tubing, the stomach was emptied and rinsed with water until no further residue was obtained. A recovery test was done for control of the tube tip position (Hassan & Hobsley, 1970). Forty-five minutes after gastric aspiration, a liquid test drink (composition described below) was administered in the stomach through the tube. Subsequently the double sampling technique determining gastric emptying rate of a test drink was applied. This method was described in detail previously (Beckers *et al.*, 1988; Beckers *et al.*, 1991; George, 1968). According to this technique, 188 mg of the inert marker Polyethylene Glycol(PEG; PEG 4000, Sigma, Germany) was added to each test meal. After ingestion of the test drink, gastric content was mixed thoroughly by aspiration and reinjection of gastric content during 1 minute and 5 mL of gastric contents were sampled for measurement of total gastric content volume. At each 10-min interval, 2.5 mL of the gastric content was aspirated, and 5 mL PEG solution (6 g/100 mL water) was added to the gastric content via the nasogastric tube. Subsequently, gastric content was mixed thoroughly, and 2.5 mL was aspirated. This protocol was followed at 10 min, 20 min and 30 min respectively after administration of the test drink. The PEG concentration before and after addition of the PEG solution was measured. From these data the total volume of gastric contents at each time point was calculated (George, 1968). Also, the amount of test meal still present in the stomach and the amount of gastric secretion per 10-min intervals was calculated using calculations as described by Beckers *et al.* (Beckers *et al.*, 1988). In the samples obtained at 10-min intervals before adding the PEG solution, the amount of intact lactoferrin was determined as described below. After aspiration, each sample was put directly into liquid nitrogen and stored at -80°C until analysis to stop degradation processes after sampling.

Test drinks

Three test drinks were investigated in a randomized cross-over order. Each beverage consisted of a 300 mL 80 g/L maltodextrin (MD20, AVEBE, Veendam, The Netherlands) solution in water with 4.5 g bovine lactoferrin (LF) (DMV international, Veghel,

The Netherlands) dissolved. The lactoferrin had an iron saturation degree of approximately 20 % (apolactoferrin) and 100 % (hololactoferrin) respectively. Apolactoferrin was ingested on two occasions. Once without a buffer and once in the presence of a citrate buffer in order to buffer gastric pH. The citrate buffer was made by mixing 0.1 mol/L citric acid monohydrate with 0.1 mol/L trisodiumcitrate dihydrate in a volume ratio of 56:44 mL/L test drink respectively. Citric acid was purchased from Merck (Darmstadt, Germany), trisodiumcitrate was purchased from Ferak (Berlin, Germany).

Analytical methods

PEG concentration in the gastric juice samples was determined spectrophotometrically using a spectronic 1001 device (Bausch en Lomb, Bergen op Zoom, the Netherlands). Carbohydrates and proteins were removed by precipitation. In the test drinks and in each sample pH was measured using a standard pH analyzer (radiometer, Copenhagen, Denmark).

A new method was developed and validated to measure LF and LF digestion fragments in gastric samples. This method uses gel permeation chromatography under denaturing conditions. We found that LF degradation may proceed on the column in samples containing a relatively large proportion of gastric juice compared to residual test drink. When the sample contained more than 50 % gastric juice, the elution from the column had to occur within 5 hours to prevent LF degradation by gastric pepsin at the low pH of the elution buffer.

Sample pretreatment: 100 μ L gastric juice or LF standard are mixed with 450 μ L 8 M urea (Merck, Germany), pH 8.5. After solubilization 450 μ L of a mixture of H₂O / acetonitrile / trifluoroacetic acid (600/ 400/ 1 v/v) is added. The resulting solution is then filtered over 0.2 μ m PVDF filter (Gelman Sciences). 20 μ L is subsequently applied to the column (Shodex KW-803; Showa Denko) and eluted with the mixture of water / acetonitril / TFA in 25 min at a flowrate of 0.6 mL/min (Pharmacy / LKB HPLC). Proteins and peptides are detected at 220 nm. Intact LF elutes after about 11 min, whereas LF fragments elute later. Calibration of counts for intact LF was done with concentrations between 1.3 and 13 mg/mL and a straight line was obtained. Lactoferrin fragments between 9 and 80 kDa fitted a straight line obtained with protein MW markers (LF, BSA, β -lactoglobulin, α -lactalbumin and glycomacropeptide).

Calculations

Calculations of gastric emptying and secretion rates were based on the formulas proposed by Beckers *et al.* (Beckers *et al.*, 1988). For these calculations, PEG concentrations in the gastric juice samples were used to determine gastric content volume at each time point and gastric secretory rate at 10-min intervals. A gastric emptying curve was constructed according to a non-linear regression formula using specialized software (GraphPad Prism, San Diego, USA). From this curve, gastric emptying half time of the test drink was determined.

The amount of intact lactoferrin entering the intestine was determined by calculating the area under the curve (AUC) of the gastric emptying curve and gastric lactoferrin

concentration. The AUC of the gastric emptying curve was considered to represent emptying of the ingested amount of 4.5 g lactoferrin into the small intestine. Hence, gastric lactoferrin breakdown was calculated by subtracting the AUC of lactoferrin breakdown from the AUC of the gastric emptying curve. Lactoferrin delivery to the intestine was calculated by subtracting the gastric lactoferrin breakdown from the ingested amount of 4.5 g lactoferrin.

Statistics

Differences in gastric emptying and in gastric pH were analyzed using one-way ANOVA for repeated measures with Scheffé post-hoc testing. Differences in lactoferrin appearance in the small intestine between the three drinks were analyzed using ANOVA factorial analysis with the amount of lactoferrin as the dependent variable, test drink as factor and volunteer as covariate. Differences were considered as being significant at the probability level of $P < 0.05$. All statistical analyses were carried out using Statview software (SAS institute, Cary, NC, USA)

Results

Gastric emptying rate is expressed as gastric emptying half time ($t_{1/2}$) of the test drink. Gastric emptying half times were 6.79 ± 2.36 , 8.46 ± 4.07 and 7.03 ± 2.14 min for apoLFbuf, apoLF and holoLF, respectively (mean $\pm$ sd). Gastric emptying rate did not differ between any of the test drinks (One-way ANOVA).

The starting pH of the apolactoferrin test drink in the presence of the pH buffer was 5.95, the starting pH of the apolactoferrin solution without buffer was 7.12 and starting pH of the hololactoferrin solution without buffer was 7.10. Intragastric pH of the apolactoferrin solution with the citrate buffer during the period of testing was significantly lower directly after ingestion of the test drink compared to both the apo- and the hololactoferrin solution without buffer. Ten minutes after ingestion intragastric pH was still lower following ingestion of the apolactoferrin solution with buffer compared to the hololactoferrin, but not compared to the apolactoferrin drink. Twenty and thirty minutes after administration, no differences were observed in intragastric pH between any of the test drinks. Also, no differences in pH were observed at any time between the non-buffered test drinks (Figure 1).

After 30 min, all test drinks were nearly completely emptied into the intestine. During this period 64, 62 and 79 % respectively of the lactoferrin present in the apoLFbuf, apoLF and holoLF test drinks entered the intestine in the intact form. No statistical differences in lactoferrin survival were observed between any of the test drinks although the hololactoferrin solution tends to be more resistant to degradation than the apolactoferrin solution ($P = 0.09$) (Figure 2).

Figure 1. Acidity of gastric fluid

Intra-gastric pH after ingestion of 4.5 g bovine apolactoferrin with citrate buffer (◇), 4.5 g bovine apolactoferrin without buffer (■) and 4.5 g bovine hololactoferrin without buffer (Δ). All drinks contain 80 g/L maltodextrin in 300 mL water. Values are means + sd, n = 12. * *P* < 0.05, drink 1 vs. drink 2; # *P* < 0.05, drink 1 vs. drink 3.

Figure 2. Lactoferrin emptied from the stomach

Total amount of intact bovine lactoferrin emptied from the stomach, expressed as a percentage of the amount of bovine lactoferrin intragastrically administered. Values are means + sd, n = 12 (all n.s.).

Discussion

All test drinks were emptied from the stomach at the same rate. Within about ten min half of all beverages was already emptied. Because of this rapid gastric emptying of the lactoferrin during which only a small portion of the lactoferrin was digested a significant amount of lactoferrin entered the small intestine in the intact form.

Iron binding was shown previously to stabilize the bovine and human lactoferrin molecule (Brines & Brock, 1983; Brock *et al.*, 1976), causing hololactoferrin to be less susceptible to degradation than apolactoferrin. This was shown after incubation with trypsin and chymotrypsin. We did not observe a significant difference in degradation rate between any of the test drinks although hololactoferrin tended to be more resistant to degradation than apolactoferrin ($P = 0.09$). This indicates that the observed difference in susceptibility to degradation between apo- and hololactoferrin from these *in vitro* studies is not reflected in the same manner in our *in vivo* study of intragastric degradation of lactoferrin, in which pepsin probably is the major cause of LF degradation. One has to bear in mind that the apolactoferrin used in the present study was 20 % iron-saturated. Considering that iron binding to lactoferrin causes a diminished susceptibility of lactoferrin to digestion, apolactoferrin containing no iron may be digested in the stomach more rapidly than the apolactoferrin used in the present study. Other investigations showed a minor degradation of lactoferrin both *in vivo* in the entire digestive tract in newborns (Spik *et al.*, 1982) as well as *ex vivo* in gastric juice of preterm infants (Britton & Koldovsky, 1989), although the latter study did show some degradation. In the present study we show partial lactoferrin degradation in the adult gastrointestinal tract *in vivo*.

Intragastric pH was higher during the first ten minutes after ingestion of both the apo- and the hololactoferrin drink without citrate buffer compared to the apolactoferrin solution with addition of a 0.1 mol/L sodiumcitrate buffer. The buffer was responsible for keeping intragastric pH relatively constant throughout the test. After twenty minutes, most of the citrate buffer was emptied from the stomach in concordance with the rest of the beverage, causing a decrease in buffering capacity and consequently intragastric pH was determined mainly by gastric acid secretion. From *in vitro* work done at our lab we know that lactoferrin is resistant to degradation at a pH above 4. We observed this in an experiment in which we incubated lactoferrin in gastric juice *ex vivo*. This can partly be explained by the previous finding that iron is released from lactoferrin at pH lower than 4.0 (Mazurier & Spik, 1980). Iron is known to stabilize the lactoferrin molecule. Thus, iron release causes the lactoferrin molecule to be more prone to digestion (Brines & Brock, 1983; Brock *et al.*, 1976). Addition of the citrate buffer failed to prevent gastric breakdown of lactoferrin in the present study because the buffer was emptied before it could be effective in preventing further acidification of gastric contents. Although gastric pH did not fall below 4.0 until the last 10 min of the experiment, when most of the test drink was already emptied from the stomach, we observed a considerable lactoferrin breakdown in the stomach. Hence, intragastric lactoferrin digestion *in vivo* occurs also at a pH higher than 4.0. This indicates a difference between the results of *in vitro* and *in vivo* experiments.

Intact lactoferrin, which has a size of approximately 79 kDa consists of two lobes: the C- and the N-lobe. Incubation of lactoferrin with pepsin leads to formation of a 39.5 kDa fragment, most likely the C-terminal half of lactoferrin (Bluard-Deconinck *et al.*, 1978). After incubation of lactoferrin *ex vivo* in gastric juice of preterm infants, lactoferrin fragments of 33, 34, 41 and 42 kDa were formed, probably also representing half-molecules of the lactoferrin molecule (Britton & Koldovsky, 1989). The N-terminal half of lactoferrin was more prone to digestion by pepsin than the C-terminal half (Line, Sly & Bezkorovainy, 1976). After incubation with pepsin, the remaining C-terminal half of the lactoferrin molecule still has iron-binding capacity. However, after incubation with trypsin and chymotrypsin two fragments were obtained; the C-terminal and the N-terminal half of lactoferrin (Bluard-Deconinck *et al.*, 1978). Both of these lactoferrin fragments are still able to bind iron ions. In other studies, *in vitro* incubation of lactoferrin with trypsin causes the lactoferrin molecule to break down to fragments with molecular sizes of 20, 30, 40 and 50 kDa (Legrand *et al.*, 1990). Also, after lactoferrin ingestion in adult mice, lactoferrin fragments containing a peptide called lactoferricin were detected in the faeces of these mice. Lactoferricin, also formed after hydrolysis with pepsin, is a biological active component of the lactoferrin molecule of approximately 3.2 kDa. It was shown to have bactericidal activity against gram-positive and especially against gram-negative bacteria (Bellamy *et al.*, 1992; Yamauchi *et al.*, 1993). The lactoferricin shows a stronger antibacterial activity than the intact lactoferrin molecule, indicating a functional role of gastric hydrolysis for optimal biological activity (Bellamy *et al.*, 1992).

In our study we found lactoferrin fragments of several different sizes at ten minutes after ingestion of the test drinks. Most abundant were fragments with a molecular weight of 76 and 41 kDa, although we did not quantify these fragments. The presence of the 76kDa fragments indicated a rapid hydrolysis of small parts of the lactoferrin molecule. In the present study we did not regard lactoferrin molecules of 76 kDa or smaller as being intact lactoferrin since any biological activity of intact lactoferrin may be different from the 76 kDa fragments. The 41 kDa fragment probably is the C-terminal half of the lactoferrin molecule. We did not examine the precise nature of the fragments. With radial immunodiffusion we established that, immediately after drinking the test drinks, the recovery of intact LF was 100 % (data not shown).

When digestion of LF occurred, as evidenced from the HPLC pattern, we could demonstrate that LF fragments larger than 35 kDa still were able to bind to the antibodies directed against the intact LF (data not shown).

One has to take into account that in this study LF was intragastrically administered and that 45 min before administration the stomach was rinsed. This was necessary for the protocol used to measure gastric emptying and tube tip control. During the 45 min before test drink ingestion subjects remained seated. Considering a normal average gastric juice production of 1500 mL/day, 45 min is sufficient to restore normal resting gastric juice volume of 50 mL. Theoretically it is possible that orally administered lactoferrin is degraded differently from what we showed in the present study but we believe the protocol reflects the normal physiological digestive environment.

A number of biological effects are attributed to lactoferrin such as an antioxidative (Baldwin *et al.*, 1984; Davidson & Lönnerdal, 1989; Gutteridge *et al.*, 1981), an anti-inflammatory, a bactericidal effect (Akin *et al.*, 1994; Bellamy *et al.*, 1992; Saito *et al.*, 1994; Turchany, Aley & Gillin, 1995; Yamauchi *et al.*, 1993) and a facilitating role in iron absorption (McMillan *et al.*, 1976). To exert these effects, lactoferrin or biologically active lactoferrin fragments must survive passage through the stomach. To our knowledge this is the first study to show that a major proportion of orally administered bovine lactoferrin to survive passage through the stomach in adults. Intragastric degradation is not significantly affected by the iron saturation of the lactoferrin molecule and addition of a citrate buffer did not influence breakdown either. These findings are essential in interpreting results of studies concerning biological effects of lactoferrin in the gastrointestinal tract.

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CHAPTER 7

SURVIVAL OF ORALLY INGESTED HUMAN LACTOFERRIN AND ENDOGENOUS LACTOFERRIN SECRETION IN THE STOMACH AND SMALL INTESTINE *IN VIVO* IN WOMEN. A STUDY USING THE ILEOSTOMY MODEL

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Abstract

Background Lactoferrin (LF), a glycoprotein present in milk and other secretions, may provide a nutritional ingredient in the treatment of intestinal disorders associated with hyperpermeability, oxidative stress and inflammation. The aim of the study was to determine the survival rate of orally administered recombinant human lactoferrin (rhLF) in the upper GI tract and in the small intestine *in vivo* in humans.

Design Eight female ileostomy patients (age 44;43-57 y) ingested a test drink containing 5 g rhLF and collected full ileostomy output for 24 h. Test drink passage was monitored using radio-opaque markers. Intact LF in ileal effluent was quantified by ELISA.

Results The entire test drink passed the small bowel during the 24-h ileal effluent collection period. LF was excreted at a low but constant rate (0.35;0.28-0.47 μg LF/2h). A correlation between gastrointestinal (GI) passage of the test drink and LF excretion over time in ileal effluent was not observed. In total, a median amount of 4 μg LF was excreted over the 24-h collection period whereas 5 g rhLF was ingested.

Conclusions Dietary rhLF is digested in the upper GI tract and does not reach the colon. Intact LF appearing in ileostomy effluent is likely to originate from an endogenous source.

Introduction

Lactoferrin (LF) is a protein of the transferrin family. It is present in milk, saliva, tears, bile, blood plasma, mucosal and genital secretions. LF is able to bind ferric iron (Fe^{3+}) with a high affinity at two different lobes. A number of physiologic effects are attributed to LF. Previous studies have shown bactericidal (Akin *et al.*, 1994; Bellamy *et al.*, 1992; Zagulski *et al.*, 1998a; Zagulski *et al.*, 1998b), anti-inflammatory (Nuijens, van Berkel & Schanbacher, 1996), antiviral (Ikeda *et al.*, 2000; Murphy *et al.*, 2000; Swart *et al.*, 1998) and antioxidative properties (Baldwin, Jenny & Aisen, 1984; Davidson & Lönnerdal, 1989; Gutteridge *et al.*, 1981) of the LF molecule.

It has been suggested that LF has an anti-inflammatory potential. This effect seems to be partly mediated by attenuating TNF-alpha response (Crouch, Slater & Fletcher, 1992). It is known that TNF-alpha is involved in the pathogenesis of inflammatory bowel disease (IBD) (Sandborn & Hanauer, 1999). Hence, from a theoretical point of view, LF could be a potential anti-inflammatory agent in IBD. An anti-inflammatory effect would be expected after topical mucosal administration of the drug as in mesazaline- and controlled ileal released budesonide therapy.

It is not known to what extent LF is degraded in the gastrointestinal (GI) tract *in vivo* in adults. Digestion may deteriorate bioactivity of LF in the intestine. Oral LF supplementation may provide a nutritional tool to improve gut health in humans. In this regard, the colon is of special interest. However, in order to exert any effects in the colon, LF has to survive upper GI transit. If it is digested in the stomach or in the small intestine, LF degradation should be avoided by the use of ileal release capsules or by other ways such as administration via enemas when studying its effects in the colon.

We showed previously that LF is only partly degraded in the stomach *in vivo* in adults (Troost *et al.*, 2001). The metabolic fate of orally ingested LF in the small intestine has not been investigated *in vivo* in adults previously. The present study aimed to determine the survival rate of orally administered recombinant human lactoferrin (rhLF) in the upper GI tract and in the small intestine *in vivo* in humans using the ileostomy model and to quantify the amounts of LF entering the colon *in vivo*.

Methods

Study 1. Lactoferrin supplementation

Subjects

Eight female volunteers with an ileostomy (median age 44; 43-57 y) participated in the study. In all volunteers, a total colectomy was performed at least 3 years before participation, enabling a steady state situation after the initial adaptation phase to the colectomy. Volunteers with Crohn's disease were only permitted to participate in the study if they had been in remission during the previous three years and if less than 7.5 % of the entire ileum was removed during previous surgery. All volunteers were free of any medication and had no known abnormalities of the digestive system apart from the absence of the colon. All volunteers were informed about the aims and the protocol of the study orally and in writing and signed a written informed consent before participation. The study was approved by the Ethics Committee of the University Hospital Maastricht (Maastricht, The Netherlands).

Dietary restriction

Subjects were not allowed to consume any dairy products on the day prior to and on the day of the investigation to avoid dietary LF intake. All subjects had to consume a standardized diet on each occasion. During breakfast at 0800 h and during lunch at 1300 h at the day prior to testing bread rolls of any kind and non-dairy drinks were allowed *ad libitum*. Dinner at 1800 h consisted of prepared meals, mainly consisting of chow mein (Dinner 2 today, Bami Goreng, Honig, Koog a/d Zaan, The Netherlands). On the day of testing volunteers were not allowed to consume any food until breakfast at 1000 h. Subsequently the diet was the same as described for the day prior to testing.

Study design

After an overnight fast subjects were admitted to the laboratory at 0800 h. On four separate test days, subjects had to ingest a test drink containing 5 g rhLF (produced by Aspergillus Awamori, Agennix incorporated, Houston, USA) dissolved in 0.10 L demineralised water. Immediately after ingestion of the test drink, the stoma effluent bag was trashed as performed routinely. During the following 14 h, all stoma effluent was collected by replacing the stoma bag at 2-h intervals. Each stoma bag had to be placed separately in a small plastic airtight box on dry ice. Stoma effluent was collected every 2 h during daytime until 2200 h. Subsequently, volunteers collected ileal effluent at 0300 h and at 0800 h.

Twenty radio-opaque plastic pellets (Sitzmarks Ø 2 mm, Konsyl Pharmaceuticals Inc., Fort Worth, TX) were added to the test drink. These inert pellets cannot be absorbed from the lumen and hence provide a marker for test drink recovery in the ileal effluent.

Measurements

After collection, each ileostomy effluent sample was weighed to determine each 2-h and total 24-h ileal effluent production. All samples were X-rayed to count the number of radio-opaque pellets in each sample. These parameters yield information regarding the GI transit of the test drink. In the ileostomy effluent samples, the amount of intact LF was determined by enzyme-linked immunosorbent assay (Lactof-EIA, Biotech, Ox-isresearch, Portland, Oregon). The coefficient of variation of repeated measurements of this assay was below 6% as measured in spiked ileal effluent samples. The sensitivity of the assay was 1 µg/L. Data were expressed as LF excretion over 2-h periods.

Study 2. Basal endogenous lactoferrin levels

In an additional *in vivo* pilot experiment the endogenous LF excretion from the small intestine was investigated. On a separate day, four of the above mentioned volunteers collected ileal effluent during the last five h of the night. Emptying of the ileostomy bag at approximately 0300 h is common practice for most ileostomy patients. Ileal effluent collection took place from approximately 0300 to 0800 h. On the day prior to this collection, subjects consumed the same diet as during the first *in vivo* study. In a subsample of these ileal effluent samples, the LF concentration was measured by Elisa (see above).

Statistics

The amount of intact LF excreted in the ileal effluent during the 24-h period was expressed as LF excretion over 2-h intervals. Differences were assessed using non-parametric statistics (Wilcoxon signed rank test, $P < 0.05$ was regarded as statistically significant difference). The median (10-90 percentiles) ileal effluent production and LF excretion on each time point were calculated and presented by Box-and-Whisker displays.

Results

Study 1. Lactoferrin supplementation

One hundred fifty-seven of the total number of 160 plastic radio-opaque pellets ingested with the test drinks (pooled over all experiments) were excreted in the ileal effluent over the 24-h collection period. During the first two h after ingestion, no radio-opaques were excreted. In the ileal effluent samples collected between 4 to 10 h after ingestion of the test drink, 147 radio-opaques were emptied into the stoma bags, whereas only 10 radio-opaques were excreted into the bags during the following 9 h. During the last 5-h collection period no radio-opaques were excreted (Table 1).

Ileal effluent production did not statistically differ between any of the 2-h collection periods. The median total ileal effluent production over the 24-h collection period was 616 (456-865) g (Figure 1).

Table 1. Excretion of radio-opaque pellets in ileal effluent, as indicator of test drink recovery after passage through the gastrointestinal tract in female ileostomists.

time of collection	cumulative number of recovered radio-opaques in ileal effluent
0 - 2	0
2 - 4	32
4 - 6	72
6 - 8	108
8 - 10	147
10 - 12	150
12 - 14	154
14 - 19	157
19 - 24	157

Eight subjects, each subject ingested a test drink containing 20 radio-opaque pellets.

LF content of the ileal effluent samples did not statistically differ between any of the samples. The median 2-h LF excretion was 0.35 (0.28-0.47) μg (Figure 1). In total, a median amount of 4.01 (2.75-9.97) μg LF was excreted over the 24-h period by each volunteer (Table 2).

Table 2. Lactoferrin excretion in ileal effluent after oral ingestion of 5 g recombinant human lactoferrin in female ileostomists

time of collection (h)	median cumulative lactoferrin in ileal effluent (μg)
0 - 2	0.4 (0.27-1.37)
2 - 4	0.6 (0.42-2.02)
4 - 6	0.9 (0.73-2.97)
6 - 8	1.3 (0.97-3.53)
8 - 10	1.8 (1.18-3.88)
10 - 12	2 (1.63-4.61)
12 - 14	2.2 (2.02-5.26)
14 - 19	3 (2.24-8.77)
19 - 24	4 (2.75-9.97)

Values are expressed as median cumulative excretion of lactoferrin from the small intestine after each ileal effluent collection period. The 25- and 75 percentiles are displayed in parentheses, $n = 8$.

Figure 1. ileal effluent production and lactoferrin excretion

Twenty-four h ileal effluent production and lactoferrin production of eight female ileostomists after ingestion of 5 g recombinant human lactoferrin, expressed as production/2h and excretion/2h, respectively. Median (25-75 percentiles) values are displayed in boxes, 10-90 percentiles are displayed in whiskers.

Study 2. Basal endogenous lactoferrin levels

In the absence of any dietary LF ingestion, the 5-h ileal effluent of the four ileostomy patients contained 33 (24-52) µg LF/L ileostomy fluid. In the LF supplementation study we found a median 5-h ileal effluent LF concentration of 7 (6-9) µg/L in these 4 subjects.

Discussion

We showed that small amounts of intact LF appeared in the ileostomy effluent after oral ingestion of a LF containing solution. The LF was excreted at a constant rate over the 24-h effluent collection period. We did not observe a correlation between the recovery of the transit markers of the test drink and LF excretion in ileal effluent.

Most if not all LF present in bile and intestinal secretions originates from specific granules of activated neutrophils (Birgens, 1985; Furmanski & Li, 1990; Pereira *et al.*, 1998a). Plasmatic LF is secreted through bile into the intestine (Harada *et al.*, 1999). Biliary LF, in healthy adults present at a concentration of approximately 1 mg/L, is increased to approximately 3 mg/L in patients with IBD (Pereira *et al.*, 1998b). Additionally, faecal LF can be regarded as a diagnostic marker for immune system activation and hence disease activity in IBD patients (Sugi *et al.*, 1996). In the faeces of healthy volunteers with an intact GI tract, an average LF excretion of 210 µg/d was observed previously, whereas patients with inactive ulcerative colitis or inactive Crohn's disease were reported to excrete approximately 5 to 6 mg/d (Sugi *et al.*, 1996). In the present study we found an average LF production of 4 µg/d in ileostomists. The discrepancy between these observations can be explained by the hypothesis that the major part of LF in the GI tract originates from leucocytes in the colon. This seems to be a plausible conclusion because leucocytes are the major source of LF. We propose

that the LF excreted in the ileal effluent may have originated from an endogenous source, and not from the diet.

This is confirmed by the observation of the second *in vivo* study. In these ileal effluent samples we found equal or, if any, higher LF concentrations in these background samples without prior dietary LF ingestion compared to the samples collected over the same time period during the LF supplementation study. We did not detect a statistical difference between these experiments. Although only four subjects participated in the background study, we may conclude that the LF detected in ileal effluent in both studies originates from an endogenous source.

The observation that orally ingested LF does not survive passage through the small bowel confirms the findings of a LF breakdown study in the small bowel in rats (Kuwata *et al.*, 2001). In that study it was shown that during GI digestion of bovine LF, smaller fragments are formed that exert biological activity against fungi (Wakabayashi *et al.*, 1996) and bacteria (Bellamy *et al.*, 1992). This implies that, although LF is digested completely in the upper GI tract and in the small intestine, oral LF ingestion may exert effects at any site in the GI tract through the action of LF fragments. We were not able to study the appearance of these fragments in ileal effluent because of technical limitations in the quantitative analysis of LF fragments in the complex ileal effluent-matrix. Moreover, the present study does not exclude any biological activity of intact LF in the upper GI tract before degradation.

The rate of radio-opaque excretion clearly shows that the test drink was emptied from the small intestine within the 24-h ileal effluent collection period. This confirms the validity of the ileostomy model for studying GI survival of nutrients as we did in the present study. Incomplete recovery of the markers was observed on two occasions. The omission of two markers in the ileal effluent of one volunteer and of one marker in another volunteer may be caused by a solid particle which temporarily retained in an intestinal fold. We may conclude that the test meal was completely emptied from the small intestine during the 24-h collection period.

The ileostomy model was applied because it offers the unique feature to collect complete ileal effluent after the full transit of the test meal through the upper GI tract. Hence, the bio-availability of any nutrient from the diet to the colon can be assessed. It is a well-accepted method to study digestion of nutrients and extrapolate the findings to subjects with an intact GI tract since the ileostomy patients participating in the present study have an intact upper digestive system.

A prerequisite for this technique of studying LF digestion in the GI tract in ileostomists is the stability of LF *ex vivo* after emptying into the ileostoma bag. LF may stay in the bag for maximally 2 h. Digestion may continue in the bag. Therefore we studied *ex vivo* LF degradation in ileal effluent. We did not observe any LF degradation during 2 h of incubation in fresh ileal effluent at 37°C (data not shown). This is in accordance with a previous report in which no degradation of LF was observed during two h of incubation at 37°C in faeces of healthy volunteers (Sugi *et al.*, 1996).

We studied digestion of recombinant human LF, produced in *Aspergillus awamori*. From three-dimensional structure comparisons it was shown that this type of rhLF has the same three-dimensional structure as the native human LF, present in human breast

milk (Baker & Lindley, 1992). Dynamic properties of recombinant and native human LF are also similar. The only difference between the two LF forms is the composition of the glycans attached to the molecule due to the fungal system in which the rhLF is produced (Sun *et al.*, 1999). The ELISA method used for quantification of intact LF present in ileal effluent detected native human as well as recombinant human LF. Iron binding was shown previously to affect the stability of the LF molecule. Iron-saturated LF (holoLF) is more resistant to degradation than iron-free LF (apoLF) (Brines & Brock, 1983; Brock *et al.*, 1976). The rhLF studied in the present study was free of iron. This is in contrast with studies using apoLF of animal origin in the natural state, which is known to have an iron saturation of 15-20 % (Steijns & van Hooijdonk, 2000). Therefore, rhLF used in the present study may be more susceptible to GI degradation than LF of animal origin.

We showed that recombinant human LF is completely degraded during passage through the stomach and the small intestine *in vivo* in humans. Intact LF entering the colon is unlikely to originate from an exogenous, dietary source of LF. This LF probably originates from granules of activated neutrophils in the upper GI tract. Therefore, if oral administration of LF is indicated to exert an effect in the colon, ileal-release encapsulation of LF should be considered to prevent GI degradation of LF before it enters the colon.

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CHAPTER 8

RECOMBINANT HUMAN LACTOFERRIN INGESTION ATTENUATES INDOMETHACIN- INDUCED ENTEROPATHY *IN VIVO* IN HEALTHY VOLUNTEERS

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Abstract

Objective To determine whether recombinant human lactoferrin ingestion inhibits non-steroidal antiinflammatory drugs (NSAID) -induced gastroenteropathy *in vivo* in healthy volunteers as a model for disorders associated with a rise in permeability of the stomach and the small intestine.

Design Fifteen healthy volunteers (age 23 ± 1.4 y) were tested. A sucrose and a lactulose/rhamnose (L/R) permeability test was performed to assess gastroduodenal and small intestine permeability as indicator of NSAID-induced gastroenteropathy. All subjects consumed standardized meals for two days. On the second day at time = -24h each subject ingested a drink containing 5 g recombinant human lactoferrin or placebo during breakfast. At $t=-9$ h, subjects ingested the same drink with 75 mg of the NSAID indomethacin and after an overnight fast at $t=-1$ h subjects consumed the drink and 50 mg indomethacin. One hour later, at $t=0$, a permeability test was performed.

Results Small intestine permeability after indomethacin and placebo was significantly higher (L/R ratio = 0.036; 0.014-0.092, $P<0.05$) compared to the permeability observed after ingestion of indomethacin and lactoferrin (0.028; 0.015-0.056), whereas gastroduodenal permeability did not differ between the two interventions ($P=0.3$).

Conclusion Oral recombinant human lactoferrin supplementation during a short-term indomethacin challenge reduced the NSAID-mediated increase in small intestinal permeability and hence may provide a nutritional tool in the treatment of hyperpermeability-associated disorders.

Introduction

In normal conditions, only minute amounts of pathogens permeate the intestinal epithelium that may cause a moderate local activation of the immune system. However, if the intestinal permeability is increased, excessive penetration of luminal aggressive agents and antigens may attract local and circulating neutrophils which causes the release of reactive oxygen species (Bjarnason *et al.*, 1993) and the initiation of gastro-intestinal inflammation (Söderholm & Perdue, 2001; Wallace, 1993). This inflammation increases permeability to a greater extent, resulting in permeation of luminal aggressive agents through the mucosa and aggravation of local tissue inflammation which, in turn, leads to increased permeability (Ferry *et al.*, 1989). An increase in intestinal permeability may finally result in a significant intrusion of pathogens, such as endotoxin, into the circulation and implies a serious health hazard. An increase in intestinal permeability or a decrease in intestinal barrier function is regarded as a pathophysiological factor in Crohn's disease (Meddings, 1997), celiac disease (Smeuol *et al.*, 1997), viral gastroenteritis (Isolauri *et al.*, 1989) and food allergy (Crowe & Perdue, 1992). Shortly after the administration of indomethacin, a non-steroidal anti-inflammatory drug (NSAID), small intestinal permeability is increased in the same order of magnitude as in these disorders (Bjarnason *et al.*, 1986). In the present study we administered indomethacin to healthy volunteers as a model for the gastroenteropathy associated with a rise in small intestinal permeability. Controlling permeability with specific food ingredients such as lactoferrin (LF) may provide a tool in the treatment of these disorders.

In animal studies, several beneficial effects were attributed to LF. Bactericidal (Bellamy *et al.*, 1992; Zagulski *et al.*, 1998), anti-inflammatory (Nuijens, van Berkel & Schanbacher, 1996), antiviral (Ikeda *et al.*, 2000; Murphy *et al.*, 2000; Swart *et al.*, 1998) and antioxidative activities (Baldwin, Jenny & Aisen, 1984; Davidson & Lönnerdal, 1989; Gutteridge *et al.*, 1981) were discovered previously. Concomitant ingestion of lactoferrin together with indomethacin may protect against indomethacin-induced gastroenteropathy. LF is a protein of the transferrin family. It is present in milk, saliva, tears, bile, blood plasma, mucosal and genital secretions (Britigan, Serody & Cohen, 1994; Levay & Viljoen, 1995) and released by neutrophil-specific granules (Davidson & Lönnerdal, 1989). Since LF is present in relatively high concentrations in human colostrum, it was thought to play an important role in the development of newborns. This hypothesis was strengthened by the discovery of specific LF receptors on the intestinal brush border (Kawakami & Lönnerdal, 1991; Mazurier, Montreuil & Spik, 1985), lymphocytes (Legrand *et al.*, 1992) and possibly in hepatocytes (McAbee & Esbensen, 1991). The physiological effects of LF have been reviewed by several investigators (Baker *et al.*, 1991; Britigan *et al.*, 1994; Brock, 1995; Iyer & Lönnerdal, 1993; Levay & Viljoen, 1995) but the exact mechanisms of action are still unclear. Some of the observed and hypothesized effects of LF may be regulated through binding to a specific LF receptor in the small intestine. However, binding of LF to its receptor is a species-specific process. It was shown previously that bovine LF does not recognize the human LF receptor, whereas rhesus monkey lactoferrin and human LF are structurally and functionally identical and have the same affinity for the intestinal brush-

border membrane LF-receptor (Davidson & Lönnerdal, 1986; Davidson & Lönnerdal, 1988).

We investigated whether recombinant human LF was able to attenuate NSAID-induced gastroenteropathy and may thus provide a nutritional intervention tool in the prevention or treatment of disorders associated with an increased intestinal permeability.

Methods

Subjects

Fifteen healthy non-smoking male volunteers (age 23.9 ± 2.2 y) were recruited for participation. All subjects were free of any medication and did not have a history of a gastrointestinal disorder. The study was carried out according to a double-blind randomized cross-over design. Each subject participated in two experiments with a wash-out period of two weeks in between. All subjects were informed orally and in writing about the aim and the protocol of the study and gave their written informed consent before participation. The study protocol was approved by the Ethics Committee of the University Hospital Maastricht, Maastricht, The Netherlands.

Design

To avoid high levels of dietary lactoferrin intake, subjects consumed a provided standardized diet and were not allowed to consume any dairy products on the two days prior to the day of testing. For breakfast and lunch bread rolls of any kind and non-dairy drinks were allowed *ad libitum*. Dinner consisted of a commercially available prepared pasta meal (Dinner 2 today, Bami Goreng, Honig, Koog a/d Zaan, The Netherlands). On each test day, a sucrose/lactulose/rhamnose test was carried out to as a measure of gastroduodenal and small intestinal permeability. Twenty-four hours prior to the permeability measurement, immediately after breakfast, subjects ingested a test drink. The flavoured test drink contained either 5 g recombinant human lactoferrin (rhLF, Agennix inc., Houston, TX, USA) dissolved in 100 mL water or flavoured placebo (100 mL water), present in a masked flask. In the evening, exactly nine hours prior to the permeability measurement, subjects ingested a test drink and 75 mg indomethacin (Genfarma bv., Maarssen, The Netherlands) and after an overnight fast, one hour before permeability assessment, subjects ingested a test drink and 50 mg of indomethacin. One hour later, after voiding, a hyperosmolar drink containing 20 g sucrose, 5 g lactulose (Genfarma bv., Maarssen, The Netherlands) and 0.5 g L-rhamnose (ICN Biomedicals Inc., Aurora, OH, USA) dissolved in 100 mL water was ingested. Subsequently, urine was collected for five hours. Subjects stayed in the lab and were not allowed to eat during the time of urine collection. Consumption of water was allowed *ad libitum* during the last two hours of urine collection. The urine volume was determined. Urine aliquots were taken and stored at -80°C until analysis. Two weeks after the first experiment, subjects commenced the same protocol with the complementary test drink.

In an additional study to the effects of rhLF on small intestinal permeability, 5 of the above subjects ingested a lactulose/rhamnose/sucrose solution as described above after an overnight fast and after voiding, with concomitant ingestion of either 5 g rhLF, dissolved in 100 mL water, or 100 mL water in masked flasks on two separate days. After ingestion of the beverage, volunteers collected urine for 5 h as described above. The experiments were carried out to conform to a randomized cross over design.

Gastrointestinal permeability

Urinary sucrose excretion provides an estimation of the permeability of the stomach and the proximal duodenum (Meddings *et al.*, 1993). Urinary sucrose was determined with a Dionex ion chromatography system (Dionex Corporation, Sunnyvale, CA). All samples were spiked with sucrose to enable identification of sucrose in the complex urine matrix. Samples were put on a column (Dionex PA1 and guard column PA1) with a sodium hydroxide – sodium acetic acid gradient. Sucrose was detected using pulsed electrochemical detection.

Intestinal permeability was assessed using the lactulose/rhamnose gut permeability test. This test is based on the comparison of intestinal permeation of molecules of different sizes. The urinary lactulose / rhamnose excretion ratio is considered to be an accurate parameter for small intestinal permeability (van Nieuwenhoven *et al.*, 1999). Lactulose and rhamnose were determined by fluorescent detection high-pressure liquid chromatography (HPLC). All samples were spiked with lactose as an external standard that does not interfere with determination of lactulose and rhamnose. Samples were incubated overnight with derivatization reagent (9-fluorenylmethyl chloroformate hydrazine) at 65°C. After cooling to room temperature the reaction mixture was injected into a dedicated HPLC-system. The fluorescence was detected with a fluorescence detector (B&L systems, Zoetermeer, The Netherlands). The protocol for lactulose and rhamnose analysis was well validated and described in detail previously (Rooyackers, van Eijk & Deutz, 1996).

In vitro lactoferrin-indomethacin binding study

An *in vitro* binding study to examine the ability of rhLF to bind indomethacin was performed. A water solution containing 25 mg/L indomethacin and 2.5 g rhLF was incubated for 7 and 22 minutes respectively. Subsequently, 0.5 mL of the rhLF/indomethacin solution was put over a 30 kDa micropore filter and the amount of indomethacin present in the filtrate solution was determined by HPLC.

Statistics

Urinary sucrose excretion is presented as median excretion (mg). Five-hours urinary excretion of lactulose and rhamnose are presented as lactulose/rhamnose (L/R in g/g) ratios. Differences in urinary sucrose, L/R ratio's and lactulose- and rhamnose recovery respectively between the lactoferrin- and the placebo intervention were assessed using non-parametric statistics (Wilcoxon signed rank test, $P < 0.05$ was regarded as a statistical significant difference). Data are presented by Box-Whisker plots.

Results

None of the subjects suffered from gastrointestinal discomfort of any kind during the test days. No side effects of lactoferrin ingestion were observed.

The median urinary sucrose excretion was lower in the LF intervention (8.86; 3.61-25.4) compared to placebo (13.75; 2.89-24.62) but this difference did not reach statistical significance ($P = 0.31$; Figure 1).

Figure 1. Urinary sucrose and lactulose/rhamnose excretion

Five-h urinary sucrose and lactulose/rhamnose excretion after ingestion of two dosages of indomethacin (75 mg at 9h and 50 mg indomethacin at 1h, respectively, before ingestion of a sucrose/lactulose/rhamnose solution) with or without ingestion of 5 g recombinant human lactoferrin at 24h, 9h and 1h, respectively, before ingestion of the sucrose/lactulose/rhamnose solution. Median (25-75 percentiles) values are displayed in boxes, 10-90 percentiles are displayed in whiskers. (Sucrose; $P=0.31$, lactulose; $P<0.05$, $n=15$, Wilcoxon signed rank test). The grey area indicates the average ± 2 sd urinary lactulose/rhamnose ratios observed in control experiments in previous investigations ($n=31$) as the 95% reliability interval for normal values in healthy subjects.

The L/R ratio after ingestion of rhLF and indomethacin was significantly lower (0.028 (0.015 - 0.056); $P<0.05$) than after the indomethacin challenge with placebo (0.036 (0.014 - 0.092)) (Figure 1). In Figure 2 the mean urinary lactulose / rhamnose ratio ± 2 standard deviations as the 95 % reliability interval in healthy controls is depicted (0.014 $\pm$ 0.016, $n = 31$). These values were obtained from control experiments in previous studies with healthy volunteers using the same protocol and analysis equipment.

The observed difference in L/R ratio between the two interventions was due to a lower urinary lactulose recovery after lactoferrin administration compared to the placebo administration (0.52% (0.40-0.99) and 0.74% (0.26-0.92) respectively; $P = 0.054$). Urinary rhamnose recovery did not differ between the lactoferrin- and placebo administration (21.08% (9.12-28.56) and 21.00% (12.31-45.53) respectively; $P = 0.78$).

The urinary lactulose/rhamnose ratio after ingestion of 5 g rhLF (0.027; 0.02-0.03) did not differ from the urinary lactulose/rhamnose ratio after ingestion of water (0.03; 0.02-0.03).

***In vitro* lactoferrin-indomethacin binding study**

After 7 minutes of incubation, no indomethacin was bound to LF: indomethacin recovery in the filtrate was 100 %. After 22 minutes of incubation, 76 % of the indomethacin appeared in the filtrate.

Discussion

It is well established that the administration of indomethacin in dosages comparable to that used in the present study increases small intestinal permeability about 2-3 fold (Bjarnason *et al.*, 1993; Bjarnason *et al.*, 1989; Bjarnason *et al.*, 1986), which is in line with our results. Intestinal permeability after taking indomethacin for seven days reverts back to normal within one week (Bjarnason *et al.*, 1991). Hence, in the present study the washout period of two weeks was sufficient to exclude any carry-over of the effects of indomethacin. Concomitant LF administration with indomethacin ingestion significantly attenuated the urinary lactulose/rhamnose ratio compared to placebo with indomethacin from 0.036 to 0.028, indicating that LF attenuated indomethacin-induced small intestinal permeability by a factor 1.4. Hence, LF administration does not completely prevent indomethacin-induced enteropathy. The observed lactulose/rhamnose ratio of 0.028, however, is within the normal range as measured previously in control experiments in our lab, whereas 0.036 is above the normal range.

The difference in urinary lactulose/rhamnose ratios was caused by a lower urinary lactulose recovery after LF ingestion compared to placebo. Lactulose permeates the intestinal epithelium paracellularly and hence, urinary excretion after ingestion of the sugar drink provides an estimation of the paracellular permeation route. Rhamnose excretion is predominantly a parameter of transcellular permeation. Since we did not observe a difference in rhamnose excretion, the observed effect of LF on NSAID-induced hyperpermeability can be solely attributed to its protective effect on the paracellular permeation route. In the second *in vivo* study we showed that LF did not affect small intestinal permeability in the unstressed small intestine. Hence, ingestion of LF does not inhibit normal small intestine permeability in humans, but provides an effective tool in the prevention of enteropathy in the small bowel.

Lactoferrin tended to exert a protective effect on gastroduodenal permeability as measured by urinary sucrose excretion. The observed difference between LF and placebo did not reach statistical significance due to a wide range in results. It has been shown previously applying the urinary sucrose excretion method that aspirin administration increased gastroduodenal permeation (Lambert *et al.*, 2001), indicating that NSAIDs inflict damage to gastric and duodenal mucosa. The observed trend in the present study warrants further investigation with sufficient power in order to elucidate the extent and pathophysiology of LF-associated protection of NSAID-induced damage to the stomach and the proximal duodenum.

Colostrum contains relatively large amounts of lactoferrin. A protective effect of a bovine colostrum preparation on indomethacin-induced increase in small intestinal permeability was previously observed. Colostrum supplementation during 7 days inhibited the indomethacin-induced hyperpermeability of the small intestine seen after 5 days of indomethacin intake compared to placebo. The placebo solution was an iso-proteinaceous whey solution free of a number of growth factors that are present in the colostrum (Playford *et al.*, 2001). No information was available about lactoferrin contents of the solutions used in this study. The results of the present study indicate that these effects could at least partly be attributed to LF present in the colostrum.

As will be explained below, the observed effect of LF in attenuating paracellular epithelial damage may be due to a protective effect on the regulation of the paracellular permeation route. LF may also interfere with another direct or indirect pathophysiological process by which indomethacin administration leads to gastroenteropathy such as the uncoupling of mitochondrial respiration, mediation of cytokine production, inhibition of cyclooxygenase-mediated prostaglandin production or induction of intracellular transcription factors.

It is well established that NSAID administration causes gastroenteropathy, thereby damaging the tight junctions between enterocytes. This has a detrimental effect on the intestinal barrier function. NSAIDs may induce enteropathy by several distinctive mechanisms.

NSAIDs are able to uncouple oxidative phosphorylation, which results in reduction of intracellular ATP levels and, as a consequence, loss of control of the integrity of tight junctions. This is regarded as the first step in the development of gastrointestinal injury (Somasundaram *et al.*, 1995; Somasundaram *et al.*, 1997). This uncoupling causes efflux of hydrogen and calcium ions from mitochondria and promotes oxygen radical damage (Bjarnason *et al.*, 1993; Carafoli, 1987). Due to this oxidative stress, tight junctions are damaged and paracellular permeability increases. LF is a well-recognized antioxidative agent because of its strong iron chelating capacity. Iron ions bound to LF are not able to participate in Fenton Chemistry in which the highly reactive hydroxyl radicals are produced. The chelation of iron by LF diminishes the production of hydroxyl radicals and consequently oxidative stress (Britigan *et al.*, 1994). This may contribute to the observed protective effect of oral LF ingestion since indomethacin-induced enteropathy is in part caused by oxidative stress in the epithelium. The ferrochelating properties of LF also result in antimicrobial activity of LF in human plasma serum as iron deprivation inhibits bacterial growth (Aguila *et al.*, 2001). Additionally, LF exerts bactericidal activity against antibiotic-resistant bacteria *in vitro* independent of its iron-binding capacity (Bellamy *et al.*, 1992; Nibbering *et al.*, 2001). These findings indicate that LF may protect against indomethacin-induced enteropathy by preventing intrusion of bacteria in the epithelium of the small intestine, which occurs when the intestinal epithelium becomes more permeable and results in a further progression of the damage. Because the ferrochelating properties of LF may explain its protective effect on indomethacin-induced enteropathy, iron-free LF (apoLF) as used in the present study, may be more effective to protect against enteropathy than iron-saturated LF (holoLF). ApoLF has a maximal capacity to bind iron whereas holoLF has a limited capacity to bind free iron, although some iron binding can still occur.

The observed protective effects of oral LF ingestion may partly be due to the regulatory function of LF in the immune system. Oral lactoferrin ingestion decreases spontaneous and lipopolysaccharide-induced production of the pro-inflammatory cytokines IL-6 and TNF α by peripheral blood cells (Zimecki *et al.*, 1999; Zimecki *et al.*, 1998). Moreover, LF and LF-derived peptides can stimulate the release of IL-8 from polymorphonuclear leukocytes, which activate neutrophils (Shinoda *et al.*, 1996) and inhibit lymphocyte production of TNF α , IL-1 and IL-2 *in vitro* (Crouch, Slater & Fletcher, 1992). This mediating effect of LF on the local and systemic immune response may

inhibit the proceeding of free radical-induced damage caused by local activation of the immune system due to NSAID-induced enteropathy. Regulation of cytokine production may be an important mechanism of action of LF resulting in protection of the integrity of the epithelial barrier, although data are equivocal. It was previously shown that oral LF administration stimulates the production of the pro-inflammatory interleukin-18 (IL-18) in the mucosa of the small intestine in rodents with experimental metastasis (Kuhara *et al.*, 2000). No data are available regarding the effects of oral LF ingestion on IL-18 production in any other physiological conditions.

It may be that the effect of LF on cytokine production is a receptor-mediated process. In that case, the origin of the rhLF administered in the present study may be essential. LF administration of animal origin may not exert the same effects as those observed in the present study since LF binding to its receptor is a species-specific process.

It is generally recognized that NSAIDs inhibit cyclo-oxygenase (COX) activity. This results in a decrease of prostaglandin (PG) synthesis and consequently a further deterioration of the epithelial barrier (Bjarnason *et al.*, 1989; Hawkey & Rampton, 1985). It is unlikely that the protective effect of LF on the integrity of the epithelial wall involves PG production because existing knowledge does not provide any indications for a mediating effect of LF on PG production.

Hypothetically, binding of indomethacin to LF would prevent indomethacin-induced gastroenteropathy because indomethacin bound to LF may lack biological activity. In the *in vitro* LF / indomethacin binding study we showed that indomethacin did only bind LF in minute amounts at a slow rate. After 7 minutes of incubation, no indomethacin-LF binding was observed and after 22 minutes 76 % of the indomethacin was recovered. This implies that at least 76 % of the indomethacin was present as free indomethacin after 22 minutes. In previous studies we showed that after 7 minutes almost half of the test drink ingested is emptied from the stomach, while after 22 minutes most if not all of the test drink is emptied from the stomach into the small intestine (Troost *et al.*, 2001). Hence, in the present study indomethacin entered the small intestine predominantly unbound to LF. The observed protective effect of oral LF administration can probably not be explained by LF-indomethacin binding. We previously showed that the major part of orally ingested LF survives gastric passage *in vivo* in adults, leaving most of the LF intact until it enters the intestine (Troost *et al.*, 2001).

The observed effects cannot be attributed to the presence of a protein load in the test drink. In contrast to LF, oral administration of casein leads to aggravation of NSAID-induced damage to the epithelial barrier, probably because transepithelial movement of casein induces enhanced mast cell activation and consequently mucosal injury (Miller *et al.*, 1991).

Intestinal permeability is increased in numerous diseases. Approximately 50 % of patients with active Crohn's disease have an increased small intestinal permeability (Bjarnason *et al.*, 1983). Moreover, it has been shown that an increase in intestinal permeability precedes clinical manifestation of Crohn's disease (May, Sutherland & Meddings, 1993). A compromised intestinal barrier function is regarded as an etiological factor in Crohn's disease (Meddings, 1997), celiac disease (Smecuol *et al.*,

1997), viral gastroenteritis (Isolauro et al., 1989) and food allergy (Crowe & Perdue, 1992). This implies that controlling permeability is important in the control of these diseases. Ingestion of the two dosages of indomethacin as carried out in the present study increased small intestinal permeability approximately 2.5-fold, comparable to the increase in permeability observed in Crohn's disease (Wyatt et al., 1993). Hence, the protocol as used in this study may serve as an appropriate model for a variety of disease states in which small intestinal permeability is increased.

We conclude that orally administered rhLF exerts biological activity in the small intestine, resulting in inhibition of indomethacin-induced enteropathy. The exact mechanism of action of LF in attenuating enteropathy remains to be elucidated. It seems plausible that the antioxidative capacity of LF is involved in the attenuation of the indomethacin-induced stress. Binding of LF to its receptor, which may play a role in the pathophysiology of the observed effect of LF in the small bowel, is a species-specific process. Therefore the results of the present study may be species-specific, depending on the origin of the LF. It seems less likely that LF interferes with the effects of indomethacin on PG synthesis or prevents the biological activity of indomethacin by binding it. Lactoferrin administration may be beneficial in the control of oxidative stress-induced damage of the small intestinal epithelium and especially in patients using NSAIDs by its attenuating effect on increased small bowel permeability.

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CHAPTER 9

GENERAL DISCUSSION

Discussion

An important function of the small intestine is its role in the defence network against pathogens, such as bacteria, viruses and (pro)oxidants. Although the role of the small intestine as an immunologically active organ is well recognized and its direct responses to pathogens are under extensive investigation, little is known about the role of dietary components in mucosal interactions or about the capacity of the small intestine to prevent damage to the intestinal mucosa. Detailed information about defence mechanisms of the human small intestine against oxidative stress and the effects of food ingredients on the integrity of the mucosal barrier is important, as this may result in the development of nutritional tools to prevent the initiation of intestinal and systemic inflammatory responses, initiated by luminal and mucosal challenges. The studies described in this thesis were designed to investigate nutrient-gut interactions and regulatory mechanisms in the small intestinal mucosa *in vivo*.

The studies described in chapters 3 and 4 were funded by the 5th Framework of the European Union (EU). This project, entitled 'Evaluation of the safety and efficacy of iron supplementation in pregnant women', with EU reference 'FeMMES QLK1-1999-00337', aimed to examine the effects of iron supplementation in humans, and to explore the mechanisms involved using cell culture and animal models. The participation in this project, in addition to the unique properties of iron in the intestine (participation in free radicals generating cascades, micronutrient interactions, and iron absorption), evolved in special emphasis of this thesis on the effects of iron administration in the human small intestine.

Iron

Iron-induced damage

Iron participates in many biological and biochemical processes. Among other effects, iron ions catalyze a cascade of reactions, generally referred to as Fenton chemistry, which results in the production of highly reactive hydroxyl radicals. This phenomenon was described in the introduction of this thesis. Hydroxyl radicals cause oxidative damage to virtually all biological molecules in their direct environment. Previous studies show that iron supplements induce oxidative stress in the intestine (Lund *et al.*, 1999; Srigiridhar & Nair, 1998; Srigiridhar *et al.*, 2001). The extent and location of iron-induced damage *in vivo* in humans has not been investigated to date. It is important to study site-specific damage in the GI tract, because the consequences of lipid peroxidation to compounds in the intestinal lumen or to enterocytes at the tip of the villi may differ from oxidative damage to enterocytes in the base of the villi, to tight junctions or to the structures underneath the epithelial layer. Furthermore, the adverse effects induced by stress in one intestinal segment presumably differ from those observed in other segments.

The antioxidant defence network in the small intestine may provide a system against intestinal oxidative stress, although data about endogenous antioxidant capacity in the intestine are scarce. In chapters 3 and 4, we showed that iron administration in medi-

cally prescribed dosages into the proximal small intestine induced lipid peroxidation in the lumen of the small intestine. This occurred in a dose dependent fashion. Iron-induced lipid peroxidation was accompanied by the release of, yet unidentified, antioxidants into the small intestinal lumen. This observation was consistent throughout all experiments. The antioxidant response occurred also in a dose-dependent way. It was evident that the antioxidant response to oxidative damage was instantaneous and very powerful, especially considering the dilution of the measured intestinal fluid samples with saline, as was discussed in the appropriate chapters. The rapid release of antioxidants indicated that it, most likely, was not *de novo* synthesized, but released from a storage compartment. The question remained which compound was responsible for the observed rise in antioxidant capacity. Direct measurement of several compounds with well-established antioxidant capacity in biological systems did not elucidate this problem. In an attempt to identify the unknown compound, Nuclear Magnetic Resonance (NMR) techniques were applied to investigate compositional differences between intestinal fluid samples with high and low antioxidant capacity (data not shown). Although consistent differences between the NMR spectra of these samples were observed, we are, at this stage, not able to attribute these observations to one specific molecule. The intestinal fluid samples will, in the near future, be compared using two-dimensional NMR techniques. This might elucidate the compound(s) responsible for the differences in antioxidant capacity between the fluid samples.

The studies described in chapters 2, 3 and 4 reveal the underlying physiological response of the small intestine to iron supplementation. We showed that 4 weeks of high-dose iron therapy does not induce functional damage to the integrity of the small intestinal mucosa in iron deficient premenopausal women, nor was the mucosal integrity more susceptible to indomethacin-induced epithelial damage after one month of iron supplementation. The results of the study suggested that iron did not induce oxidative damage to the small intestinal mucosa. However, after developing a novel, accurate model to directly investigate processes in the lumen of the small intestine *in vivo*, we showed that iron induced lipid peroxidation in healthy volunteers. The data showed that iron administration initially causes oxidative stress in the small intestine, which confirms previous data from animal experiments (Fodor & Marx, 1988; Srigriridhar & Nair, 1998; Srigriridhar & Nair, 2000). However, it was shown for the first time that intestinal oxidative stress activated a powerful antioxidant network. The data from the studies described in chapters 2, 3 and 4 suggested that, despite initial oxidative damage upon iron administration, the antioxidant response in the small intestine presumably prevents impaired mucosal integrity of the small intestine during periods of iron therapy. However, the studies described in this thesis focused on intraluminal effects of iron administration and on the mucosal barrier function. The impact of intestinal iron administration on other properties of the small intestinal mucosa, such as enzyme activities in the brush border, or intraepithelial processes *in vivo* in humans remains to be investigated.

Iron-mediated changes in gene expression patterns

The study described in chapter 4 showed that iron or iron-induced oxidative stress mediated the expression of many genes in small intestinal mucosa. In an attempt to

identify specific biological pathways, mediated at the transcriptomic level by the iron interventions, the study results were analyzed using public, shared databases. This approach revealed that iron or iron-induced oxidative stress strongly affected several distinct processes in the mucosa of the small intestine. Iron mediated several G protein coupled receptor (GPCR) associated processes. GPCRs comprise the largest superfamily of proteins in the body. They serve functions in a wide variety of biological pathways, which are generally related to intracellular signalling routes. The observation that three out of the six processes most strongly mediated by the iron interventions involved GPCRs suggests an important, previously unknown, stress response mechanism, which should be investigated in detail. The other three processes identified by the method used to allocate the observed changes in gene expression patterns to biologically pathways, namely cell cycle, calcium channels and complement activation, also warrant further investigation.

The genome-wide microarray scans contain a lot of data. They provide detailed information about baseline gene expression profiles in the healthy human small intestine, some mediated by iron or iron-induced oxidative stress, some involved in many specific biological pathways, and other genes with a yet unknown function. Many changes in gene expression profiles failed to reach significance, in part owing to the design of the microarray experiment. Mucosal tissue samples were obtained approximately 120 minutes after the start of iron perfusion. Recent literature shows that significant changes in gene expression occur approximately 180 minutes after oral iron administration to rats (Frazer *et al.*, 2003). Therefore, the timing of tissue sampling may not have been optimal for gene expression experiments. However in the original design a genome wide microarray was not included since the technology was still under development at that time. This aspect should be considered in the design of future gene expression experiments.

The variety of individual genes and biological pathways that were mediated by the iron perfusion experiments, confirms the importance of implementing genome-wide gene expression experiments in the investigation of poorly understood phenomena, such as intestinal oxidative stress, but also in many (intestinal) diseases. The microarray technology provides a sophisticated tool to obtain information about the complex dynamic equilibrium between a wide variety of processes. It can be applied to identify new signalling pathways in health and disease and, additionally, it has great potential to be implemented in scientific research as a 'hypothesis generating' tool, because it provides information about virtually all dynamic processes in the body. Due to the huge amount of sometimes contradictory data, it may not provide definitive answers to specific hypotheses. For that purpose, implementation of dedicated complementary deoxyribonucleic acid (cDNA) microarrays, with a limited number of genes to be studied, may be indicated, in addition to other techniques such as proteomics and biochemical analyses of biological samples.

Considerations of iron supplementation

Iron supplementation affects the human small intestine in several ways. This thesis shows that iron given as single iron dosages of 80 or 400 mg do not induce major oxi-

ductive damage to the small intestinal mucosa. The long-term effects of iron on mucosal gene expression and its consequences will need to be established. The question remains whether the initial iron-induced, oxidative damage observed in the intestinal perfusion experiments, affects other structures or processes in the small intestine. It may be suggested that free radicals pass the epithelium and induce damage to the structures underneath. This may affect the microvasculature or the lymph system in the mucosa, or damage the enteric nerve system. Furthermore, free radicals, produced in the lumen of the small intestine, may cross enterocyte membranes and attack other compounds in these cells. Polyunsaturated fatty acids (PUFAs) are vulnerable to free radical-induced damage, due to the presence of multiple double bonds. Therefore, mitochondrial outer membranes with large concentrations of PUFAs appear to be especially vulnerable to oxidative damage. Moreover, the nucleus of epithelial cells may be attacked, which may induce DNA strand breaks or other molecular damage. These issues need to be addressed in future studies of the effects of iron and oxidative stress in the small intestine *in vivo*. Furthermore, the experiments described in this thesis were carried out in relatively healthy volunteers. In people at higher risk of intestinal oxidative stress, such as in the malnourished, newborns, the elderly, or patients suffering from GI diseases, therapeutic amounts of iron may lead to more severe damage.

Chapter 5 showed that iron supplements inhibit the absorption of zinc, when taken in a water solution. This phenomenon was already present with moderate amounts of supplemental iron, suggesting that long-term iron supplementation may impair body zinc status. If the cause of iron deficiency anaemia is malnutrition, as is observed regularly in developing countries, inhibition of zinc absorption is a severe health threat and, hence, deserves special attention in the medical treatment of those patients (Allen, 1994; Solomons & Ruz, 1997). Copper absorption was not impaired by the presence of iron and, hence, should not need special attention. The studies described in this thesis show that usual, medically used amounts of iron can safely be prescribed to iron-deficient but otherwise healthy patients, if care is taken regarding the patients zinc status. It should, however, be considered that high dosage of iron may induce damage to the intestinal submucosa, as described above. Hence, iron therapy should be limited to the replenishment of body iron stores.

Lactoferrin

Gastrointestinal digestion of lactoferrin

The studies described in chapters 6 and 7 aimed to investigate the metabolic fate of the glycoprotein lactoferrin in the gastrointestinal tract. Several effects have been attributed to this protein. Its capacity to bind iron with high affinity suggests a role in the defence against microbes, viruses, inflammation and oxidative challenges, whereas the presence of specific lactoferrin receptors in the apical membrane of the intestinal mucosa suggests functions in enterocyte-specific processes, such as iron absorption. The potential functional roles have resulted in the development of oral lactoferrin supplements, which claim to be beneficial to human health and are commercially available as over-the-counter supplements.

Detailed information about lactoferrin degradation, prior to studying its effects in various sites of the GI tract, was needed to design subsequent studies of the effects of lactoferrin supplementation. Chapter 6 describes lactoferrin survival during gastric transit. Up to 79 % of intact lactoferrin, ingested in a sugar solution into the stomach of healthy volunteers, entered the small intestine as intact lactoferrin. Hence, most lactoferrin, either as apo- or as hololactoferrin, enters the small intestine in its original, intact form. The study described in chapter 7 showed that recombinant human lactoferrin is fully degraded in the small intestine. In this study applying the ileostomy model, trace amounts of lactoferrin appeared in the ileostomy effluent. These minute amounts originated most likely from an endogenous source. We suggest that neutrophils, attracted by immune activation owing to pathogenic challenges in the lumen of the small intestine, degranulate upon infiltration into the mucosa or after release into the lumen of the small intestine, thereby releasing endogenously produced lactoferrin (Furmanski & Li, 1990; Pereira *et al.*, 1998). A low level of immune activation is part of normal intestinal physiology. The low lactoferrin concentrations in ileostomy effluent should, therefore, not be considered as indicators of gastrointestinal disease activity. High faecal lactoferrin levels can be found in subjects with an intact gastrointestinal tract. It was shown that faecal lactoferrin can be regarded as a marker of disease activity in Inflammatory Bowel Disease patients (Sugi *et al.*, 1996). The data from chapter 7 suggest that faecal lactoferrin predominantly originates from the colon, and not from the small intestine.

The lactoferrin used in the studies described in chapters 6 and 7 was isolated from bovine and recombinant human sources, respectively. Lactoferrin binding to its specific receptors is a species specific process. Therefore, it is important to carefully consider the origin of the lactoferrin used in the design of studies of lactoferrin in the gastrointestinal tract. Despite the different origin of the lactoferrin used in chapters 6 and 7, the molecular structure of bovine and (recombinant) human lactoferrin are largely identical; bovine lactoferrin consists of 689 amino acids, whereas human lactoferrin contains 691 amino acids. The three-dimensional structure of these lactoferrins is largely the same (Baker & Lindley, 1992; Steijns & van Hooijdonk, 2000). Therefore, the assumption that the different isoforms of lactoferrin are digested at approximately the same rate seems to be justified.

The results from chapters 6 and 7 indicate that oral lactoferrin supplements deliver intact lactoferrin to the small intestine without the need to protect against gastric degradation. Functional effects of lactoferrin supplementation may therefore be expected in the small intestine. If lactoferrin administration is indicated for application in the colon, the use of ileal release-encapsulation or enemas should be considered.

Functional application of lactoferrin supplements

In chapter 8, the non-steroidal anti-inflammatory drug indomethacin was administered to induce standardized mucosal damage in healthy volunteers. Synchronous supplementation with lactoferrin inhibited the indomethacin-induced rise in small intestinal permeability. This was the first study to show a beneficial effect of lactoferrin administration on gut function in humans. The underlying mechanism of action is not clear.

Although it seems plausible to attribute the protective effects of lactoferrin to its antioxidant capacity, this explanation is not supported by the outcome of the studies described in chapters 3 and 4. In these studies, oxidative stress in the small intestine was provoked by local administration of acute dosages of ferrous iron. Iron initiated lipid peroxidation in the intestinal lumen. In all experiments, this rise in lipid peroxidation was accompanied by a synchronous rise in antioxidant capacity. From those studies we concluded that the detrimental effects of oxidative stress in the human small intestine were inhibited by the release of a powerful antioxidant into the lumen of the small intestine. Considering the large antioxidant capacity available to counteract the oxidant challenges, it is unlikely that administration of an exogenous antioxidant, such as lactoferrin, would provide an additional protection against acute intestinal oxidative damage. Hence, the observed beneficial effect of lactoferrin administration, described in chapter 8, was probably not induced by the antioxidant capacity of lactoferrin, but by another mechanism of action.

This does not exclude a beneficial role of antioxidant supplementation in other situations associated with impaired gut function. Chronic oxidative stress in the intestine may result in depletion of the endogenous antioxidant capacity of the intestinal secretions. Moreover, malnutrition may impair the endogenous antioxidant defence network, and, hence, antioxidant supplementation may be indicated to prevent intestinal oxidative damage. Furthermore, in patients with a so-called 'compromised gut', an additional, oxidative stress may induce enteropathy, whereas this may be prevented by dietary antioxidant supplementation or by dietary components that stimulate the generation of a capable antioxidant network.

Considerations for future research

The studies described in this thesis were designed to investigate responses of the human small intestine *in vivo* to dietary factors, such as iron and lactoferrin. Future studies should be designed to further investigate nutrient-mucosal interactions in the small intestine. Information about the causal relation between intestinal oxidative stress and inflammation, about the interaction between dietary components and the mucosal barrier, and about environment-gene interactions in intestinal mucosa will contribute to the understanding of gut function in health and disease, and may provide nutritional tools to optimize gut health. Specific aims, which should be investigated, are to identify the antioxidant which was released upon oxidative damage in the small intestine, to investigate the possibilities of increasing the endogenous antioxidant depot in patients with low antioxidant levels, as was observed in several GI disorders, to investigate the impact of iron administration in the small intestine on sub-mucosal structures and to elucidate the consequences of the observed changes in gene expression patterns following iron administration in the small intestine.

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SUMMARY

Summary

The small intestinal mucosa forms a functional, tightly controlled barrier against intrusion of a wide variety of pathogenic components. Its integrity may be compromised by factors such as dietary pathogens and toxic food ingredients. In this thesis research is focussed on two food ingredients with positive but potentially also negative health aspects; iron and lactoferrin. Iron is an important micronutrient, due to its participation in many biochemical processes, however, free iron ions are known to catalyze the formation of highly reactive hydroxyl radicals. These radicals are extremely reactive, and may cause damage to all biological molecules in the body. Hence, iron ingestion may lead to undesirable side effects in the gastrointestinal tract and also, iron may compete for absorption with other micronutrients, especially divalent cations such as zinc and copper. The presence of high amounts of dietary iron may, therefore, impair the bioavailability of zinc and copper. In this thesis we investigated the deleterious effects of iron administration on the integrity and biochemistry of the small intestinal mucosa, and on the bioavailability of zinc and copper. The milk protein lactoferrin is a food ingredient with potential positive effects on the intestinal mucosa due to its biological activity, among others the antioxidant and iron binding capacity. We investigated effects of lactoferrin in the small intestine as a second food ingredient.

Chapter 2 describes a study of the effects of iron supplementation on the integrity of the small intestinal mucosa. In this placebo-controlled study, we showed that small intestinal permeability, a well-accepted indicator of the integrity of the small intestinal mucosa and a parameter of epithelial damage, was not affected by oral iron supplementation at daily dosages of 100 mg iron for 28 days. These findings suggested that, in contrast with previous data from *in vitro* and animal studies, iron did not induce oxidative stress in the intestine. To investigate iron-mediated biochemical processes in the small intestine *in vivo*, we developed an intestinal perfusion technique. **Chapter 3** describes the results of the first study applying this novel technique. We administered iron into the proximal small intestine, and sampled small intestinal fluid. We showed that iron administration induced lipid peroxidation, a parameter of oxidative damage, in the lumen of the small intestine. This was accompanied by a momentary rise in total antioxidant capacity of the samples, suggesting that oxidative stress triggered the release of a powerful but yet unidentified antioxidant in the small intestine. In this study, we were unable to investigate the effects of iron administration on the small intestinal mucosa. Therefore, in the study described in **chapter 4**, the intestinal perfusion technique in humans was combined with gastroduodenoscopy to obtain tissue samples from the small intestinal mucosa. The effects of different dosages of iron on oxidative stress and antioxidant capacity in small intestinal secretions and on gene expression profiles in small intestinal mucosa were investigated. We showed that iron administration in the small intestine caused oxidative damage and the release of antioxidants in a dose-dependent manner. Also, the iron intervention resulted in changes in the expression of eighty-nine genes. Functional mapping of genome-wide microarray experiments, carried out to investigate changes in gene expression profiles, identified six biological processes that were significantly mediated by the iron intervention. Although it is clear that several of these processes were associated with cell signalling

pathways, the biological implication of these results will have to be elucidated in future studies, specifically aiming to investigate these processes.

A study of the interaction between iron, zinc and copper was described in **chapter 5**. The investigation was designed to quantify zinc and copper absorption in the small intestine in the presence of different amounts of iron. We showed that a single dosage of iron significantly inhibited zinc absorption. The rate of inhibition of zinc absorption was not dependent on the dosage of iron. Copper absorption was not affected by the presence of iron.

The milk protein lactoferrin is suggested to exert many different biological activities in the intestine, in part due to its strong iron-binding capacity. Antioxidative, antiviral, anti-inflammatory and bactericidal properties were previously attributed to the molecule. Prior to studying its possible application as a functional food ingredient to decrease or prevent gastrointestinal disorders, it is important to obtain detailed information about its gastrointestinal survival rate. Gastric digestion of bovine lactoferrin was investigated in the study described in **chapter 6**. We showed that more than 60 % of all lactoferrin administered to the stomach survived gastric transit. It was previously suggested that iron-saturated lactoferrin (hololactoferrin) was less susceptible to gastrointestinal degradation than the iron-unsaturated form (apolactoferrin). However, this study showed that gastric digestion of holo- and apolactoferrin *in vivo* did not statistically differ. The metabolic fate of lactoferrin after oral administration, and after transit through the stomach and the small intestine, was investigated in the study described in **chapter 7**. We demonstrated in this study applying the ileostomy model that orally administered recombinant human lactoferrin is completely digested before it is emptied from the small intestine. We observed small but relatively constant amounts of intact human lactoferrin appearing in the ileal effluent, and suggest that these trace amounts of lactoferrin originated, most likely, from an endogenous source, such as the granules of infiltrating neutrophils in the small intestine.

We investigated the capacity of lactoferrin to prevent or inhibit mucosal damage in the stomach and in the small intestine *in vivo* in the study described in **chapter 8**. The non-steroidal anti-inflammatory drug indomethacin was administered to healthy volunteers to provoke damage to the intestinal epithelium. This caused standardized, well-investigated and reproducible gastrointestinal damage, which was quantified by measuring the permeability of the small intestine using the lactulose/rhamnose gut permeability test. Oral lactoferrin supplementation reduced the indomethacin-induced damage to the mucosa of the small intestine. This is the first study to show a functional, beneficial effect of lactoferrin supplementation in humans, and justifies the design of further studies to investigate the application of lactoferrin supplements in patients suffering from gastrointestinal disorders, as well as healthy people, in order to prevent gastrointestinal discomfort.

SAMENVATTING

Samenvatting

De binnenwand van de dunne darm, de darmmucosa, vormt een functionele verdediging tegen het binnendringen van ziekteverwekkers. De integriteit van de darmmucosa kan verminderen onder invloed van verschillende factoren, bijvoorbeeld ten gevolge van pathogene bacteriën of toxische componenten die via de voeding de darm binnen komen. In dit proefschrift worden twee voedingscomponenten bestudeerd. Enerzijds is het onderzoek gericht op het melkeiwit lactoferrine als voorbeeld van een voedingsingredient met mogelijke positieve eigenschappen ten aanzien van de integriteit van de darm. Anderzijds is speciale aandacht besteed aan het micronutrient ijzer, dat een belangrijke functie heeft in vele biochemische processen. Het is echter bekend dat de aanwezigheid van ijzer kan leiden tot een snelle vorming van schadelijke hydroxyl radicalen. Deze radicalen zijn zeer reactief, en kunnen schade berokkenen aan alle biologische molecule in het lichaam. Dien tengevolge kan de inname van ijzer leiden tot het optreden van ongewenste bijwerkingen in het spijsverteringskanaal. Bovendien kan ijzer de opname van andere micronutrienten, zoals zink en koper, remmen omdat deze metalen voor een deel via een identiek mechanisme worden opgenomen vanuit de darm. Hierdoor ontstaat een competitie voor opname. De aanwezigheid van grote hoeveelheden ijzer in de voeding kan dus wellicht de biologische beschikbaarheid van zink en koper remmen.

In dit proefschrift hebben wij enerzijds de schadelijke effecten van ijzertoeiening op de integriteit van de darmmucosa en op biochemische processen in de dunne darm bestudeerd, alsmede de invloed van de aanwezigheid van ijzer op de opname van zink en koper. Anderzijds is onderzoek verricht naar het afbraak in en de effecten van lactoferrine in het spijsverteringskanaal.

In het onderzoek dat in **hoofdstuk 2** beschreven staat is het effect van ijzersuppletie op de integriteit van de dunne darm onderzocht. We hebben aangetoond dat de doorlaatbaarheid, ofwel de permeabiliteit, van de dunne darm, hetgeen een maat is voor integriteit van de darmwand en gebruikt kan worden als een parameter van darmschade, niet beïnvloed wordt door ijzersuppletie. Deze bevinding duidt er op dat ijzer geen schade aan de darmwand veroorzaakt. Dit is in tegenspraak met de gegevens die uit eerder gepubliceerd reageerbuis- en proefdieronderzoek beschikbaar zijn. We hebben een darmperfusietechniek ontwikkeld om het mogelijk te maken de invloed van ijzer op biochemische processen in de darmholte te bestuderen. Hierbij wordt bij gezonde mensen een deel van de dunne darm doorgespoeld met een vloeistof en kunnen darmsapmonsters worden afgenomen. In **hoofdstuk 3** wordt het eerste onderzoek beschreven waarin gebruik is gemaakt van deze nieuwe techniek. Hierbij hebben we het eerste deel van de dunne darm doorspoeld met een ijzerhoudende oplossing. Tijdens deze perfusie zijn darmsapmonsters afgenomen. De resultaten van dit onderzoek laten zien dat toediening van ijzer in de darm leidt tot een snelle vorming van vetzuurperoxides. Dit is een maat voor oxidatieve schade. We hebben bovendien aangetoond dat deze schade samenging met een zeer sterke stijging van de antioxidatieve capaciteit in de darmsapmonsters. Deze gegevens duiden op het feit dat oxidatieve schade in de dunne darm van mensen leidt tot een directe vrijmaking van antioxidanten. Onderzoek naar de identiteit van deze antioxidant heeft tot nu toe geen duidelijke identificatie opgeleverd. In dit onderzoek waren wij niet in staat om

de effecten van ijzertoediening in de dunne darm op de darmmucosa zelf te onderzoeken, omdat slechts darmvloeistof en geen darmweefsel werd afgenomen. Daarom hebben we in het onderzoek dat staat beschreven in **hoofdstuk 4** de darmperfusietechniek gecombineerd met het afnemen van darmweefsel door middel van gastroduodenoscopieën. In dat onderzoek zijn de effecten van verschillende doseringen ijzer op oxidatieve schade, op antioxidatieve capaciteit en op de genetische aansturing, ofwel de genexpressie, van cellen in de darmmucosa onderzocht. Dit onderzoek bevestigde dat ijzertoediening in de dunne darm oxidatieve schade en de vrijmaking van antioxidanten veroorzaakt. Beiden waren afhankelijk van de hoeveelheid ijzer dat werd toegediend. De toediening van ijzer veroorzaakte een verandering in de expressie van 89 genen. Na functionele analyse van deze genexpressie resultaten bleek dat zes biologische processen significant waren beïnvloed door de ijzertoedieningen. Enkele van deze processen waren betrokken bij de overdracht van signalen. Toekomstig onderzoek dat specifiek gericht dient te zijn op de bestudering van deze processen moet de biologische betekenis van deze resultaten ophelderen.

In **hoofdstuk 5** wordt de invloed van verschillende hoeveelheden ijzer op de opname van zink en koper beschreven. De resultaten van dit onderzoek laten zien dat de aanwezigheid van ijzer de opname van zink in de dunne darm remt. Hierbij maakt het niet uit of er een kleine of grote hoeveelheid ijzer aanwezig is. De opname van koper werd niet beïnvloed door de aanwezigheid van ijzer.

Het melkeiwit lactoferrine heeft, vooral door zijn sterk ijzerbindende capaciteit, verscheidene eigenschappen die de darmgezondheid kunnen verbeteren. Aan het molecuul worden onder andere antioxidatieve, antivirale, anti-inflammatoire en antibacteriële eigenschappen toebedeeld. Voordat kan worden onderzocht of er een rol voor lactoferrine in voedingssupplementen is weggelegd, dient eerst informatie verkregen te worden omtrent de afbraak van lactoferrine in het spijsverteringskanaal. In het onderzoek dat beschreven is in **hoofdstuk 6** is de afbraak van lactoferrine, afkomstig uit koemelk, in de maag onderzocht. Meer dan 60 % van het lactoferrine dat in de maag werd gebracht bleek onveranderd vanuit de maag in de dunne darm te worden geledigd. Voorgaand onderzoek liet zien dat lactoferrine dat verzadigd is met ijzer beter bestand zou zijn tegen afbraak in het spijsverteringskanaal dan onverzadigd lactoferrine. Dit werd echter niet bevestigd in ons onderzoek. In het volgende onderzoek, beschreven in **hoofdstuk 7**, hebben we de afbraak van recombinant humaan lactoferrine na passage van de maag en de dunne darm onderzocht met behulp van het ileostoma model. Het ingenomen lactoferrine werd volledig afgebroken tijdens de passage door de maag en dunne darm. De zeer kleine hoeveelheden lactoferrine die met een constante snelheid werden uitgescheiden uit de dunne darm waren zeer waarschijnlijk afkomstig uit endogene lactoferrine productie.

Naar aanleiding van de eerder gepubliceerde aanwijzingen dat lactoferrine de gezondheid op verschillende manieren kan bevorderen hebben we een onderzoek uitgevoerd naar het vermogen van lactoferrine om schade aan de darmmucosa te voorkomen. Voor dit onderzoek, dat beschreven staat in **hoofdstuk 8**, hebben we het medicijn indomethacine aan gezonde vrijwilligers gegeven. Indomethacine wordt in de medische praktijk voorgeschreven om pijn en ontstekingsreacties te bestrijden. Het heeft als bijwerking dat de darmmucosa wordt beschadigd, waardoor de darmdoor-

DANKWOORD

Het is af, het proefschrift is klaar. In een dankwoord wordt een passende beschrijving van de afgelopen promotieperiode verwacht, eventueel aangedikt met een aardige anekdote, geschreven vanuit de achtergrond van de auteur. Verwachte cliché's, zoals verhalen vanuit een wielerspectief, zal ik u echter besparen. Hora est, nietwaar? U heeft immers net vol overgave het proefschrift gelezen. Ik zal dit dankwoord dan ook beknopt houden.

Tijdens mijn stage-verblijf in Pittsburg, USA, in 1997 bereikte mij per E-mail reeds het bericht dat er bij de capaciteitsgroep Humane Biologie van de Universiteit Maastricht een AiO positie aan zat te komen en dat ik er, eenmaal terug in de lage landen, goed aan zou doen daar op te solliciteren. Ik ben niet lang daarna aan de slag gegaan met het AiO project, waarvan bij aanvang slechts vast stond dat het onderzoek een brug zou moeten vormen tussen de gastroenterologie en het humane voedingsonderzoek. Het eindresultaat van dat project is dit proefschrift.

Voor het tot stand komen van dit proefschrift ben ik met name mijn twee promotores veel dank verschuldigd. Wim, ik ben je buitengewoon erkentelijk voor de wijze waarop je mijn promotie-onderzoek als promotor hebt begeleid. Met name jouw netwerk aan contacten binnen de universiteit en met industrie was essentieel voor verschillende studies, evenals je inzicht in de 'hot topics' in de levenswetenschappen. Daarnaast heb je een grote bijdrage gehad in het opzetten van verschillende onderzoeken. Met name in de beginperiode van het promotieproject heb je, met je soms confronterende kritiek, mijn artikelen in een leesbaar formaat gebogen. Ook je positie als tweedehands-auto verkoper, sponsor van mijn fanclub tijdens de Ridderronde in Maastricht en van luttele meters bier tijdens weer eens een feestavond met de capgroep, evenals je medewerking aan promotievideo's heb ik zeer gewaardeerd. Tot op de dag van vandaag blijf je me verbazen met je werklust (ik herinner me de E-mail die je me schreef toen je tijdens je vakantie in Canada op de boot stond te wachten, en dus toch niets te doen had ...).

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CURRICULUM VITAE

Curriculum vitae

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