Institutionen för Fysiologi och Farmakologi

Diet and inflammation
The role of nitrate and conjugated linoleic acid

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av

Cecilia Jädert

Huvudhandledare:
Professor Jon Lundberg
Karolinska Institutet
Institutionen för Fysiologi och Farmakologi

Bihandledare:
Docent Mia Phillipson
Uppsala Universitet
Institutionen för Medicinsk Cellbiologi

Sara Borniquel
Karolinska Institutet
Institutionen för Fysiologi och Farmakologi

Fakultetsopponent:
Dr. Andrew Webb
King's College London
Department of Clinical Pharmacology

Betygsnämnd:
Docent Markus Sjöblom
Uppsala Universitet
Institutionen för Neurovetenskap

Professor Lennart Lindbom
Karolinska Institutet
Institutionen för Fysiologi och Farmakologi

Professor Jesper Haeggström
Karolinska Institutet
Institutionen för Medicinsk Biokemi och Biofysik

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From the Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

DIET AND INFLAMMATION

THE ROLE OF NITRATE AND CONJUGATED LINOLEIC ACID

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Till min familj

ABSTRACT

A diet rich in vegetables and unsaturated fatty acids is associated with a lower risk of major diseases including cardiovascular disease, type 2 diabetes and chronic inflammation. Yet, despite extensive research, the active component(s) responsible for these effects has not been pinpointed and studies with single nutrients have been largely unsuccessful. Recent research from our laboratory and elsewhere suggests that the inorganic anion nitrate (NO3-), especially abundant in green leafy vegetables, is converted in our bodies to nitrite (NO2-) and then further to nitric oxide (NO). The latter is a central signalling molecule with a number of beneficial effects in the cardiovascular- and gastrointestinal systems. Ingested nitrate is absorbed to the blood and mixed with nitrate from endogenous sources formed by NO synthases (NOS). Circulating nitrate is actively transported and accumulated in the salivary glands and excreted with saliva. Oral commensal bacteria play a surprisingly important role in nitrate bioactivation by reducing salivary nitrate to the more reactive nitrite anion. The nitrate-nitrite-NO pathway is now emerging as a significant source of NO, in addition to classical endogenous formation of this gas by NOS.

In this thesis we have specifically explored the role of nitrate and nitrite in modulation of inflammation and in regulation of gastrointestinal mucus formation. In addition we have also studied effects of conjugated linoleic acid (CLA), another dietary constituent with proposed anti-inflammatory effects.

The results show that nitrite and dietary nitrate can reduce leukocyte recruitment during acute inflammation in the microcirculation. Dietary nitrate also prevented NSAID-provoked small intestinal inflammation in a process dependent on oral nitrate-reducing bacteria. Although strong anti-inflammatory effects were observed with dietary nitrate, the ability to clear an infection was not impaired. Dietary nitrate, nitrite and CLA were further demonstrated to alleviate inflammation in a mouse model of colitis. The protective effect seen with CLA involved upregulation of trefoil factor 3 (TFF3) expression through activation of peroxisome proliferator-activated receptor gamma (PPARγ) in the colon mucosa. Furthermore, dietary nitrite also had therapeutic effects in already established colonic inflammation, possibly mediated by maintaining the colonic mucus layer and promoting healing of colon epithelial cells. Finally, we demonstrate that the firmly adherent gastric mucus layer, normally present in conventional mice, was almost absent in germ free mice. In addition, a reduced gastric mucus layer was also observed in mice treated with broad spectrum antibiotics. While treatment with nitrate increased the mucus thickness further in conventional mice it had no effects in germ free mice, again demonstrating the essential role of oral bacteria in bioactivation of nitrate. Remarkably however, when the germ free mice were fed a low dose of nitrite, resembling what would normally be generated in saliva by bacteria, the gastric mucus thickness increased dramatically. This suggests that commensal oral bacteria modulate gastric homeostasis via physiological recycling of nitrate, originally derived from NOS.

In conclusion, the studies in this thesis demonstrate that dietary nitrate, nitrite and CLA play a direct role in the regulation of inflammatory responses, both locally in the microcirculation and in the gastrointestinal tract. These results may have implications for future dietary recommendations in prevention and treatment of inflammatory disorders.

LIST OF PUBLICATIONS

I. **JÄDERT C**, Petersson J, Massena S, Ahl D, Grapensparr L, Holm L, Lundberg JO and Phillipson M. *Decreased leukocyte recruitment by inorganic nitrate and nitrite in microvascular inflammation and NSAID-induced intestinal injury*. Free Radic Biol Med. 2012 Feb 1;52(3):683-92.

II. Borniquel S, **JÄDERT C** and Lundberg JO. *Dietary conjugated linoleic acid activates PPARγ and the intestinal trefoil factor in SW480 cells and mice with dextran sulfate sodium-induced colitis.* J Nutr. 2012 Dec;142(12):2135-40.

III. **JÄDERT C**, Phillipson M, Holm L, Lundberg JO and Borniquel S. *Preventive and therapeutic effects of nitrite supplementation in experimental inflammatory bowel disease.* Redox Biology. 2014 Jan; 2:73-81.

IV. Petersson J*, **JÄDERT C***, Phillipson M, Borniquel S, Lundberg JO and Holm L. *Physiological recycling of endogenous nitrate by oral bacteria regulates gastric mucus release.* Manuscript.

*equal contribution

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LIST OF ABBREVATIONS

cGMP	cyclic guanosine monophosphate
CLA	conjugated linoleic acid
Conv	conventional
DAI	disease activity index
DSS	dextran sulfate sodium
GC	goblet cell
GF	germ free
GI	gastrointestinal
HCl	hydrochloric acid
IBD	inflammatory bowel disease
ICAM-1	intracellular adhesion molecule type 1
iNOS	inducible NOS
i.v.	intravenous
LA	linoleic acid
L-NAME	N_{ω}-Nitro-L-arginine methyl ester
MIP-2	macrophage inflammatory protein 2
MPO	myeloperoxidase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
NSAID	nonsteroidal anti-inflammatory drug
OA	oleic acid
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
$ONOO^-$	peroxynitrite
PCR	polymerase chain reaction
PPAR-γ	peroxisome proliferator-activated receptor gamma
RNI	reactive nitrogen intermediate
sGC	soluble guanylyl cyclase
TFF	trefoil factor
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
UC	ulcerative colitis

1 INTRODUCTION

1.1 Diet and health

The link between diet and health has achieved increased attention during the recent decades and has led to a growing consumer awareness regarding the nutritional value of food. As with the Mediterranean or the traditional Japanese diet, a high intake of fruit, vegetables and unsaturated fatty acids is associated with a lower risk for cardiovascular disease (CVD) and type 2 diabetes mellitus (1-6). The mechanisms of the protective effects were initially explained by the abundance of vitamins and antioxidants in these foods (7), but the active nutrients have not yet been determined. The strongest associations with protective effects have interestingly been found with diets high in green leafy vegetables (1, 4, 8). Paradoxically, these vegetables also have the highest concentrations of inorganic nitrate which is just about the only natural substance in vegetables that has been pointed out as being harmful. This is due to the suggested link between dietary nitrate and increased risk of cancer, first reported more than 50 years ago. However, these proposed harmful effects have not been proven by any human epidemiological studies (9). At the same time, green leafy vegetables have recently been ranked as one of the healthiest choice of vegetables, again supposedly due to their high content of vitamins and antioxidants (10). An emerging field of research on dietary nitrate and nitrite during the last decade has demonstrated highly surprising beneficial effects of these anions, thereby slowly changing the negative overall view. These salutary effects are due to the conversion of nitrate and nitrite in our bodies to nitric oxide (NO) and other bioactive nitrogen oxides with physiological effects. In this thesis we have continued to explore health effects of dietary nitrate, nitrite and fatty acids, with a focus on control of inflammation. Inflammation in various forms represents a major global health problem and is the underlying cause of a number of major chronic diseases.

1.2 Endogenous formation of nitric oxide

Before the recent discovery that nitrate and nitrite from the diet can be reduced to NO, endogenous production of NO by specific enzymes was thought to be the only pathway of formation. NO is produced by the nitric oxide synthases (NOSs), a family of enzymes that exist in three isoforms, neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) (11). These enzymes produce NO from the amino acid L-arginine with molecular oxygen as a co-substrate (Fig. 1). NO mediates a broad spectrum of signalling and regulatory functions in mammalian biology, such as nerve transmission, host defence and vasoregulation (11-13). The classical signalling mechanism of NO is mediated through binding to the heme-containing guanylyl cyclase (sGC) with subsequent increased formation of the second messenger 3',5'-cyclic guanosine monophosphate (cGMP). In turn, this leads to decreased intracellular calcium and relaxation of vascular smooth muscle cells (14, 15). The discovery of NO as a signalling molecule in the cardiovascular system elicited the Nobel Prize in Physiology or Medicine in 1998. However, prior to the identification of NO as an endogenous vasodilator, the endpoint had been utilized for more than a century with the use of organic nitrates, such as glyceryl trinitrate (GTN) to treat angina pectoris and high blood pressure. Organic nitrates ($RO\text{-}NO_2$) release NO and have potent acute vasodilatory effects. A drawback with longterm use of these substances

is the development of tolerance and possible development of endothelial dysfunction with increased production of reactive oxygen species (ROS) (16, 17).

NO is a very short-lived molecule due to its high reactivity with other molecules, and is rapidly oxidized into nitrite and nitrate in blood and tissues (Fig. 1). With the formation of nitrite and nitrate, the metabolism of NO was thought to be completed and these were for a long time considered to be inactive end products. It has been known since the 1970s that nitrate (NO_3^-) could be reduced to nitrite (NO_2^-) by oral bacteria (18). However, a novel discovery was made in the mid-1990s by two independent research groups (19, 20) who demonstrated that NO could be formed by reduction of nitrite in the acidic stomach. Thus, nitrate and nitrite tuned out not to be merely inert end products but actually substrates for the regeneration of NO. The formation of gastric NO from nitrite is suggested to occur in a series of non-enzymatic chemical reactions, with the intermediate formation of nitrous acid (HNO_2) and dinitrogen trioxide (N_2O_3) (20).

1. $NO_2^- + H^+ \rightarrow HNO_2$
2. $2HNO_2 \rightarrow H_2O + N_2O_3$
3. $N_2O_3 \rightarrow \cdot NO + \cdot NO_2$

Soon after this discovery, the conversion of nitrite to NO was observed also in the ischemic heart (21). This led to the important understanding that alternative routes of NO-formation existed in addition to the generation from NOSs; both in the gastric compartment and systemically (Fig. 1).

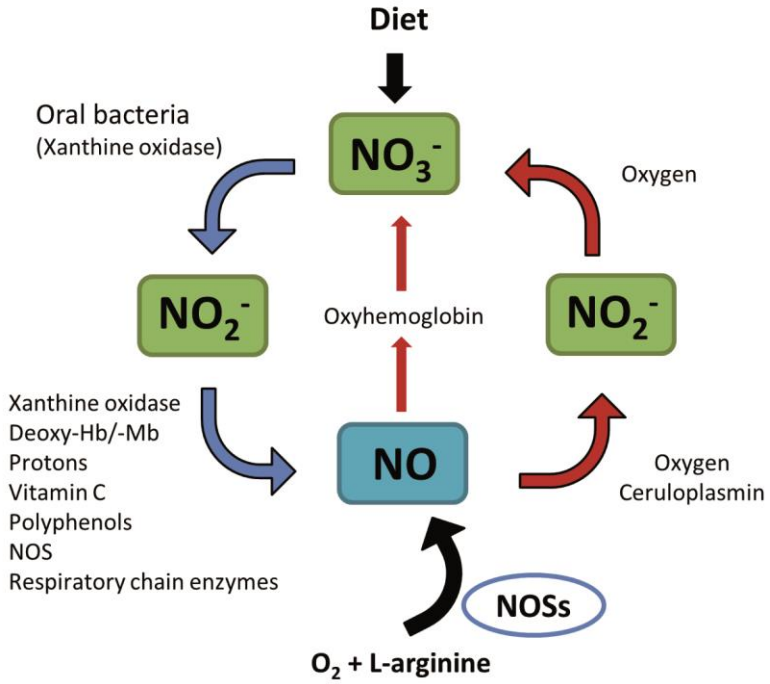

Figure 1. Overview of a mammalian nitric oxide (NO) cycle with oxidation and reduction of nitrate, nitrite and NO.

1.3 Dietary sources of nitrate and nitrite

Various forms of nitrogen are essential in the biology of plants and all other living organisms, and the process of nitrogen cycling in nature is heavily dependent on bacteria. Atmospheric nitrogen gas (N_2) undergoes fixation by bacteria and is converted into ammonium (NH_4^+) which is further oxidized to nitrite and nitrate. Nitrate in the soil is then taken up by plants and transported from the roots to the leaves. The levels of nitrate in plants are dependent on many factors, including the amount of sunlight, temperature and access to nitrogen. Therefore, the amount of nitrate absorbed by different plants varies greatly with origin and season of growth (22). Vegetables are the dominant source of nitrate ingested by mammals which correspond to 60-80% of nitrate intake (23), and green leafy vegetables have particularly high nitrate concentrations. The highest concentrations are found in rucola, spinach and beet root, which contain on average well over 100g/kg in fresh weight. Also celery, fennel, rhubarb and radish are high in nitrate. Potatoes have quite low levels of this anion, but may contribute substantially to the total nitrate intake due to the high consumption of this vegetable in a normal Western diet (22). Since nitrate is water soluble, processing vegetables in various ways including washing, peeling and cooking can substantially reduce the nitrate content (23). In contrast to nitrate, the intake of nitrite originates mainly from foods where it is added as a preservative. Even with this ingestible source, around 80% of our exposure to nitrite is dependent on *in vivo* conversion of ingested nitrate (24).

1.3.1 Circulation of dietary nitrate

As in the circulation of nitrogen in plants described above, bacteria play a crucial role in the reduction of nitrate and nitrite also in mammalian biology. Ingested nitrate is efficiently absorbed from the upper gastrointestinal (GI), with a bioavailability of around 100% (25). In the blood, nitrate originating from dietary sources and from oxidation of endogenous NO by NOSs is then mixed. Fasting plasma levels of nitrate are around 20-40μM, but increases several fold after nitrate intake (26). A major part of the nitrate is excreted by the kidneys, though up to 25% is actively absorbed and accumulated in the salivary glands (27). This leads to a 10-20 times higher concentration of nitrate in human saliva than in plasma (26) (Fig. 2). The reason and the mechanism behind this accumulation of salivary nitrate are still not fully understood, but it seems to involve a specific transport protein named sialin, functioning by co-transporting NO_3^-/H^+ (28). Secreted salivary nitrate is reduced to nitrite in the oral cavity in a process dependent on commensal oral bacteria (29, 30). The swallowed nitrite is then further non-enzymatically metabolized to NO and other nitrogen oxides by the low pH in the stomach, a process that is enhanced by polyphenols and ascorbate (19, 31-33). However, much of the nitrite also escapes this reduction and enters the systemic circulation where it is mixed with nitrite generated by NOS. The plasma concentration of nitrite is around 50-100nM during fasting conditions and is also increased substantially after nitrate intake (26). Circulating nitrite can then be reduced to NO by various enzymes in blood and tissues, including haem-proteins and xanthine oxidoreductase (XOR) (Fig. 1) (22, 34, 35). Even though some nitrate can be reduced systemically to nitrite by mammalian processes (34), the bacterial conversion seems to play a more effective role in nitrate metabolism (29, 36).

The now acknowledged alternative generation of NO from the nitrate-nitrite-NO pathway is suggested to function as an important back-up system for NOS-dependent generation of this gas. Especially during conditions of low pH and hypoxia, this pathway will ensure sufficient formation of NO to maintain vascular homeostasis. In fact, while the oxygen-dependent NO generation from NOS is compromised under hypoxia, the nitrate-nitrite-NO pathway is instead greatly enhanced (37, 38).

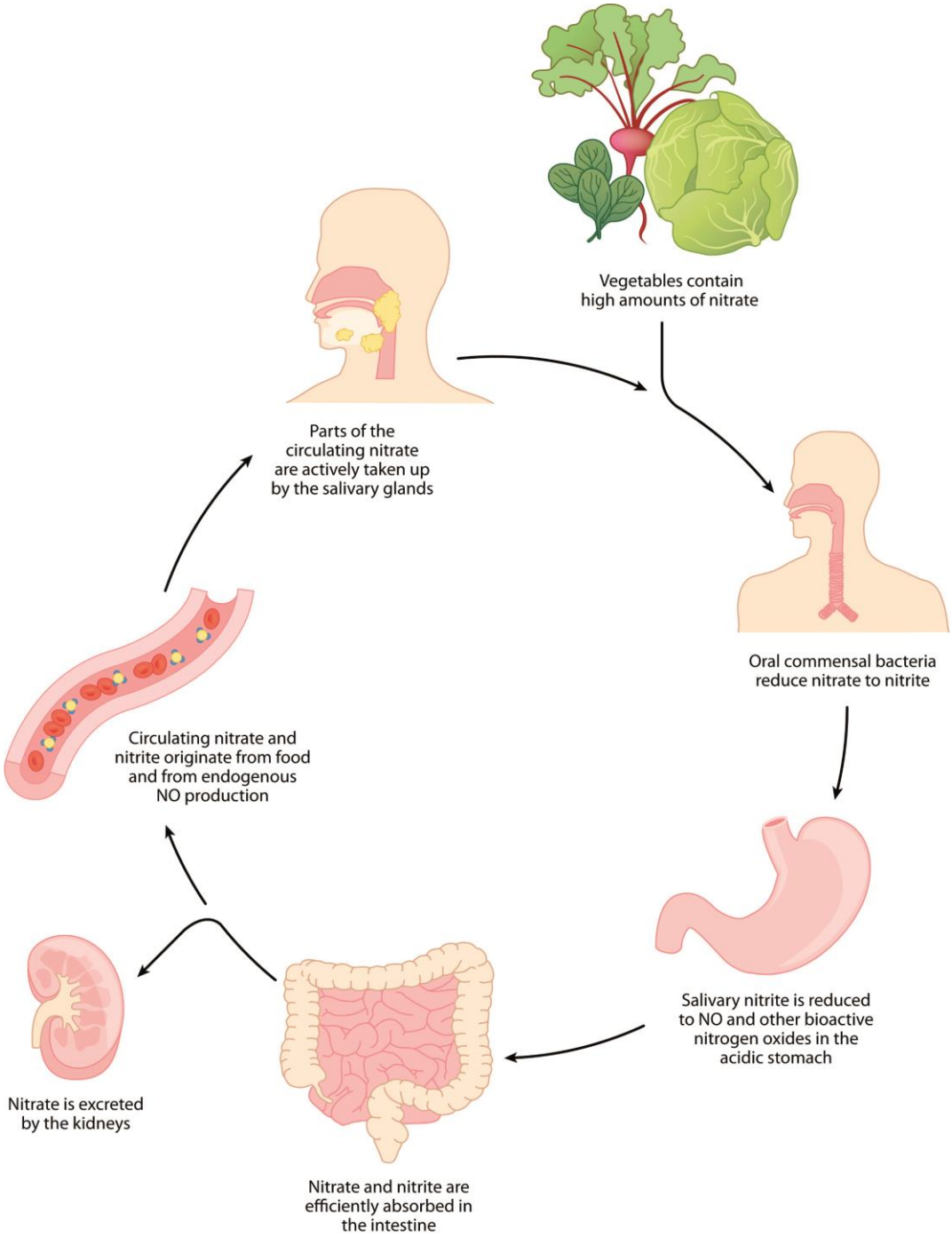

Figure 2. The enterosalivary circulation of ingested nitrate in humans. Figure adapted from (22).

1.3.2 Adverse effects of nitrate and nitrite

The knowledge about inorganic nitrate and its potential effects in improving cardiovascular disorders has been utilized since 700 BC by the Chinese. In the western world, the practice of using inorganic nitrate and nitrite in CVD started in the late 19th century and lasted until the early parts of 20th century. However, this therapeutic regimen was abandoned with the view that nitrite might have toxic effects by reacting with secondary amines to form nitrosamines (16, 17). Animal experiments with long-term exposure to nitrosamines had earlier been shown to give rise to an increased incidence hepatic tumours (39). With this, the concerns arose that this reaction might also occur in humans. Although numerous studies have examined the risk of nitrate/nitrite in the development of human tumours, including gastric cancer, the results are still not clear. A number of international and governmental agencies have now concluded that there are inadequate evidence for carcinogenicity of dietary nitrate in humans or animals (9, 23). However, there is still no consensus regarding the risk of cancer from nitrite in food (9, 24). Other adverse effects with ingestion of high doses of nitrate are the formation of metheamoglobin in infants, named the "blue baby syndrome". With the reduction of nitrate to nitrite, the ferrous iron in oxyhemoglobin is oxidized, thereby forming metheamoglobin which has less ability to bind oxygen, leading to reduced oxygen delivery to tissues. Infants under six months of age are more susceptible to this condition due to lower levels of the reducing enzyme which converts metheamoglobin back to oxyhemoglobin (16). With reports of methemoglobinemia in infants and the potential carcinogenic effects, major restrictions have been made by governmental agencies to decrease the amounts of nitrate and nitrite in the drinking water. All this has also contributed to an overall negative view of nitrate and nitrite. However, with the last two decades of intensive research on the health benefits of inorganic nitrate, the therapeutic potential off this anion against disease has become evident. Focus is now slowly shifting from the toxic properties of nitrate to its desirable biological effects.

1.3.3 Emerging health effects of nitrate and nitrite

Numerous studies have reported therapeutic effects of nitrate and nitrite administration since the existence of a complete reverse pathway of nitrate being converted to NO was proven (22, 26). The effects of nitrite in the cardiovascular system has especially been investigated (40). In particular, nitrite has been shown to protect against damage caused by ischemia-reperfusion in various organs (8, 41), as first shown in the heart by Webb and colleagues (42). Dietary nitrate has also been increasingly studied, demonstrating reduction in blood pressure in healthy volunteers, both with sodium nitrate (43) and a natural source in the form of beet root juice (44). A surprising effect of increased athletic performance has also been observed after ingestion of nitrate in humans, which is suggested to be a result of a reduction of mitochondrial oxygen consumption (45, 46). In animal studies, a great number of biological effects have been demonstrated by nitrate and nitrite administration (22). In the GI-tract, gastric nitrite has been demonstrated to increase gastric mucosal blood flow and mucus thickness (47). Along with this, dietary nitrate administration has shown to be protective against experimentally induced ulcers (48, 49). The anti-inflammatory effects of nitrate and nitrite have also recently started to be investigated. It was shown in the early 1990s that NO had an inhibitory effect on leucocyte adhesion (50, 51), an event probably mediated by activation of sGC (52).

Similar effects were seen by Stokes et al (53) with dietary nitrite after inducing vascular inflammation in mice by a high-cholesterol diet. Indications of reduced kidney inflammation with dietary nitrate have also been demonstrated in rats subjected to a high-salt diet mediating renal damage and hypertension (54). Furthermore, dietary nitrate has been shown to reverse features of the metabolic syndrome that develop in mice lacking endogenous NO synthesis by eNOS, such as body weight, visceral fat, improved glucose metabolism and plasma triglycerides (55).

Many of the biological effects seen with nitrate and nitrite are associated with formation of NO and NO-related substances. However, it has also been suggested that nitrite itself might mediate biological effects (56). A variety of different nitrogen oxides can be formed in the acidic gastric milieu and elsewhere which can mediate NO-like signalling. Thiol groups (-SH) in cysteine residues of proteins might undergo S-nitrosation by nitrite, generating S-nitrosothiols which can act as NO donors (57) or generate post-translational modifications that can alter protein function (58). Other responses might be mediated by the generation of electrophilic nitro-fatty acids (NO_2-FA). Nitration of unsaturated fatty acids can be induced by nitrite-derived nitrogen oxides (NOx), especially the nitrogen dioxide radical ($\cdot NO_2$), formed in high amounts during gastric acidification of nitrite (59). The electrophilic properties of nitrated fatty acids can mediate anti-inflammatory signalling through activation of nucleophilic proteins such as the peroxisome proliferator activated receptor-γ (PPAR-γ) and suppress inflammatory NF-kB signalling. Although endogenous formation of nitro-fatty acids occurs, the levels found in plasma are in a low range, and some authors have questioned if they play a role in normal physiology (60, 61).

1.4 Unsaturated fatty acids

Fatty acids are an essential part of the inflammatory response, by modulating inflammatory processes and by acting as substrates for the generation of signalling molecules (62). Poly- or mono- unsaturated fatty acids (PUFA, MUFA) contain one or more double bonds in the carbon chain and ingestion of these compounds have been linked with health benefits (63). However, the effects are heavily dependent on the type of fatty acid ingested. The general consensus is that the typical western diet contains high amounts of n-6 PUFA and lover levels of n-3 PUFA, leading to an unbalanced fatty acid ratio, which is linked with many inflammatory diseases including obesity, rheumatoid arthritis and inflammatory bowel disease (IBD) (63, 64). The major n-6 PUFA from vegetable sources is linoleic acid (LA, 18:2n-6), which is a precursor for arachidonic acid (AA) that is subsequently converted into inflammatory eicosanoids, such as prostaglandins and leukotrienes. On the other hand, the major vegetable source of n-3 PUFA is α-linoleic acid (ALA, 18:3n-3), which can modulate the inflammatory response by mediating competitive inhibition of the inflammatory eicosanoids (63-65). However, the role of PUFAs in mediating an overall pro- or anti-inflammatory effect is still unclear. Unsaturated fatty acids can also be nitrated by reactions with nitrogen oxide species as mentioned above. These reactions result in formation of for example nitro linoleic acid and nitro oleic acid, both of which have anti-inflammatory effects by inhibiting pro-inflammatory cytokines (66).

1.4.1 Conjugated linoleic acid (CLA)

Conjugated linoleic acids (CLA) are natural fatty acids found in egg, meat, cheese and other dairy products. It is a group of positional isomers of linoleic acid [18:2], which is typically present in plants (67). Up to twenty-eight different isomers are included in the CLA family, but the two most abundant isoforms of CLA found in foods and supplemental preparations are *cis*-9,*trans*-11 (c9t11) and *trans*-10,*cis*-12 (t10c12) (Fig. 3). These two forms are considered to be bioactive (67). The two double bonds found in CLA are separated by one single bond, hence the term conjugated. From dietary sources, up to 90% of the total CLA intake consists of the c9t11 isoform (68), which is suggested to have regulatory effects of the immune response, while t10c12 is more involved in regulation of body fat mass (69, 70). CLA is formed in the rumen of ruminant animals as an intermediate product of complete bacterial biohydrogenation from linoleic acid to steric acid [18:0], thereby changing the double bonds into a saturated carbon chain (71) (Fig. 4). The rumen bacterial conversion was earlier assumed to be the major source of CLA isomers in dairy products. However, another intermediate in this process is *trans*-vaccenic acid [18:1], a form of fatty acid that can be incorporated into tissues of the animal and be converted back to c9t11 by the enzyme delta-9 desaturase. This is now considered to be the major pathway of c9t11 formation, explaining the high levels of CLA found in milk and beef (72). The formation of c9t11 by delta-9 saturation has also been observed in humans and rodents (73). The mechanistic explanation for CLA-mediated protection against inflammation is still largely unknown. However, there have been indications that CLA might enhance the production of the anti-inflammatory cytokine IL-10 in dendritic cells and in macrophages, which in turn reduces NF-kB activity (74, 75). Furthermore, macrophage activity seems to be influenced by CLA treatment by responding with a decreased production of proinflammatory cytokines and other inflammatory mediators such as COX-2 as well as inhibition of iNOS via a PPAR-γ mediated mechanism (76). The intake of CLA from natural sources has been estimated to be around 150 - 440 mg/day (67, 77, 78), a dose that is very low compared to dietary supplementation studies made with this fatty acid (70). When studying the health effects of CLA, supplementation is usually administrated by a mix of isomers c9t11 and t10c12 in ratio 1:1, either as free fatty acids or as incorporated in phospholipids. At present, both the US Food and Drug administration and the European Food Safety Authority has approved a supplemental dose of 3g/day CLA, with an of intake up to 6 months (79).

Due to the presence of double bonds in CLA, radical reactions are possible that might lead to the formation of nitrated fatty acids. In a recent study, CLA was even demonstrated to be a preferential substrate for endogenous nitration in favour of other fatty acids due to the specific location of the double bonds. Nitrated CLA has also been detected in tissues of mice supplemented with nitrite and CLA and in plasma of healthy humans (80).

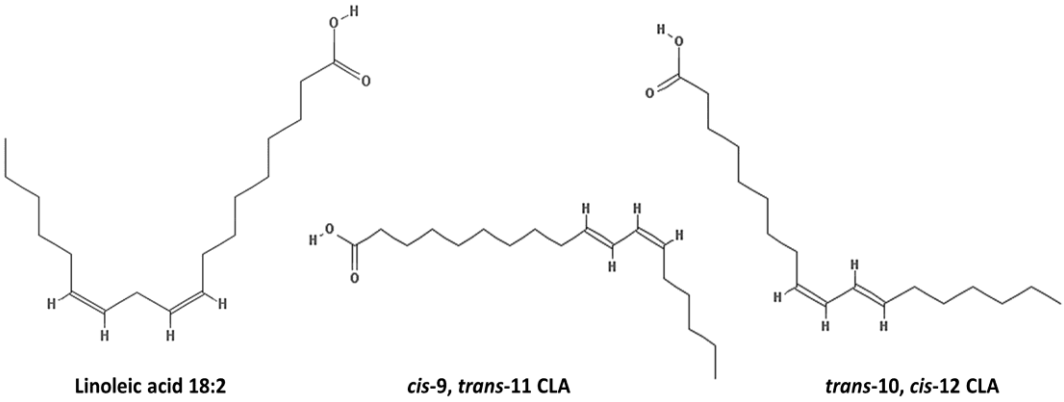

Figure 3. Chemical structure of linoleic acid and the two most abundant isoforms of conjugated linoleic acid.

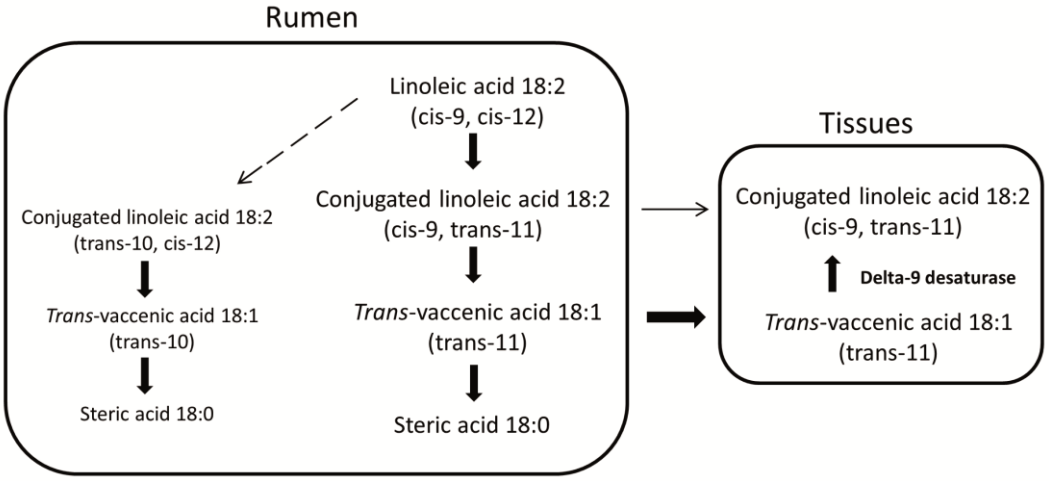

Figure 4. Pathways for rumen and tissue synthesis of *cis*-9, *trans*-11 CLA and alteration in the biohydrogenation pathway of linoleic acid into synthesis of *trans-10, cis-12* in the ruminant animal. Figure modified from (81).

Over the past two decades, the intake of *trans* fatty acids (TFAs) have decreased significantly due to the reported increased risk of CVD (82). The group of TFAs is characterized by containing one or more double bond in *trans* configuration, and TFA can be divided into two different groups; artificial (industrial) and natural (ruminant) trans fatty acids. The industrial TFAs are formed from partly hydrogenated vegetable oils and can be included in foods in order to replace animal fats and to increase shelf life of the products (83). In spite of the negative view of *trans* fatty acids on human health, the naturally formed *trans* fatty acid CLA has been shown to reduce body weight, have effects against cancer and cardiovascular diseases and to modulate inflammatory responses in various animal models of disease (70). For that reason, all isoforms of CLA are excluded in the definition of TFA (79). However, the effects of CLA in humans are not always as conclusive as in animal models and the mechanisms of action are often unknown (67). A recent overview of clinical studies with natural TFA, shows that moderate intake does not increase the risk for cardiovascular diseases (79). To the contrary, there are also indications that the intake of CLA, especially as a supplement in

higher amounts, may have similar unfavourable effects on lipoprotein levels as other *trans* fatty acids (82). More safety studies on the intake of CLA in relation to other TFA are needed to delineate the difference in effect and mechanisms of actions.

1.5 Gastrointestinal mucosal defence

The mucosal membrane lining the gastrointestinal tract is an important barrier against external threats as well as endogenously secreted factors such as gastric acid and degrading digestive enzymes. Pathogens accompanying the intake of food and fluids or inhabiting the colon may cause damage to the host. The mucosa consists of a single layer of epithelial cells covering the basement membrane, the lamina propria and a thin layer of smooth muscle cells (Fig. 5). The entire mucosa of the gastrointestinal tract is covered by a secreted mucus layer, which in conjunction with a tight epithelium, resident immune cells and well-functioning mucosal blood flow protects the mucosa from damage.

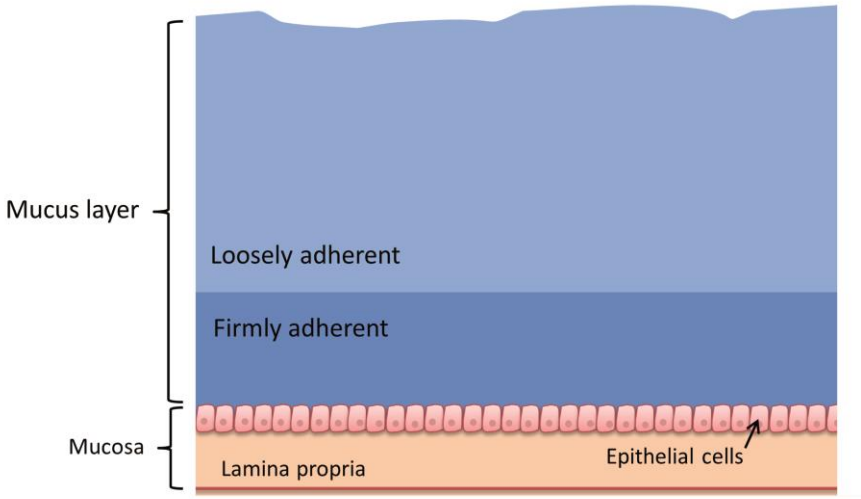

Figure 5. Schematic picture of the gastrointestinal mucosa and the luminal mucus layer.

1.5.1 The gastrointestinal mucus layer

The well-organized protective layer of mucus covering the surfaces of the gastrointestinal tract consists of secreted gel-forming mucins. These are highly glycosylated large glycoproteins originating from the MUC genes (84). In the small and large intestine, the major secretory mucin synthesized and secreted by intestinal goblet cells is Muc2 (85, 86), while Muc5ac and Muc6 are the most important mucins forming the cross-linked mucus layer in the stomach (87). Both the colon and the stomach have a protecting mucus layer organized in a two layer system; the loosely adherent layer adjacent to the lumen, and the inner firmly adherent layer (Fig. 5). The latter is strongly adherent to the epithelium and cannot be easily removed, while the outer layer can be ablated by suction (88). The mucus barrier is not static, but results from the release of mucins by epithelial mucus-producing cells, as well as degradation by proteolytic enzymes which is involved in formation of the two layers (89). A range of hormonal, neural and inflammatory substances regulate mucus formation and release including NO, histamine, prostaglandins, cytokines, hydrogen chloride and acetylcholine (90).

There is a great diversity in the functional aspects of the different parts of the GI-tract, and the mucus layer also has location dependent roles. In the stomach, production of hydrochloric acid by the parietal cells is of importance for degradation of food but also to limit growth of pathogens. However, since this is associated with a very low gastric pH (pH 1-2 in fasting humans), the mucus layer has an important function in protecting the epithelial cells from damage by maintaining neutral pH at the epithelial cell surface through the formation of a pH-gradient (91). The colonic mucus layer is suggested to be a functional barrier against luminal bacteria, thereby limiting bacteria-triggered inflammation. An absent colonic mucus layer has been observed in $Muc2^{-/-}$ mice (86), which are very sensitive to chemically induced colitis and are prone to develop spontaneous inflammation and cancer with increasing age (92, 93). Also, in different mouse models of colitis and in patients with inflammatory bowel disease, the colonic mucus layer has been reported to be more permeable (94), thereby increasing the risk of bacterial translocation and subsequent inflammation. This suggests that the colonic mucus layer is essential to maintain colonic homeostasis and protect against acute and chronic inflammatory disease.

1.5.2 The epithelial cell barrier

An intact epithelial barrier is essential for the integrity of the gastrointestinal mucosa. Insults to the epithelial cells, such as bacterial penetration, might result in an inflammatory response via rapid expression and secretion of pro-inflammatory cytokines and chemoattractants. Resident immune cells together with the epithelium thereby alert the innate immune system to the presence of foreign substances which provide signals for the onset of further inflammatory responses (95). Minor disruptions of the surface layer are frequent due to friction by the large amounts of materials passing through, as well as the acidic environment and degrading enzymes present. More severe injuries as a result of illness or pharmacological side effects might also occur. Both smaller and more serious damage to the epithelial cell layer require rapid repair, starting with restitution followed by regeneration. Restitution is the spread of epithelial cells over the basement membrane to restore the cell-cell contact in order to prevent bacteria or harmful antigens from infiltrating the underlying compartments. This process is mediated by many factors, however trefoil factors (TFFs) have attained increased attention in recent years and are suggested to play a key role in epithelial restitution (96, 97). Three isoforms of trefoil factors have been identified; gastric peptide (pS2/TFF1), spasmolytic peptide (SP/TFF2) and the intestinal trefoil factor (ITF/TFF3). The two former are produced in the gastric and duodenal mucosa (98-100) while TFF3 is localized to the small and large intestine and produced by the goblet cells and excreted with mucins (101). All TFFs have conserved motifs of 6 cysteine residues called the “trefoil” domain; resulting in inter-disulfide bonds that mediate a compact structure which probably mediates the resistance to protease degradation (97). Many studies have demonstrated the important effect of these peptides in gastrointestinal mucosal homeostasis (102, 103), which are suggested to stabilize the mucus structure and are also highly involved in wound healing (97, 104-106). Specific deletion of the TFF3 gene in mice increased the susceptibility to colonic injury in various experimental models of colitis (107), which could be reversed by additional administration of TFF3 (104).

Another important protein in gastrointestinal epithelial cells is the nuclear receptor PPAR-γ, which is one of three members in the PPAR subfamily. These are conserved ligand activated transcription factors, stimulated by small hydrophobic signalling molecules such as hormones, steroids and fatty acids. Upon ligand binding, the receptor can regulate gene expression by activation or repression (108). PPAR-γ is highly involved in metabolic regulation, participating in energy storage and glucose homeostasis. Expression of PPAR-γ is high in colonic epithelium and in activated immune cells (61, 109), and is suggested to also be an important mediator in several anti-inflammatory pathways. Activation of PPAR-γ has been shown to alleviate inflammation in various disease models including experimental models of colitis (61). PUFAs are known ligands for PPAR-γ (110), which can reduce expression of NF-kB (61) by a suggested suppression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) (111). Nitrated fatty acids are also candidates ligands to PPAR-γ (112), and supplementation with nitrated oleic acid has been proven to be protective in an experimental model of colitis (113). CLA isomers are other proposed activators of PPAR-γ, and have also been demonstrated to alleviate inflammation in experimental colitis (114-116). The presence of a PPAR-γ-binding site in the TFF2 gene has been proposed, which may mediate the anti-inflammatory effects of CLA (117). A similar binding site might exist on the TFF3 gene and therefore mediate protective effects. However, little is known about the effects of PPAR-γ ligands and the regulation on TFF3 genes in colitis.

1.5.3 The inflammatory response

The immune system has an essential role in the defence against pathogens, protecting us from severe infections. In general, the immune system is divided into cells active in the innate or the adaptive system with different cells and signalling cascades. The acute inflammatory response is initiated by a rapid recruitment of leukocytes from the blood to the afflicted site, primarily involving neutrophils which are a part of the innate immune system. In humans, ~60% of all circulating leukocytes are granulocytic neutrophils, compared to only ~10% in mice (118). These short-lived immune cells are a vital part of our immune system. In the intestinal vasculature and in other microvascular beds in the body, the endothelium regulates the entry of leukocytes in a multi-step mechanism (Fig. 6). The recruitment cascade is initiated by upregulation of selectins on the activated endothelium, e.g. P-selectin, which induces leukocyte rolling along the blood vessel wall. Next, leukocyte binding of chemokines presented on the endothelium induces integrins on the surface of the leukocytes and mediate adhesion, crawling and finally transmigration through of the vessel wall. The transmigration of leukocytes involves complex binding of endothelial ligands, where a key adhesion molecule is the intracellular adhesion molecule type 1 (ICAM-1). Subsequently, leukocyte binding to components in the endothelial junction and the perivascular basement membrane further progresses the transmigration (119-123). The direction of leukocyte movement in this process of crawling and transmigration is governed by an intravascular chemokine gradient originating from the afflicted tissue (124). Accumulation of infiltrated leukocytes is a key feature in IBD, and an abnormal microvascular leukocyte recruitment might lead to impaired mucosal healing and chronic inflammation (95).

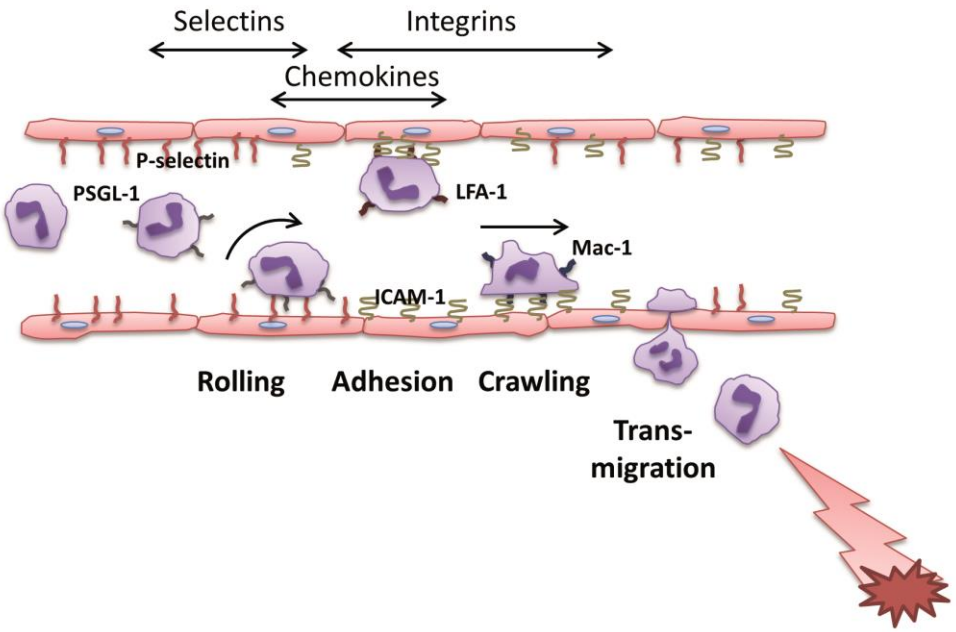

Figure 6. Schematic illustration of the leukocyte recruitment cascade in acute inflammation.

1.6 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic and relapsing inflammatory illness of the gastrointestinal tract, comprising mainly Ulcerative Colitis (UC) and Crohn´s disease (CD). These diseases are thought to be due to a dysregulated immune response to the commensal microflora, which has a genetic link and can be developed by environmental triggers (95). CD is generally displayed by an irregular, transmural inflammation, most commonly observed in the ileum and colon, but can affect any part of the GI-tract. UC on the other hand is usually affecting the colon and rectum in a more continuous pattern, and the inflammation is typically limited to the mucosa (95, 125). The pathogenesis of the disease is not fully understood but the incidence of both UC and CD has steadily increased in the western countries since the mid-1900s (126). The highest prevalence of IBD is reported in Europe and North America (127) and approximately 0.5-1% of the Swedish population is affected (128). There has also been an increase in the incidence and prevalence of IBD during the past decade in Asia, particularly in East Asia (129) (Fig. 7). The reason for the increased incidence is not entirely clear, but diet and lifestyle factors, which have dramatically shifted over the last decades, have been discussed (126). The interactions between the host and the intestinal microbiota are in many cases beneficial in modulating the immune system, regulating energy metabolism and in extracting important nutrients (130). However, interactions with bacteria can also be deleterious to the host by disrupting the mucosal barrier and initiating an uncontrolled inflammatory response which might lead to the development of IBD (131). As mentioned above, an altered mucus layer has been demonstrated in patients with UC with indications of bacterial penetration of the inner mucus layer (94, 132). Bacterial penetration can lead to infiltration of immune cells and cause destruction of the protective epithelial cell layer. Injuries of the epithelial cell layer are a prominent feature in UC and this damage is

resembled in some animal models of inflammation including DSS-induced colitis (dextran sulfate sodium, see below) (133, 134). Hence, stimulation of mucosal healing is an important target in modern therapeutic strategies for UC and a key end-point in many clinical trials today (133). By obtaining mucosal healing, the long-term development of the disease appears to be improved (135).

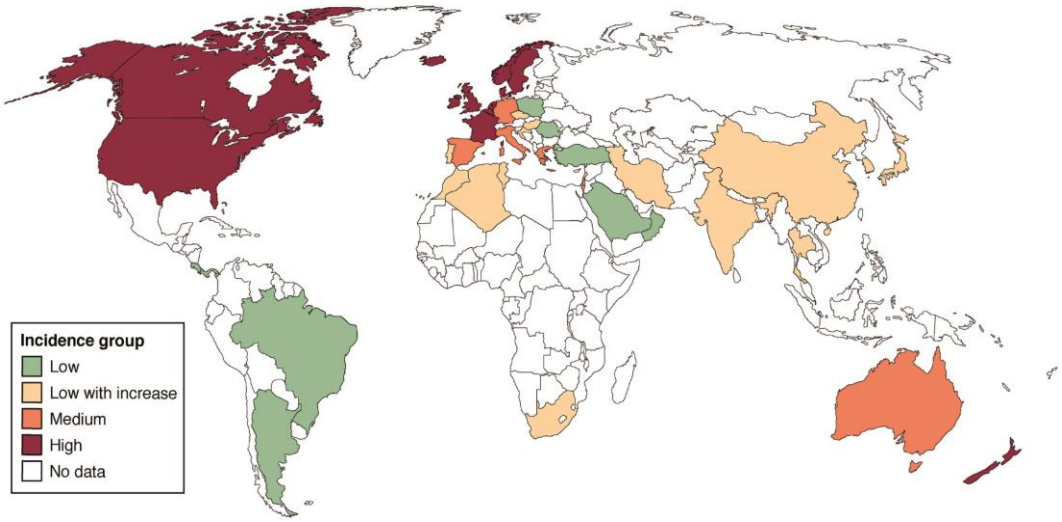

Figure 7. Worldwide annual incidence of inflammatory bowel disease. Red refers to incidence greater than 10 per 100,000, orange to incidence of 5–10 per 100,000, green to incidence less than 4 per 100,000 and yellow to low incidence that is continuously increasing. Figure adapted from (136).

UC is a life-long disease with a relapsing nature, which adversely affects the quality of life (125). No curative treatment exists for these patients today. Instead, the therapy aims to induce remission by anti-inflammatory and immunosuppressive drugs. However, most of these have serious side-effects in long-term management of the disease, often due to the immunosuppression (137). More recent therapeutic approaches under development are focusing on inhibiting specific proinflammatory cytokines, thereby preventing immune cell activation and proliferation (95). However, approved biological therapies available today are limited to antibodies directed towards tumour necrosis factor alpha (TNFα), and a large part of patients are non-responders (138). Furthermore, in patients with more severe relapses and with poor effects of medical treatment, surgery is often the only option (125). The development of new treatments and nutritional strategies to better control the disease is important, in combination with further research to understand the disease mechanisms.

1.6.1 Experimental models of colitis

At present, there are over 50 mouse models of intestinal inflammation, often divided into acute or chronic models of colitis. Oral administration of DSS is the most common acute animal model of IBD, particularly in mice (139). The mechanism of DSS-induced colonic injury is still largely unknown. However, the acute inflammatory reactions are believed to occur by non-specific damage to the colonic epithelium and heavily depend on the use of mouse strain and the molecular weight of the DSS (140, 141). Typical symptoms in animals are weight loss, bloody stools and diarrhoea (134), in combination with common

histological features such as epithelial ulceration, loss of goblet cells and infiltration of immune cells (142, 143). These pathophysiological characteristics resemble injuries that can be observed in human UC (134, 142). In many other aspects, this model is considered to be a relatively poor surrogate for the human disease, i.e. in complexity of the inflammatory response. The pathology of DSS mainly involves the innate immune system with increased infiltration of macrophages and polymorphonuclear leukocytes (PMNs), with little contribution from the adaptive system of T- and B-cells (144). Nevertheless, since DSS is thought to have direct toxic effects on epithelial cells in combination with immune cell infiltration, the DSS-model is suggested to be quite useful in studies of mucosal wound repair (139).

2 AIMS

The overall aim of this thesis was to study the metabolism of dietary nitrate, nitrite and conjugated linoleic acid (CLA) and their effects on inflammatory processes. The more specific objectives of this thesis were:

Paper I: To investigate the effects of nitrite and dietary nitrate on leukocyte recruitment in microvascular inflammation and in NSAID-induced intestinal injury.

Paper II: To examine the anti-inflammatory effects of CLA in DSS-induced colitis and the involvement of PPAR- γ and TFF3 activation.

Paper III: To investigate the preventive and therapeutic effects of dietary nitrate and nitrite in DSS-induced colitis and the regulation of colonic mucosal integrity.

Paper IV: To determine the involvement of the oral microflora and nitrite in physiologic regulation of gastric mucus formation.

3 MATERIALS AND METHODS

Brief descriptions of the methods used in this thesis are described below. For more detailed information, please see the material and methods section for each specific paper.

3.1 Experimental animals

The following strains of mice were used in these studies; male C57/Bl6 (B&K Universal, Stockholm, Sweden and Taconic M&B, Ejby, Denmark), female BALB/c (Scanbur AB, Sollentuna, Sweden), male NMRI (Scanbur AB, Sollentuna, Sweden) and male germ free NMRI mice (germ free animal facility, Karolinska Institutet, Sweden). All mice weighed 18-45 g and were housed under standardized conditions of temperature (21-22°C), humidity and illumination (12-h light/darkness). Male Sprague-Dawley rats (Charles River, Scanbur AB, Sweden), weighed 210-310g and were kept under the same standard conditions as the mice. All animals were allowed to adjust to the cage environment at least 7 days before experimental start with free access to pellet food and tap water. All experiments were approved by the Ethics Committee for Animal Experiments at the Karolinska Institute and Uppsala University.

3.2 Animal treatments

3.2.1 Nitrate supplementation (Paper I, III, IV)

In the animal experiments of paper I, III and IV, mice and rats were given sodium nitrate (10 mM; Sigma-Aldrich) in the drinking water for 7 days before start of experiments. This concentration of sodium nitrate resulted in a daily intake of around 140 mg/kg body weight for mice and 85 mg/kg body weight for rats.

3.2.2 Nitrite administration (Paper I, III, IV)

In paper I, sodium nitrite (1.3 mg/kg; Sigma-Aldrich) was injected i.v. to mice in a bolus dose (0.1 ml) through a cannulated jugular vein. In paper III, mice were administrated sodium nitrite (1 mM) in the drinking water for 7 or 4 days, resulting in a daily intake of 10 mg/kg body weight. Conventional and germ free mice in paper IV, were supplemented with sodium nitrite (1mM, 0.25mM or 50µM) in the drinking water for 7 days, mediating a daily intake of about 11 mg/kg, 2.75 mg/kg or 0.55 mg/kg body weight, respectively.

3.2.3 CLA supplementation (Paper II)

Mice in paper II were supplemented with CLA (50:50 *cis*-9,*trans*-11 and *trans*-10,*cis*-12, purity >99%; Nu-Chek Prep) in their chow to obtain a daily intake of 100 mg/kg.

3.2.4 Induction of gastrointestinal NSAID-injury (Paper I)

NSAID-induced small intestinal injury was induced in paper I. Mice were administrated diclofenac (60 mg/kg) via gavage after having fasted 6 h before and 3 h after administration. N^G-nitro-L-arginine methyl ester (L-NAME, 1g/L; Sigma–Aldrich) was added to the drinking water 24 h before diclofenac gavage, and given as an additional bolus dose (10 mg/kg) with the diclofenac. Examination of the intestines was performed 17-19 h later. In separate experiments, rats were gavaged with diclofenac (30 mg/kg) after being fasted 4 h before and investigated 17-19 h later.

3.2.5 Chlorhexidine mouth spray (Paper I)

In the rat experiments of paper I, the oral microflora was suppressed in some animals. Rats were treated twice daily with 0.3 ml chlorhexidine mouth spray (2 mg/ml, CorsodylR, GlaxoSmith-Kline) applied topically onto the dorsal part of the tongue for a period of 7 days with or without nitrate supplementation.

3.2.6 Induction of experimental colitis (Paper II, III)

Experimental colitis was induced in female BALB/c mice in paper II and III. DSS (2 or 2.5%, 45 kDa; TdB Consultancy, Uppsala, Sweden) was added in the drinking water for 7 days. To evaluate the severity of the disease the Disease Activity Index (DAI) was assessed daily. The score is based on three parameters; the degree of body weight loss, diarrhoea and faecal haemorrhage, earlier described by Ito *et al* (145). On day 7, mice were sacrificed by cervical dislocation under induced anaesthesia with isoflurane (Abbot, Scandinavia, Sweden). The presence of blood in stools was evaluated by analysis of Hemoccult (Beckman Coulter), the colon length was measured and plasma and other tissues were snap frozen and kept in -80°C until further analysis.

3.2.7 Antibiotic treatment of mice (Paper IV)

To obtain a clinically relevant model of microflora elimination, mice were treated with broad spectrum antibiotics in paper IV. An antibiotic cocktail consisting of ampicillin (1 g/L, Sigma), vancomycin (500 mg/L, Hospira), neomycin sulfate (1 g/L, Gibco), and metronidazole (1 g/L, Braun) was added to the drinking water for four weeks prior to further experiments. The antibiotic cocktail was replaced with a freshly made solution every 2-3 days. The method has been used earlier for depletion of gut microbiota (146). Bacteria from colonic faeces (aerobic and anaerobic) and bacteria scraped from the oral cavity (aerobic) were cultivated for 72 h and inspected for growth. The nitrate reducing activity of oral bacteria on the plates was investigated. Colonies were grown over night in Mueller Hinton broth and separate incubations were made with 1 mM sodium nitrate (Sigma-Aldrich) for 0, 1, 2, 3, 4, 5 and 6 h. The reaction was stopped by adding methanol (1:2) and freezing at 20°C until analysis. The incubated samples were centrifuged and the nitrate and nitrite concentrations were measured using the highly sensitive HPLC method (ENO-20; Eicom Japan).

3.3 *In vivo* animal protocols

3.3.1 Leukocyte recruitment in microvascular inflammation (Paper I)

Leukocyte recruitment in microvascular inflammation was explored through intravital microscopy in paper I (Fig. 8). Mice were anesthetized by spontaneous inhalation of isoflurane (Abbot, Scandinavia, Sweden) and the left cremaster muscle was gently opened and mounted for observation of leukocytes in postcapillary venules through bright-field intravital microscopy (Leitz Ortholux II with 25x/0.6W objective, connected to a Hamamatsu C3077 camera). Throughout the experiment, the muscle was constantly kept moisture by superfusion of bicarbonate-buffered saline (pH 7.4, 37°C). Induction of neutrophil recruitment in the cremaster muscle was made by adding murine macrophage inflammatory protein 2 (MIP-2, CXCL2; R&D Systems, England) to the buffer. The MIP-2 superfusion (0.5 nM) continued for 90 min. A 5-min base line recording of chosen vessel was done before the MIP-2 superfusion and further recordings were made at 30, 60

and 90 min at the same spot. In separate experiments, the sGC inhibitor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris Bioscience, USA) was added to the buffer (100 µM) to investigate potential NO-sGC-cGMP aspects of leukocyte recruitment. Neutrophils were analysed in the recordings for number of rolling cells (number of leukocytes passing per min), rolling velocity (average time of 10 first cells passing over a 100 µm segment), adhesion (cells stationary for more than 30 s) and emigration (cells in the extravascular space within the field of view 0.05 mm^2).

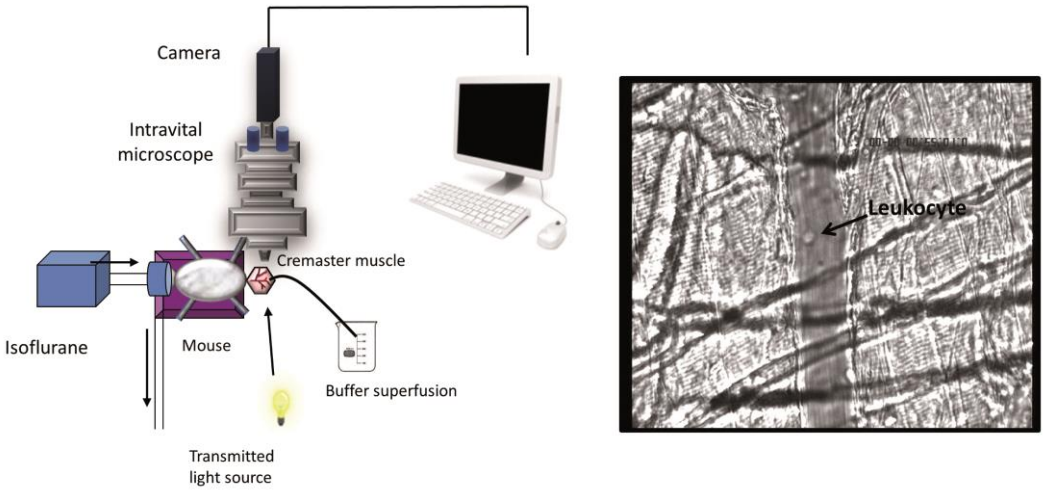

Figure 8. Left: The experimental set-up of the cremaster muscle for *in vivo* imaging of leukocyte recruitment in microvascular inflammation. Pre-warmed bicarbonate-saline was superfused over the muscle to keep it moist and MIP-2 and ODQ was added to the buffer. Right: 5 min recordings of the selected postcapillaty venule were made before and at time 30, 60 and 90 min of MIP-2 superfusion and later analysed for number of rolling, adherent and emigrated leukocytes.

3.3.2 Blood flow measurements (Paper I, IV)

To measure organ blood flow in the small intestine of rats in paper I, a microsphere technique was used. Rats were anesthetized by an intraperitoneal injection of inactin (thiobutabarbital sodium 120 mg/kg; Research Biochemicals, USA) and catheters were inserted in the femoral and carotid artery. During the entire experiment, the blood pressure was monitored. When arterial blood pressure was stable, the diluted nonradioactive microspheres (~300 000, 15µm in diameter) (E-Z Trac Ultraspheres; IMT, Stason Laboratories, USA) were injected through the carotid catheter over 10 s. A reference blood sample was withdrawn from the femoral catheter with a constant rate (~0.55ml min^{-1}) staring 10 s before injection of microspheres until 60 s after injection. To evaluate the organ blood flow in the small intestine the rats were sacrificed by an i.v. injection of potassium chloride and pieces of the ileum and kidneys was quickly removed and weighed (~150 mg and ~100 mg respectively). The microspheres were visualized using a method of freeze-thawing and counted in the organs and reference blood sample using a light microscope. Organ blood flow in the ileum was calculated according to the following formula; $\mathbf{Q_{org} = (N_{org} \times Q_{ref})/N_{ref}}$. Q_{org} is the organ blood flow (ml/min x g tissue), Q_{ref} is the blood flow in the reference sample (ml/min), N_{org} and N_{ref} are the total numbers of microspheres present in the organ or the reference sample, respectively. The

microspheres counted in the kidneys and the following obtained value of blood flow were used as to assure adequate mixing of the microspheres in the circulation. A difference of less than 10% in the blood flow values between the two kidneys was accepted as sufficient mixing.

In paper IV, blood flow in the exposed gastric mucosa of mice was measured using Laser-Doppler flowmetry (PeriFlux 4001 Master and PeriFlux Pf 3; Perimed, Stockholm, Sweden). With this method, the number and velocity of the red blood cells are measured by a laser light (wavelength 635 nm, helium neonlaser). The reflected light is recorded by a pair of fibers and the velocity is calculated as the change in frequency, namely the Doppler shift. The gastric mucosa in conventional and germ free mice were treated with topical administration of sodium nitrite (1mM, pH 3; Sigma-Aldrich) and the laser probe was fixed by a micromanipulator and placed at the same spot above the mucosa (0.5-1 mm) to monitor the gastric blood flow.

3.3.3 Bacterial clearance (Paper I)

In the investigation of bacterial clearance in paper I, bioluminescent *S. aureus* (strain Xen29, Caliper Life Sciences, USA) was injected subcutaneously in the back of mice. Before injection (~24h), a part of the back fur was removed with a depilatory cream. Bacteria were cultured to exponential growth level, as measured by a spectrophotometer and were diluted with a carrier of saline Cytodex beads (10mg/ml) (Sigma–Aldrich) in PBS. Approximately 1×10^6 CFU (colony-forming units) was injected into isoflurane anesthetized mice, with or without pretreatment of nitrate. Bacterial clearance was monitored by a Xenogen bioimaging device (IVIS Spectrum; Caliper Life Sciences) in mice under anaesthesia by isoflurane at 0, 0.75, 2.5, 5, 12 and 24 h and thereafter every 24 h postinoculation for 14 days. Bioluminescence was quantified using Living Image software (Caliper Life Sciences).

3.3.4 Quantification of P-selectin expression (Paper I)

Measurements of P-selectin expression in the small intestine of rats were performed in paper I, in order to evaluate the acute intestinal inflammation. Rats were anesthetized with inactin (thiobutabarbital sodium 120 mg/kg; Research Biochemicals, USA) and a dual-labelled monoclonal antibody technique was used, previously described in detail (147). In brief, catheters were inserted in the left jugular vein and right carotid artery for injection of antibodies and blood sampling, respectively. A mixture of 10 µg of ^{125}I -labelled P-selectin MAb (RMP-1) and 5 µg of ^{131}I-labeled nonbinding MAb (P-23) was injected. Blood samples were subsequently taken via the carotid artery in preheparinized rats (3,000 IU/kg), after 2.5 and 5.0 min. The animals were then drained of blood and the small intestine was collected and weighed. Quantification of P-selectin expression was determined by analysing the activity of the ^{125}I and ^{131}I antibodies in the small intestine using an LKB 1282 Compugamma (Wallac Oy, Turku, Finland).

3.3.5 Gastrointestinal mucus thickness measurements (Paper III, IV)

Colonic mucus thickness was examined in the DSS-induced colitis and nitrite pretreated mice in paper III. In paper IV, gastric mucus thickness was measured in conventional or germ free mice. The general surgery set-up and method of mucus thickness measurements is illustrated in Figure 9. Mice were anesthetized with spontaneous inhalation of

isoflurane (Abbot, Scandinavia, Sweden) through a breathing mask and the body temperature was maintained at 37°C by a heating pad underneath the mouse. Preparation of the mice was done by opening the abdomen through a midline incision. The distal colon was exposed and opened longitudinally by cautery, while the stomach was opened along the greater curvature of the forestomach, following loosening of ligaments. Both the colon and stomach were draped over a truncated cone with the mucosal side facing up. The mucosa was then exposed through a hole in a mucosal chamber (0.2 cm^2) that was placed over the tissue and sealed with silicon grease. To maintain the moistness of the mucosa, the chamber was filled with 37°C 0.9% saline solution. In some of the gastric mucus measurements, the chamber was filled with acidic saline (pH 2) and sodium nitrite (1mM) for 60 min before mucus measurements. Also, ODQ (Sigma-Aldrich) was added to the solution (1mM) dissolved in 5% DMSO in some experiments.

The mice were left to stabilize for 30-60 min after the surgery before measurements of the mucus thickness. Glass micropipettes were pulled to obtain a thin tip (1-3µm) and dipped in silicon solution for a non-adhesive surface and to avoid mucus from adhering to the glass. To visualize the luminal mucus layer, a suspension with charcoal particles was applied on the mucosa. The micropipette was connected to a micromanipulator (Leitz, Wetzlar, Germany) and pushed into the mucus gel at an angle (*a*) of 30-40° to the epithelial cell surface. The distance from the mucus surface to the epithelial cells (b) was measured with a digimatic indicator and the thickness of the mucus gel (T) was then calculated (Fig. 9). The technique has been described in detail previously (148). To measure the firmly adherent mucus layer, the outer loosely adherent layer was gently removed by suction with a thin cannula connected to a syringe. The mean value of mucus measurements at five different spots was used as one observation to obtain a more true value of the mucus thickness. The same five spots were measured again after 1h to reveal mucus dynamics.

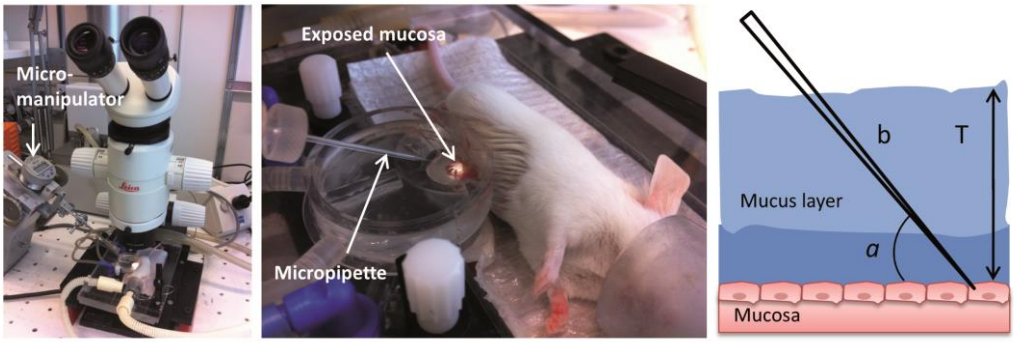

Figure 9. Left and middle: Mouse preparation for mucus thickness measurement. Right: A schematic drawing describing the mucus measurement and the values obtained for mucus thickness calculations.

3.4 *In vitro* methods

3.4.1 Cell cultures and wound repair assay (Paper I, II, III)

In paper I, human dermal microvascular endothelial cells (HDMEC) were used to study surface adhesion molecules with immunofluorescence. Human colon epithelial cells (SW480) were cultured for experiments in paper II and III for investigating gene expression by qPCR of PPAR-γ and TFF3, and to study the effect of nitrite in wound healing. The wound healing assay has previously been described in detail (149). For more detailed information about cell culture conditions and treatments, see the material and methods section for each specific paper.

3.4.2 Immunohistochemistry and histology (Paper I, II, III)

Fluorescent immunohistochemistry was performed in paper I of cultured HDMEC. Cells were stained with mouse anti-human ICAM-1 (10 μg/ml; R&D Systems) and secondary fluorescent antibody goat anti-mouse Alexa 555 antibody for ICAM-1(1:500; Invitrogen, Eugene, OR, USA) after treatment with TNF-α and sodium nitrite. Visualization and detection were made by a confocal microscope and the ICAM-1 intensity was quantified using ImageJ (1.43u; National Institutes of Health, USA).

Immunohistochemistry of frozen colonic tissue from mice treated with DSS and additional treatments of nitrate, nitrite or CLA was performed in paper II and III. Following excision of the colon, specimens from the proximal and distal part of the colon were placed in TissueTec (Oct Cryomount, Histolab, Sweden), snap frozen and kept in -80°C. Cross sections of colon tissue (6 μm) were fixed in 1% paraformaldehyde (Sigma-Aldrich) for 10 min and washed with PBS. Blocking of endogenous peroxidases with H_2O_2 (Sigma-Aldrich) was done for 10 min followed by PBS washing and applying the avidin-biotin system (Vector Laboratories, USA) for reducing the non-specific background, 15 min each. The specimens were incubated with primary antibody (see specific antibodies in each specific paper) over night at +4°C. Further blocking of non-specific binding with bovine serum albumin (Sigma-Aldrich) for 1 h prevented the secondary antibody from cross reacting with endogenous immunoglobulins in the tissue. Incubation with secondary biotin-conjugated antibody (Jackson Immuno Research, USA) was done for 30 min followed by applying the avidin biotinylated enzyme complex (Vectastain ABC kit, Vector Laboratories, USA) and 3,3- diaminobenzidine (DAB; Vector Laboratories, USA) was used to detect the antibody. Sections were counterstained with hematoxylin (Sigma-Aldrich) for visualization of tissue structure. The expression score of each protein was expressed using a graded scale from 0-3, reflecting the intensity of staining, judged by a researcher blinded to the protocol.

Histological analysis of colonic tissue from DSS-treated mice was performed in paper III to investigate tissue morphology and inflammatory status. Intact mice colons were embedded in paraffin and cut in longitudinal sections (4 μm). Staining with hematoxylin and eosin (H&E) was done to analyse inflammatory status and tissue destruction, and alcian blue–periodic acid-Schiff (PAS) staining was done to visualize colonic mucin. Disease severity and abundance of goblet cells (mucin) was evaluated by a score of 0-3 by a researcher blinded to the section protocol.

3.4.3 mRNA isolation and qRT-PCR (Paper II, IV)

Total mRNA was isolated from colonic epithelial cells or snap-frozen stomach tissue by homogenization in Trizol reagent in paper II and IV, respectively. The relative gene expression levels of PPAR-γ, TFF3, HO-1, Muc5AC and Muc6 were determined by real-time PCR using ABI 7500 Fast Real-Time PCR system (Applied Biosystem, Sweden) and β-actin was used as standard control. Amplification was carried out by SYBR Green PCR Master Mix (Applied Biosystem, Sweden). See information about primer sequences and thermal conditions in the methods section for each specific paper.

3.4.4 Intestinal neutrophil infiltration (Paper I)

To study neutrophil infiltration in intestinal tissue from mice treated with diclofenac and nitrate, a quantitative measure of myeloperoxidase (MPO) was performed with enzyme linked immunosorbent assay (ELISA). See specific details about the protocol in the methods section for paper I.

3.4.5 Nitrate, nitrite and RXNO measurements in plasma (Paper I)

Plasma levels of nitrate, nitrite and RXNO were measured from mice directly after i.v. injection of sodium nitrite (1.3 mg/kg) in paper I. Blood samples were collected and mixed with 100 mM N-ethylmaleimide/40 mM EDTA solution to prevent degradation of anions. The samples were immediately centrifuged (6500 rpm) for 5 min at 4 °C and stored at −80 °C until analyzed. Nitrite, nitrate, and S-nitrosothiols were measured by highly sensitive chemiluminescence (77 AM; EcoPhysics, Duernten, Switzerland) and HPLC methods (ENO-20; Eicom Japan). The method has been described in detail previously (26).

3.5 Statistical analysis

Single comparisons between two parameters were evaluated by a two-tailed paired Student's t-test, were the animals served as their own control. Unpaired two-tailed Student's t-test was used to compare values between two groups. For multiple comparations, significance was evaluated by analysis of variance with one-way ANOVA with the Tukey's post hoc test. For changes within groups, repeated-measures ANOVA with Dunnett's or Bonferroni's multiple comparison test was used. Statistical analyses were performed with GraphPad Prism software 4.0 or 5.0 (GraphRad Software, La Jolla, CA, USA). All data are shown as means ± standard error of the mean (SEM). Values of $P < 0.05$ were considered significant.

4 RESULTS AND DISCUSSION

With the discovery that nitrate and nitrite are not solely inactive NOS-derived end-products of NO metabolism, but also constitute a reservoir for NO in blood and tissues (26), an extensive search for the role of these anions in health and disease has begun. Since NO has a fundamental role in blood flow regulation, many of the studies have focused on cardiovascular effects of inorganic nitrate and nitrite. These include protective effects in ischemia reperfusion injury (42), lowering of blood pressure (43), reversal of endothelial dysfunction and inhibition of platelet aggregation (44). During recent years, an interest also in anti-inflammatory effects has emerged since NO has well-known effects in reducing leukocyte activation and adhesion (50, 51). Fatty acids are also involved in the regulation of inflammation. However, there are great variations in the action of these substances depending on structure and interaction with other signalling proteins (64). CLA is one particular fatty acid that has received increased attention in recent years due to its ability to modulate inflammatory reactions (67).

This thesis investigates both systemic and local anti-inflammatory effects of different components of our everyday diet; nitrate, nitrite and CLA using different *in vivo* models enabling studies of immune cell behaviour and mucosal defence mechanisms of the GI-tract.

4.1 Paper I

Decreased leukocyte recruitment by inorganic nitrate and nitrite in microvascular inflammation and NSAID-induced intestinal injury

Recruitment of immune cells from the circulation to tissues is fundamental for host defence against pathogens, but accumulation of leukocytes can also lead to tissue damage and chronic inflammation when dysregulated. If the functional balance between pro- and anti-inflammatory mediators is lost, e.g. with low or too high NO-levels, the interaction of neutrophil and endothelium might result in a change to a pro-inflammatory state. Such changes could contribute to many widespread chronic diseases in the Western world, including atherosclerosis and inflammatory bowel disease. These common disorders are associated with sustained low-grade inflammation, which results in aggravation of the disease (95, 150). Furthermore, neutrophil recruitment to adipose tissue during increased energy intake has also recently been indicated to play an important role in tissue inflammation and in the development of insulin resistance (151). Another large clinical problem involving upregulated inflammatory reactions is the adverse effects of various commonly used drugs. An example is the adverse effects from the widespread use of NSAIDs, inducing gastrointestinal injury (152). The initial mechanism of mucosal damage in response to NSAIDs is believed to be the result of increased leukocyte activation and adhesion prior to activation of the adaptive immune cells (153). With the notion that the immune response is a fundamental biological function, the question is how to manipulate these interactions without creating severe immunosuppressive consequences for the individual. NO has previously been shown to reduce leukocyte activity and adhesion (50, 51), and nitrite has been indicated to have similar results (53). In this study, we investigated if dietary nitrate affects leukocyte recruitment and in

addition, whether the ability to eliminate bacterial infection is affected by nitrate treatment. We also aimed to further elucidate the underlying mechanisms.

4.1.1 Acute nitrite treatment attenuates leukocyte recruitment

The effect of an intravenous injection of sodium nitrite on leukocyte behaviour during MIP-2-induced acute inflammation was investigated in the mouse cremaster muscle by intravital microscopy. A bolus dose of nitrite (1.3 mg/kg) given before initiation of MIP-2 superfusion, decreased neutrophil adhesion and emigration by 50-70% after 60 and 90 min compared to control mice. This dose of nitrite has previously been demonstrated to have protective effects in a sepsis-induced model (154), and was chosen as an initial dose to see if effects on leukocyte recruitment could be observed. Another recent study has indicated decreased leukocyte recruitment by a similar single high dose of nitrite by intramuscular injection in an acute lung injury model induced by chlorine toxicity (155). In line with this study, our results suggest that nitrite can mediate a rapid protective effect against an acute inflammatory response by reducing infiltration of leukocytes. Also lower doses of nitrite administered in the drinking water for 3 weeks have earlier been shown to decrease leukocyte adhesion and emigration in a model of microvascular inflammation induced by high cholesterol diet (53). The reduced recruitment of neutrophils in response to MIP-2 in our study are most likely not due to lower amount of circulating leukocytes or altered blood flow, since neither rolling flux (cells/min) or rolling velocity (μm/s) was affected by the nitrite treatment. In accordance with this, Stokes and colleagues (53) observed no difference in leukocyte count, wall shear stress or blood pressure in oral long-term administration of nitrite. Also, Ahluwalia and colleagues (52) have previously shown that there are no differences in erythrocyte velocity or shear stress in mesenteric postcapillary venules of eNOS-/- mice, or in mice receiving the sGC inhibitor ODQ or an NO-donor in the superfusate. Instead, the reduced recruitment might, at least partly, be a result of a nitrite-dependent modulation of vascular adhesion molecules. Indeed, we noted that in TNF-α stimulated endothelial cells, the expression of ICAM-1 was downregulated in the presence of nitrite (100 μM). When investigating the mechanistic effects of nitrite-mediated reduction in leukocyte recruitment during acute inflammation, ODQ was added to the MIP-2 superfusion buffer in order to explore if the effect was cGMP dependent. While ODQ did not affect nitrite-dependent reduction in neutrophil emigration, neutrophil adhesion was partly inhibited compared to what was observed in mice treated with only nitrite. This suggests that nitrite may interfere with the two different steps in the recruitment cascade via different signalling pathways. Besides feeding the established sGC-cGMP pathway via NO formation, nitrite may also signal by other mechanisms, for example by S-nitrosation of proteins. However, since the effect of ODQ on adhesion was apparent only at the last measurement of superfusion, continued measurements might have resulted in a more clear effect. It is also possible that ODQ did not block all sGC enzymes by 90 min, since this preparation in whole muscle can create a diffusion barrier. For the very same reason, a somewhat high concentration of ODQ was chosen compared to an earlier study of intravital microscopy in the mesentery (52).

4.1.2 Dietary nitrate decreases leukocyte emigration and reduces NSAID-induced injury

Since it had become apparent that dietary nitrate could have NO-like effects in various physiological situations (43, 49, 55), we further investigated whether a dietary approach with nitrate could have similar effects as seen with nitrite. A dose of nitrate that resembles a high intake of nitrate-containing vegetables was given via the drinking water one week before the induction of acute inflammation in the cremaster preparation. These experiments revealed that leukocyte emigration was reduced to a similar level as with nitrite injection, while no effect on leukocyte adhesion was observed. Thus, emigration efficacy was decreased while adhesion was not affected, again indicating separate signalling pathways for these steps of the recruitment cascade. Adherent cells crawl before they transmigrate out of vasculature (156), and whether nitrate and nitrite affect this process remains to be studied. Furthermore, nitrate treatment might also affect the endothelial junction proteins, thereby closing the sites for leukocyte transmigration.

Decreased leukocyte emigration by nitrate was further studied in another inflammatory model by diclofenac-induced intestinal damage. Using this model of GI-injury, nitrate pretreatment resulted in similar observations as in the acute model with MIP-2, demonstrated by lower levels of intestinal MPO and reduced P-selectin expression. In agreement with previous studies, a crucial role of oral bacteria in conversion of nitrate to nitrite was demonstrated by administrating antiseptic mouthwash during the week of nitrate treatment (29). This resulted in loss of nitrate-induced reduction of P-selectin expression. These results further show that the nitrate-nitrite-NO pathway indeed has profound effects on leukocyte function, a finding that could be of considerable importance for several diseases involving inflammation. However, apart from the oral bacterial conversion of nitrate, other nitrite reductases might also be important in the reduction of nitrite to NO in the intestine (Fig. 1). Nitrate did not increase luminal NO levels, which suggests that the effects on leukocyte recruitment are primarily mediated by an accumulation of nitrite and other reactive nitrogen intermediates delivered via the blood and adjacent cells. Multiple pathways exist for nitrite reduction in tissues and blood, including XOR, eNOS and deoxygenated haemoglobin/myoglobin (22). To pinpoint the most important reductases of critical importance in this model is indeed interesting and will be a goal for future studies.

4.1.3 Dietary nitrate is not immunosuppressive

With the reduced leukocyte emigration after nitrate pretreatment observed in both the acute and intestinal model of inflammation, we further investigated if the local anti-inflammatory effects could translate to systemic immune suppression. Neutrophils are dominant in the defence against *S. aureus* infections, so we therefore used these bacteria to investigate bacterial clearance. Our results demonstrated that nitrate treatment did not reduce the ability to clear subcutaneous *S. aureus* infections compared to untreated mice. This could be explained by the diverse chemotactic gradients that leukocytes are exposed to during bacterial infections. A hierarchical relationship between chemotactic factors released by bacteria, activated endothelium or leukocytes determine what signalling pathways that are induced and thereby the action of the immune cell. Thus, the anti-inflammatory signals of nitrate and nitrite in response to the low-grade inflammatory

stimuli by MIP-2, seems to be totally overridden by the signals from the bacterial infection. Hence, nitrite and nitrate can thereby potentially mediate an anti-inflammatory condition without the adverse effects associated with immunosuppression therapies.

4.2 Paper II and III

Anti-inflammatory effects of nitrate, nitrite and CLA in experimental colitis

The anti-inflammatory effects of nitrate and nitrite demonstrated in **Paper I**, were further tested in another model of intestinal inflammation by DSS-induced colitis, to further study the potentially protective effects of these anions. Anti-inflammatory effects of CLA have previously been demonstrated *in vitro* and *in vivo*, often partly mediated through activation of PPAR-γ (69, 116). Both nitrite and CLA have previously been demonstrated to ameliorate experimental colitis (115, 157). However, the clinical relevance of these observations in IBD can be questioned, since the treatment is initiated before the induction of disease and the levels of treatment are often above recommended intake or not achievable by a dietary intake (115, 157). The experimental design of paper II and III instead allowed studies of both preventive and therapeutic effects of dietary CLA, nitrite and nitrate during DSS-induced colitis. The treatments and disease were induced simultaneously, or alternatively the treatment was initiated after DSS induction.

4.2.1 Preventive effects of nitrate, nitrite and CLA on DSS-induced colitis

We first investigated if nitrate, nitrite and CLA could have preventive effects on inflammation in a model of mild colitis initiated by concurrent administration of 2% DSS to Balb/C mice for 7 days (Fig. 10 A). Using this approach, the markers of inflammation measured by colon length and DAI-score were clearly improved by nitrite (1mM) in the drinking water, and CLA (100mg/kg/d) through the diet. However, administration of nitrate (10mM) in the drinking water only demonstrated a reduction in DAI-score while no prevention of colon shortening could be observed. Additional investigation of key inflammatory markers in the colonic tissue indicated a reduction in the levels of iNOS for all treatments studied, while the NF-κB subunit p65 was reduced with nitrate and CLA. This further establishes the anti-inflammatory effects seen by the improved colon length and DAI-score.

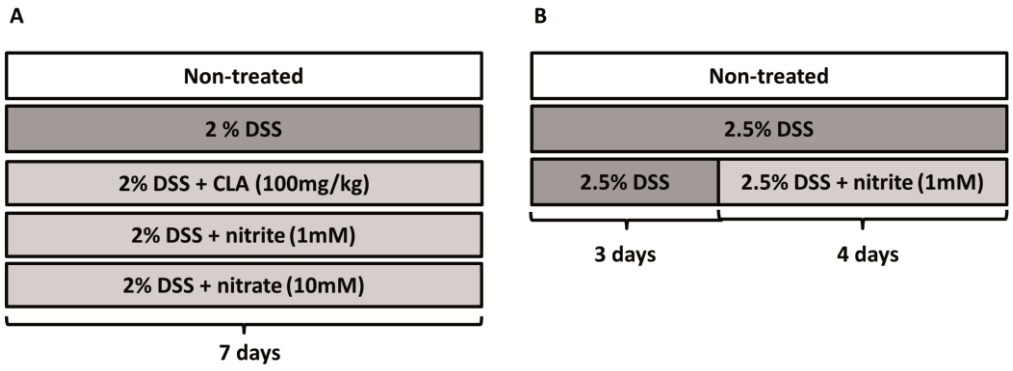

Figure 10. Experimental protocol for investigations of (A) the preventive or (B) therapeutic effects of CLA, nitrite and nitrate in DSS-induced colitis in mice.

4.2.2 Nitrite has therapeutic effects in established colitis

Very few studies with anti-inflammatory therapeutic agents have reported efficacy in reversing an already existing intestinal inflammation (139). Based on the strong anti-inflammatory effects of nitrite in the preventive model, we wanted to study if this anion could also be protective in an already established disease. DSS (2.5%) treatment was initiated for 3 days to induce the disease in mice, followed by 4 days with additional nitrite supplementation (Fig. 10 B). After 3 days treatment with DSS, the disease onset was determined by a significant aggravation of colitis clinical symptoms, such as diarrhoea and rectal bleeding. These markers have previously shown good correlation with inflammation in experimental colitis (158). Even with the delay in nitrite treatment, improved results of colon length and lower DAI-score was observed, and were similar to the effects seen in the preventive experiment of concurrent nitrite administration with DSS. The observed anti-inflammatory effects were further confirmed by histological examination of the colonic tissue, which revealed that nitrite could partly prevent histopathological injuries (colon crypt loss, immune cell influx, and ulceration of the epithelial surface) compared to the DSS-treated group. These results are in line with the findings in **Paper I**, indicating that nitrate and nitrite can prevent leukocyte infiltration in acute inflammation. Inflammatory responses with enhanced leukocyte and platelet aggregation, leading to defects in the microcirculation, have been postulated to play a central pathogenic role in development of IBD and in DSS-induced colitis (159, 160). Since this model of colitis in some ways resembles human UC, our findings of nitrite alleviating DSS-induced colitis may be of clinical importance to ease the inflammatory activity and subsequently reduce symptoms (125), although it is generally difficult to translate findings in experimental animal models to what is occurring in patients. These results suggest that nitrite has an ability to alleviate inflammation even in an initiated state of inflammation, thus not only functioning in a preventive manner.

4.2.3 Insights on the mechanisms behind the anti-inflammatory effects of nitrite and CLA

We continued our studies of nitrite by investigating the effect on colonic mucus thickness in DSS-induced colitis, since this layer represents an essential barrier in preventing translocation of bacteria and toxins from the colonic lumen to the mucosa (161). This protective barrier has been shown to be reduced in patients with IBD and in DSS-treated mice (94), indicating that it could be an important target for colitis treatment. Even with the delayed administration of nitrite, we observed a complete preservation of the colonic mucus thickness compared to the DSS group, reaching the same levels as the control mice. This preservation of the mucus barrier could be one of the nitrite-induced protective mechanisms by stimulated mucus secretion, and could be mediated by nitrite itself or nitrite-derived NO. Previous studies have shown that gastric mucus release is stimulated by NO-donors (162, 163), via a mechanism involving activation of sGC and subsequent cGMP formation (164). Investigation of the mechanism behind the nitrite mediated mucus release needs to be elucidated in further studies.

To investigate whether the anti-inflammatory effects of nitrite and CLA could be related to an increased mucosal healing, we used different approaches. Using colon epithelial cells, gene expression of PPAR-γ and TFF3, respectively involved in broad anti-

inflammatory effects and epithelial restitution, were investigated after treatment with different fatty acids (oleic acid, linoleic acid and CLA). An increased expression of TFF3 and PPAR-γ was observed after treatment with CLA (2.5 µM). Furthermore, pre-treatment with the PPAR-γ inhibitor GW9662 reduced the CLA-induced expression of both PPAR-γ and TFF3. This indicates that CLA induces PPAR-γ which in turn regulates TFF3. In an *in vitro* model of wound healing in colon epithelial cells, nitrite (100 µM) improved healing 24 h after wound incision of a confluent monolayer, indicating that nitrite has the ability to increase epithelial restitution. With the demonstrated effect of nitrite to sustain mucus thickness after DSS-injury, and since mucins and TFF3 are released in co-exocytosis (101), we also investigated whether the expression of TFF3 in colonic tissue was induced after nitrite and DSS-treatment. Similar to treatment of CLA, nitrite demonstrated a trend towards induced colonic expression of TFF3 (Fig. 11). This suggests that upregulation of this peptide might play a role in the anti-inflammatory effect seen with both CLA and nitrite.

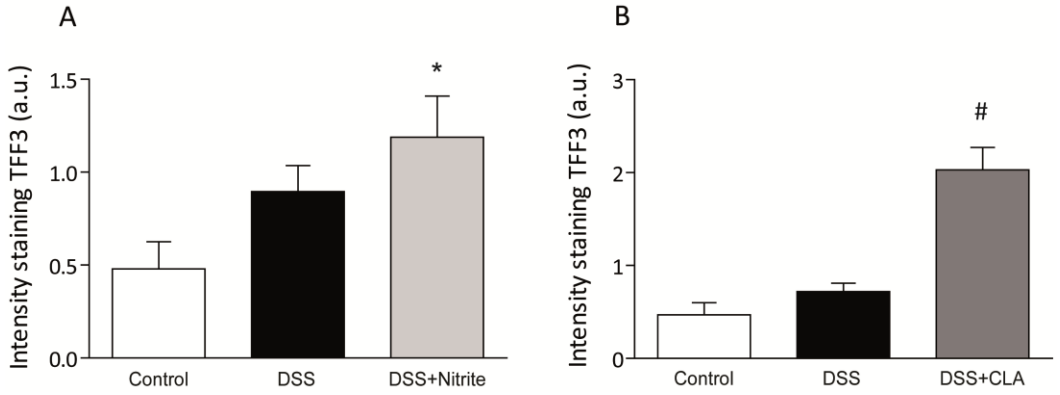

Figure 11. Quantification (arbitrary units) of colonic TFF3 expression by immunohistochemistry staining of mice, (A) treated with or without 2% DSS and nitrite (1 mM) or (n=12) (B) treated with or without 2% DSS and CLA (100mg/kg) (n=8) for 7 days. ($P < 0.05$,* vs. Control and # vs Control and DSS). Please note that B is taken from paper II, (Figure 3D).

Although both nitrite and CLA demonstrate promising results, a great limitation with using a chemically-induced model of colitis is that very few substances with anti-inflammatory effects show similar protection in a more chronic mouse model (139). To further investigate the potential protective effect of nitrate, nitrite and CLA in IBD, it is necessary to study these substances in more advanced chronic inflammatory models of colitis. Also, the effects on the adaptive immune system need to be explored in order to evaluate protective properties on more complex and chronic stages of inflammation. A recent study demonstrates that NO mediates a suppression of Th17 cells, which are associated with the pathogenesis of several autoimmune diseases including IBD (165). This indicates that NO and possibly nitrate and nitrite treatment might have effects also on other immune cells and in more complex inflammatory models. Furthermore, simultaneously with the publication of **Paper II**, the effect of oral CLA administration was investigated in a clinical trial of patients with mild or moderate Crohn's disease (166). The intake of 6g/day CLA decreased the disease activity and resulted in reduced lymphocyte production of pro-inflammatory cytokines after 12 weeks. Interestingly, this

dose is equivalent per kilogram body weight to the one we administered by the diet to the mice. Future studies are needed to investigate the importance of TFF3-induction and eventual mucosal healing by CLA in IBD. Figure 12 summarizes the suggested protective pathways mediated by CLA and nitrite in DSS-induced colitis.

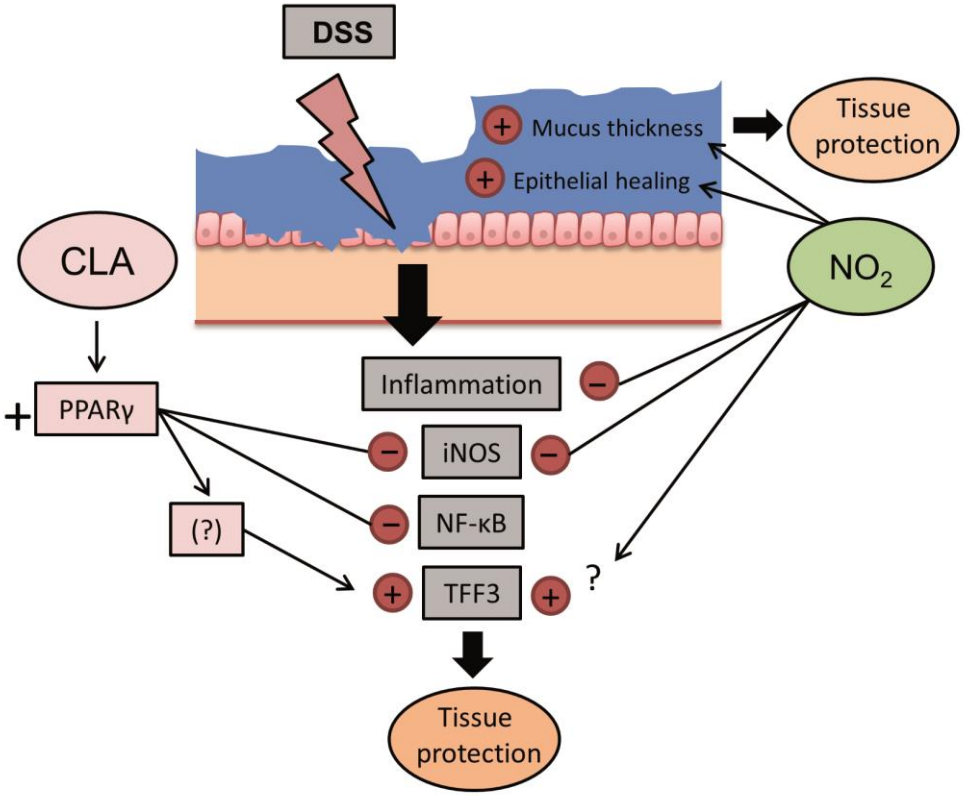

Figure 12. Proposed pathways of protection by CLA and nitrite in DSS-induced colitis.

4.3 Paper IV

Physiological recycling of endogenous nitrate by oral bacteria regulates gastric mucus thickness

The observation of nitrate accumulation in saliva from the circulation, and its reduction to nitrite by the oral bacteria to generate NO in the acidic gastric lumen, suggests an important role for maintaining homeostasis also in the upper GI tract. However, the physiological role of endogenously produced nitrate and nitrite coming from NOS is still not clear. It is tempting to speculate that the entero-salivary nitrate cycle described above serves as a salvage pathway to regenerate NO that would otherwise be lost through metabolism and excretion. In an attempt to study this, we used germ free mice and studied the gastric mucus generation in response to nitrate and nitrite.

4.3.1 Recycling of endogenous nitrate is important for regulation of the gastric mucus layer

Earlier studies have indicated that both the oral and gastrointestinal microflora have a central role in regulating generation of gastric NO (29, 167). The results from this study clearly show that even nitrite, at levels simulating those originating from NOSs alone, has profound effects on the gastric mucosa by increasing the firmly adherent mucus layer. While untreated germ free mice almost lacked adherent mucus layer, nitrite in doses ranging from high dietary to physiological levels increased mucus thickness to the same extent. In contrast, nitrate administration to germ free mice had no effect on regulation of mucus thickness, again demonstrating the pivotal role of bacteria in bioactivation of this anion. The importance of recycling endogenous nitrate has recently been investigated in a human study, where the subjects were treated with antibacterial mouthwash together with a nitrate low diet (36). The results showed that the plasma levels of nitrite decreased in correlation with an increase in blood pressure, indicating that endogenous nitrate recycling is essential for physiological control of blood pressure. Furthermore, a very recent study suggests that a cross-talk between the nitrate-nitrite-NO pathway and the NOS-dependent pathway exist in the vasculature (168). This would favour the control of vascular NO homeostasis and maintain levels of NO at physiological concentrations.

We now have indications that broad spectrum antibiotics also reduce the adherent gastric mucus layer although the effect was not as dramatic as in germ free mice. Treatment with antibiotics completely eradicated the colonic microflora, while the oral flora was still present and able to reduce nitrate to nitrite. This might be the explanation to the observed relatively thick gastric mucus layer compared to germ free mice. However, the consequence of a reduced gastric mucus layer against induced injury has not been fully elucidated.

Our present results show that regulation of the firmly adherent gastric mucus layer is influenced by signalling mechanisms through nitrite generated by the oral microflora. Interestingly, the effect of nitrite in the regulation of gastric mucus thickness does not seem to be mediated by the activation of the sGC-cGMP pathway, which is opposite to what has been observed in its regulation of gastric mucosal blood flow (47). The cGMP-independent mechanism observed in this study might instead favour the existence of alternative nitrite signalling pathways that can only be speculated on at this stage.

Alterations of protein function could be mediated by nitrite through the formation of S-nitrosothiols or by nitration through the nitrogen dioxide radical (58, 169). It would be interesting to measure the levels of these substances in the mucus from nitrite treated animals to see if this might be a relevant signalling pathway. However, the amount of mucus that can be obtained from a mouse is extremely small, and the S-nitrosothiols formed are very reactive and volatile. Another interesting signalling mechanism to explore is whether nitrite itself, like some other bacterial products, can mediate intracellular signalling through toll-like receptors (TLRs). TLRs are important not only in protection against pathogenic infections. In fact, it has been demonstrated recently that normal commensal bacteria are recognized by TLRs, which in turn serve to mildly activate the immune system thereby regulating epithelial homeostasis and protecting against gut injury (146). By the use of a TLR4 receptor agonist in germ free mice or mice deficient in MyD88 or TLR4, such possible involvement of nitrite in gastric mucus regulation could be addressed.

5 CONCLUSIONS

- Inorganic nitrate and nitrite reduce leukocyte recruitment during acute inflammation.
- Dietary nitrate has potent anti-inflammatory effects in NSAID-provoked intestinal injury in a process involving reduction of MPO and P-selectin levels.
- Nitrate effects proceed via intermediate formation of nitrite, a reaction involving oral nitrate-reducing bacteria.
- Despite being anti-inflammatory, dietary nitrate does not impair bacterial clearance.

- Dietary CLA attenuates DSS-induced colitis, partly mediated by an upregulation of TFF3 expression through activation of PPARγ in colon epithelial cells

- Inorganic nitrate and nitrite alleviate DSS-induced colitis.
- Dietary nitrite has therapeutic effects also in already established colonic inflammation.
- Dietary nitrite mediates the protective effects by prevention of DSS-induced thinning of the colonic mucus layer, and promotes healing of colon epithelial cells.

- Germ free mice are almost completely devoid a firmly adherent gastric mucus layer.
- Physiological levels of nitrite restore gastric mucus thickness through a cGMP-independent mechanism.
- The gastric mucus layer is reduced by treatment with broad spectrum antibiotics in mice.

6 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The fact that green leafy vegetables, which contain high amounts of nitrate, are now considered to be the top choice of vegetables for improved health is intriguing since nitrate ironically has been targeted as the only harmful natural substance in this food group. Emerging data from animal and human studies support the hypotheses that nitrate is an active substance in vegetables contributing to their positive health effects. In addition to this, the still unknown reason for the active accumulation of nitrate in saliva observed in the 1970s suggests that there is indeed something important with this anion for general physiological regulation and homeostasis.

In this thesis I have explored the new area of anti-inflammatory effects by nitrate, nitrite and CLA. The present studies demonstrate that specific components in our diet play a direct role in regulating acute inflammatory responses, both in the microcirculation and in the gastrointestinal tract. Therapeutic opportunities for dietary nitrite have been further indicated in a model of already established colonic inflammation, an effect partly mediated by nitrite-derived NO. We also show the potential importance of recycling endogenously produced nitrate in regulating gastric homeostasis, a process dependent on oral nitrate reducing bacteria. These results lead to additional interesting questions to be addressed, and further studies of nitrate, nitrite and CLA in inflammatory reactions are called for. Can these therapies also affect adaptive immune cells and chronic inflammation? Furthermore, several studies indicate that nitrate and nitrite have profound effect on gastric mucosal homeostasis, and are important for maintaining a proper gastric mucus layer. Exactly how salivary-derived nitrite regulates gastric mucus secretion is still unknown, as is to which extent the gastric mucus layer is essential for protection of gastric injury.

There are promising future therapeutic opportunities for dietary nitrate and nitrite in various conditions. The most appreciated areas for therapy have been correlated with cardiovascular disorders such as hypertension, endothelial dysfunction and ischemia-reperfusion injury. However, other pathophysiological conditions where nitrate and nitrite might have beneficial effects are also receiving increased attention, including infection, inflammation and metabolic diseases such as diabetes (53-55, 170-172). Importantly, although many initial studies show promising therapeutic applications with dietary nitrate (44, 173), there are also examples in clinical trials where this type of treatment does not seem to have an effect (174). There is a future need to delineate how nitrate and nitrite are affecting different patients and determine which factors are important for successful treatment. Also, the importance of vegetables high in nitrate needs to be further explored. Larger long-term clinical studies are clearly needed in order to further examine the potential for nitrate and nitrite in prevention and treatment of diseases. Even with the great potential of possible important preventive effects for entire populations, these costly studies are difficult to conduct in reality mainly due to the lack of interest from by the pharmaceutical industry. The intellectual property is hard to defend since these anions are chemically simple, cheap and readily available. However, the value of health improvements for the society as a whole might be extensive and should not be neglected.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

7.1 Bakgrund

Det är idag ställt utom allt tvivel att maten vi äter har en mycket stor påverkan på vår hälsa. En traditionell medelhavskost med stort intag av bl.a grönsaker och omättade fetter har visat sig kunna skydda mot uppkomst av en rad sjukdomar inklusive hjärtkärlsjukdomar och typ 2-diabetes. Det är dock fortfarande inte klarlagt vilka delar i kosten som ger detta skydd. Antioxidanter, fibrer, mineraler och vitaminer tros ha betydelse, men studier av enskilda näringsämnen har hittills inte gett tydliga resultat.

Vissa typer av grönsaker innehåller stora mängder nitrat (NO_3^-), såsom rödbetor, sallad och spenat. Merparten av det nitrat vi äter tas upp i tunntarmen och transporteras sedan vidare till blodet. Därifrån koncentreras nitrat till spottkörtlarna via en aktiv process vilket medför att vår saliv innehåller en mycket hög halt nitrat. Den nitratrika saliven omvandlas av bakterier i munhålan till nitrit (NO_2^-) som sväljs ner till magsäcken. Nitrit i magsäcken kommer i sin tur att reduceras till kväveoxid (NO) vid kontakt med den sura magsaften. En viss del av den nitrit vi sväljer kan också undkomma denna reduktion och istället tas upp till blodet och vävnader där nitrit kan reduceras till NO genom ett antal enzym. NO är en viktig biologisk signalmolekyl som styr många fysiologiska funktioner i kroppen, t.ex. kärlvidgning, signalering mellan nerver och immunförsvar mot infektioner. Att NO kan bildas från nitrat i kosten är en relativt ny upptäckt, medan man längre har vetat att NO kan produceras av kroppen själv genom specifika enzym, så kallade NO syntas (NOS). Detta NO kan sedan omvandlas till nitrit och nitrat i blod och vävnader och därmed blanda sig med nitrat och nitrit som härstammar från kosten. I våra kroppar finns alltså ett system där nitrat, nitrit och NO kan ombildas till varandra i ett spännande kretslopp. Att kunna öka kroppens tillgång på NO genom kosten kan vara intressant då många hjärtkärlsjukdomar delvis tros bero på en bristande NO bildning från kroppens egna NOS. En grönsaksrik kost har visat sig vara skyddande mot uppkomst hjärtkärlsjukdomar såsom hypertoni, och det har nyligen föreslagits att just nitrat i dessa grönsaker kan vara en viktig bidragande orsak. Forskningen runt kostrelaterat nitrat och nitrit har ökat enormt de senaste decennierna och främst fokuserat på sjukdomar rörande hjärtkärlsjukdomar och magsår. Studier har också visat att NO bildat från NOS har stor betydelse i immunologiska reaktioner som inflammation, men det är inte känt hur nitrat och nitrit kan påverka dessa processer. Rekrytering av vita blodkroppar (leukocyter) från blodet till angripen vävnad är en central händelse i det tidiga inflammatoriska förloppet, t.ex. vid en bakterieinfektion. Denna process sker i flera steg och styrs av olika mediatorer som frisätts från vävnaden till blodet. Detta medför att leukocyterna fastnar i blodkärlen och sedan vandrar ut från blodbanan till det inflammerade området för att bekämpa infektionen. Även om denna process är avgörande för ett välfungerande skydd mot farliga infektioner så kan det också leda till skadlig kronisk inflammation.

Förutom NO har även en rad andra ämnen en viktig betydelse vid inflammatoriska reaktioner. Ett exempel är fettsyror, som kan agera som signalämnen och förmedla pro- eller anti-inflammatoriska effekter. En särskild typ av fettsyra, konjugerad linolensyra (CLA), finns naturligt i mejeriprodukter och kött från idisslande djur och har nyligen visat sig kunna minska inflammation i experimentell tarmsjukdom. Även om anti-

inflammatoriska effekter har påvisats så vet man fortfarande lite om hur denna signalering sker.

Det övergripande syftet med studierna i denna avhandling var att undersöka hur nitrat, nitrit och CLA via kosten påverkar fundamentala funktioner och inflammatoriska processer i magtarmkanalen.

7.2 Resultat och diskussion

I det första delarbetet (**Paper I**) undersökte vi hur en nitratrik kost kan påverka rekryteringen av leukocyter till en inflammerad vävnad. För att studera detta förbehandlades möss med nitrat och nitrit följt av analyser av vita blodkroppar i blodkärl i en tunn muskel med hjälp av mikroskopi. Inflammation stimulerades genom tillsats av en inflammatorisk substans (MIP-2, macrophage inflammatory protein-2) som attraherar leukocyter. Resultaten visade att både nitrat och nitrit kunde minska aktiveringen av leukocyterna och därmed minska antalet celler som tog sig ut från blodet till vävnaden. Vidare resultat visade att detta kan bero på en nedreglering att specifika adhesionsmolekyler på blodkärlens yta (ICAM-1, intracellular adhesion molecule type 1) som annars leder att leukocyter fastnar och vandrar genom blodkärlet. Effekten av nitrat från kosten på leukocytrekrytering undersöktes vidare i en annan kliniskt relevant modell. Behandling med vissa anti-inflammatoriska läkemedel (NSAIDs, nonsteroidal anti-inflammatory drugs) medför ofta biverkningar i form av retningar och sår i magtarmkanalen. Genom att ge möss ett sådant läkemedel (diclofenac) efter förbehandling med nitrat, studerades graden av inflammation i den första delen av tunntarmen. Nitrat visade sig även här kunna minska infiltration av leukocyter till vävnad samt nedreglera andra typer av adhesionsmolekyler (P-selektin) på blodkärlens yta. Ytterligare försök där råttor gavs en antibakteriell munsköljvätska visade att denna skyddande effekt var helt beroende av bakterierna i munhålan som omvandlar nitrat till nitrit. Slutligen undersökte vi om effekten av en minskad aktivering av leukocyter efter ett inflammatoriskt stimuli vid intag av nitrat kunde medföra att immunförsvaret även blir sämre på att bekämpa en riktig bakterieinfektion. Detta studerades genom att luminicerande *Stafylococcus aureus*-bakterier injicerades under huden i kontroll- och nitratbehandlade möss, där läkningen av infektionen observerades under 14 dagar. Trots att nitrat visade sig ha en anti-inflammatorisk effekt vid mild stimulering av inflammation så gav det inte upphov till en försämrad förmåga att bekämpa en allvarlig bakterieinfektion. Slutsatsen av denna studie visar att nitrat via kosten kan ge anti-inflammatoriska effekter utan att medföra oönskade biverkningar i form av ökad risk för infektioner.

Syftet med det andra och tredje delarbetet (**Paper II, III**) var att undersöka de anti-inflammatoriska effekterna av dietärt nitrat, nitrit och CLA vid experimentell tarmsjukdom. Inflammatorisk tarmsjukdom hos människor innefattar sjukdomarna Crohns sjukdom och Ulcerös kolit. Det drabbar nästan 1% av norra Europas befolkning och klassas därmed som en folksjukdom. Detta är en livslång och idag obotlig sjukdom som ger upphov till tarminflammation och tros bero på en förlorad tolerans mot vår tarmflora. I dessa studier användes en vanlig modell av denna sjukdom där möss ges det sjukdomsinducerade medlet DSS (dextran sulfate sodium) som leder till inflammation i tjocktarmen. Resultaten visade att främst CLA och nitrit, men även nitrat till en viss grad,

kunde lindra kliniska symptom och inflammatoriska processer i denna modell av inflammatorisk tarmsjukdom. Även tjocktarmsceller odlade i kultur studerades för att lättare kunna undersöka aktivering av olika anti-inflammatoriska signalvägar. Genom att studera effekterna av CLA i både celler och tjocktarmsvävnad från möss, kunde vi se att CLA troligtvis har skyddande effekt genom en aktivering av de anti-inflammatoriska signalvägarna PPAR-γ (peroxisome proliferator-activated receptor gamma) och TFF3 (trefoil factor 3). Dessa signalvägar är essentiella i läkningsprocessen av cellskador i magtarmkanalen. För att vidare studera huruvida den anti-inflammatoriska effekten av nitrit vid tarmsjukdom även kunde skydda vid en redan etablerad inflammation, gavs behandling med nitrit till möss tre dagar efter start av DSS. Även denna fördröjning av nitritbehandling visade liknande anti-inflammatoriska effekter som när nitrit gavs samtidigt med DSS. Den skyddande effekten av nitrit visade sig kunna bero på förmågan att öka tjockleken av det skyddande slemlagret i tjocktarmen som fungerar som en barriär mellan bakterier och cellvägg i tarmen. Vidare studier på tjocktarmsceller visade att nitrit också kunde förbättra läkningen av inducerade sår, vilket tros vara en viktig faktor för att även mildra symptomen av inflammatorisk tarmsjukdom hos människa. Dessa två studier visar att dietärt CLA, nitrit och till viss del nitrat, kan lindra inflammatorisk tarmsjukdom hos möss. Nitrit visade sig även ha terapeutiska effekter i denna modell. Dessa resultat kan ha viktig betydelse för framtida kostrekommendationer i behandlingen av inflammatorisk tarmsjukdom hos människa.

Under de senaste decennierna har många studier visat effekter av ett extra tillskott av nitrat och nitrit via kosten på olika aspekter av hälsa och sjukdom. Mycket mindre studerat är hur kroppens egenproducerade nivåer av nitrat och nitrit från NOS påverkar regleringen av olika fysiologiska funktioner. I delarbete fyra (**Paper IV**) undersöktes rollen av kroppens egna nivåer av nitrat och nitrit på magslemhinnan. Dessa effekter studerades i bakteriefria möss som inte kan omvandla nitrat till nitrit via bakterier i munhålan, och därför inte heller kan bilda NO i magsäcken. Detta gör det möjligt att istället ge dessa möss olika doser av nitrit för att efterlikna det nitrit som annars normalt sväljs ner under hela dygnet. Resultaten visade att bakteriefria möss hade ett extremt tunt slemlager i magsäcken, medan även mycket låga nivåer av nitrit hade betydande effekter genom att kraftigt öka detta slemlager. Eftersom bakteriefria möss uppvisade ett mycket tunt slemlager, undersökte vi huruvida detta även kunde påvisas i en annan kliniskt relevant modell genom behandling av möss med bredspektrumantibiotika. Intressant nog visade sig dessa djur också ha ett tunnare slemlager i magsäcken jämfört med kontrollmöss, även om effekten inte var lika stor som hos de bakteriefria mössen. Behandling med antibiotika slog helt ut bakteriefloran i tarmen medan bakterierna i munhåla fortfarande existerade med förmåga att reducera nitrat till nitrit. Detta kan förklara varför antibiotikabehandlade möss fortfarande har ett relativt tjockt slemlager. Betydelsen av en minskad slemtjocklek i magsäcken för en ökad känslighet för magsår är ännu inte fastställd. Fortsatta studier bör genomföras för att utröna betydelsen av detta slemlager vid en ökad påfrestning av magslemhinnan. Dessa resultat visar att även fysiologiska nivåer av nitrit som kan bildas av orala bakterier är viktiga i regeleringen av slemtjockleken i magsäcken. Detta tyder på att återvinning av nitrat som bildas genom NO av NOS är viktigt att upprätthålla en väl fungerande magslemhinna.

7.3 Sammanfattning

Resultaten i denna avhandling pekar på att specifika komponenter i vår kost spelar en direkt roll i regleringen av inflammatoriska reaktioner, både på lokal nivå i små blodkärl och på organnivå i magtarmkanalen. Terapeutiska möjligheter har även påvisats av dietärt nitrit i en etablerad modell av tarminflammation, en effekt som delvis förmedlades av NO. Kroppseget nitrat, som från början är bildat via NOS, har också en viktig roll i regleringen av en väl fungerande magslemhinna, en process som är beroende av bakterier i munhålan som kan omvandla nitrat till nitrit.

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