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THE ROLE OF NITRIC OXIDE IN THE GASTROINTESTINAL TRACT

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To my family

A subtle thought that is in error may yet give rise to fruitful inquiry that can establish truths of great value.
Isaac Asimov

ABSTRACT

Nitric oxide (NO) is an important second messenger involved in the regulation of a multitude of mechanisms in the body, such as neurotransmission, smooth muscle contractility, host defense and immune regulation. Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic disorders affecting the gastrointestinal (GI) tract with unknown etiology. These diseases are characterized by increased NO levels in the gut lumen, aberrant leukocyte recruitment to the inflamed tissue and changed motility pattern of the intestines.

This thesis aimed to investigate NO's involvement in inflammatory reactions as well as its regulatory role on motility in the GI tract by studying NO-related gene expression in IBD, α_2 integrin antibody treatment in comparison to conventional IBD drugs in experimental colitis, neuropeptide S (NPS) effects on motility, contractility and inflammation, as well as NO's regulation of the migrating motor complex (MMC) in relation to muscarinic and 5-HT_3 receptor blockade.

Cluster analysis of NO-related gene expression in CD and UC revealed common pathophysiological processes, with hypoxia-inducible factor 1 (HIF-1) as a central regulator of inflammation, angiogenesis and tissue fibrosis. Moreover, interaction analysis pinpointed the association of upregulated expression of IL-8 and ICAM-1 in both diseases, highlighting an exaggerated leukocyte infiltration in the pathophysiology of CD and UC.

In comparison to conventional IBD drugs, treatment with a function-blocking anti-α_2 antibody by rectal administration showed alleviation of signs of colitis, such as reduced body weight loss, rectal bleeding, inflammation score and inflammatory biomarker expression including inducible NO synthase (iNOS). Although treatment with methotrexate also showed several signs of ameliorated colitis, these effects were not accompanied by a broad reduction in inflammatory marker expression. This study provides evidence for therapeutic use of integrin $\alpha_2\beta_1$ as a novel drug target for treatment of IBD.

Infusion with NPS prolonged the MMC cycle length and the phase III duration in upper small intestine. Contractility studies on excised human muscle strips revealed a dampening of the amplitude, with NPS acting directly on small intestine circular muscle, while this effect seems mediated by prejunctional receptors in colon. These effects of NPS on motility and contractility are in agreement with the changes seen during inflammatory reactions in the intestine. Moreover, NPS induced the expression of inflammatory markers iNOS, IL-1β and CXCL1, further supporting a role of NPS in NO-dependent induction of inflammation in the GI tract.

Studies with the NOS inhibitor L-NMMA suggested marked effects of NO on motility. L-NMMA, shown to inhibit NO, initiated phase III MMC activity, while additional muscarinic and 5-HT_3 receptor blockades revealed that the transition from phase I to phase II activity seem regulated as a balance between inhibitory nitrergic and excitatory cholinergic and serotonergic pathways.

These results demonstrate increased iNOS expression during inflammatory reactions in the GI tract, with the resulting increase of NO as a pathophysiological inhibitor of motility seen in inflammatory disorders of the GI tract.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-IV):

I. **Gillberg L**, Varsanyi M, Sjöström M, Lördal M, Lindholm J and Hellström PM.
Nitric oxide pathway-related gene alterations in inflammatory bowel disease.
Scand J Gastroenterol. 2012;47(11):1283-97.

II. **Gillberg L**, Berg S, de Verdier PJ, Lindbom L, Werr J and Hellström PM.
Effective treatment of mouse experimental colitis by alpha 2 integrin antibody: comparison with alpha 4 antibody and conventional therapy.
Accepted for publication in Acta Physiol. 2012 Sep,
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III. Rudholm Feldreich T, **Gillberg L**, Halim MA, Webb D-L, Sundbom M, Karlbom U, Broad J, Sanger GJ, Näslund E and Hellström PM.
Neuropeptide S: effects on motility, contractility and inflammation in the rat and human gastrointestinal tract.
Manuscript

IV. **Gillberg L**, Webb D-L and Hellström PM.
Nitric oxide control of the migrating motor complex in man: L-NMMA effects in relation to muscarinic and 5-HT_3 receptor blockade.
Manuscript

Related article:
Webb D-L, Rudholm-Feldreich T, **Gillberg L**, Halim MA, Theodorsson E, Sanger GJ, Campbell CA, Boyce M, Näslund E and Hellström PM.
The type 2 CCK/gastrin receptor antagonist YF476 acutely prevents NSAID induced gastric ulceration while increasing iNOS expression.
Accepted for publication in Naunyn Schmiedebergs Arch Pharmacol 2012 Nov.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

5-ASA	5-aminosalicylic acid
5-HT	5-hydroxytryptamine / serotonin
$[Ca^{2+}]_i$	Intracellular free calcium concentration
ANOVA	Analysis of variance
CaM	Calmodulin
CD	Crohn's disease
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
DAI	Disease activity index
DSS	Dextran sulfate sodium
ECM	Extracellular matrix
EFS	Electrical field stimulation
eNOS	Endothelial nitric oxide synthase
ENS	Enteric nervous system
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCS	Glucocorticosteroids
GI	Gastrointestinal
HIF-1	Hypoxia-inducible factor 1
IBD	Inflammatory bowel disease
ICC	Interstitial cells of Cajal
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
i.v.	Intravenous
L-NMMA	N^G-monomethyl-L-arginine
MMC	Migrating motor complex
NFκB	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
NPS	Neuropeptide S
NPSR1	Neuropeptide S receptor 1
O_2	Oxygen
O_2^-	Superoxide anion
$ONOO^-$	Peroxynitrite
qPCR	Quantitative polymerase chain reaction
s.c.	Subcutaneous
sGC	Soluble guanylyl cyclase
TNF	Tumor necrosis factor
UC	Ulcerative colitis

1 INTRODUCTION

1.1 NITRIC OXIDE

Before 1980, nitric oxide (NO) was considered to be merely an environmental pollutant produced by combustion of fossil fuels and involved in ozone depletion. This changed when Furchgott and Zawadzki [1] made the ground-breaking discovery that endothelial cells produce a factor (initially called endothelium-derived relaxing factor (EDRF)) required for vasodilation and relaxation of smooth muscle. This became the first description of NO as a biological second messenger. In 1987, two independent groups showed that EDRF is actually NO [2, 3]. At the same time, others showed that the bactericidal effect of macrophages is dependent on their capacity to produce nitrite and nitrate and that the cytotoxic effector is NO [4-6]. The discovery that NO act as a signaling molecule in the cardiovascular system was awarded the 1998 Noble Prize in Physiology or Medicine. NO is now an accepted biological mediator involved in several physiological and pathophysiological mechanisms, including neurotransmission, regulation of smooth muscle contractility and host defense [7]. Today, NO can also be used as a marker to objectively detect inflammation in several organ systems, i.e. asthmatic disease in the airways [8], cystitis in the urinary bladder [9] and colitis in the intestine [10].

1.1.1 The chemistry of NO

NO is a small free radical with a molecular weight of 30 Dalton, in line with other second messengers [11]. The mechanism of action of NO is based on its unpaired electron, making it highly reactive [12]. This molecule is a colorless gas that quickly reacts with oxygen (O_2) in air to produce nitrogen dioxide, a tissue damaging gas. However, this reaction is concentration-dependent, making NO stable at low concentrations seen in the physiological setting. In the absence of oxygen, NO easily dissolves in water, where it becomes stable [13]. Due to its hydrophobic and uncharged nature, NO is easily diffusible in both membranes and cytoplasm [14, 15]. Even though it only has a half-life of a few seconds [16], its free permeability through cell membranes without the need of transporters or receptors makes it possible for NO to travel in and out of cells several times throughout its lifespan [17]. Under physiological conditions, the biological effects of NO are mediated by its reactions with transition metals, such as iron, zinc and copper present in the prosthetic group of metal-containing proteins, cysteine residues in proteins forming S-nitrosothiols as well as with other free radicals such as superoxide anion (O_2^-) and molecular O_2 [18-20]. For example, many of NO's physiological functions, such as control of vascular tone and neurotransmission, are caused by its activation of soluble guanylyl cyclase (sGC), an enzyme that produces the second messenger cyclic guanosine monophosphate (cGMP), by binding to the ferrous iron contained in its heme group (Fig 1) [21, 22]. Furthermore, by S-nitrosylation, NO is known to alter protein function of transcription factors and kinases regulating signaling cascades inside the cell, such as the nuclear factor κB (NFκB) transcription factor and the c-Jun N-terminal kinase (JNK) in the mitogen-activated protein kinase (MAPK) cascade [23, 24]. O_2^- is formed as a byproduct in the mitochondrial electron transport chain [25, 26], and its reaction with

NO results in the highly reactive molecule peroxynitrite ($ONOO^-$) that can either nitrate, nitrosate or oxidize lipids, proteins and nucleic acids, forming the basis of the cytotoxic effect of NO in tissues as well as the phagocytic effect in macrophages [17, 27]. However, several factors will affect the reaction between NO and its target, such as rate of formation, distance of diffusion and reaction with other molecules in excess (i.e. depending on the partial pressure of O_2 and amount of O_2^-) [28]. In aqueous solutions, NO is auto-oxidized by O_2 into nitrite (NO_2^-) [29], whilst inside the blood vessel NO can react with oxyhemoglobin to form nitrate (NO_3^-) [30]. Together with NO, NO_2^- and NO_3^- are part of the circulatory store of NO [31, 32], although part of the NO_3^- pool is also known to be excreted in the urine [33].

1.1.2 Formation

NO can be produced in several ways in the human body (Fig 1):

Enzymatic synthesis

The NO that is not generated by non-enzymatic inter-conversion with NO_2^- and NO_3^- is produced by a group of enzymes known as NO synthases (NOSes), that in the presence of O_2 converts the amino acid L-arginine to L-citrulline and NO by performing an electron oxidation of a guanidino nitrogen in L-arginine [5, 34]. For this reaction to happen, the enzyme uses nicotinamide adenine dinucleotide phosphate (NADPH) as a co-substrate [5], together with flavin adenine dinucleotide (FAD) [35], flavin mononucleotide (FMN) [36], heme [37], tetrahydrobiopterin (BH_4) [38, 39], and calmodulin (CaM) [40] as co-factors. Three isoforms of NOS exist: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). These isoenzymes share 50-60% sequence similarity and need to form homodimers to become active [41, 42].

nNOS and eNOS are named after where they first were found located and acting [40, 43-46]. However, both these enzymes have been found to be widely distributed in many different cell types, leading to an additional nomenclature in which they also are called NOS I and NOS III, respectively, based on the historical order they were purified [47]. These two isoforms are constitutively expressed and their activity dependent on the binding of the cofactor CaM, which in turn is regulated by the physiological changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) [40, 48]. Such changes can be caused by agonist binding, for example acetylcholine binding to its muscarinic receptor expressed on the endothelial cell or action potential-stimulated release and binding of glutamate to its receptor on nerve cells [49]. Furthermore, the eNOS activity can also be affected by physical changes such as shear stress [50]. When activated, they produce low amounts (pico- to nanomolar) of NO due to their short activation periods (minutes) [16]. Both of these isoforms have been implicated in pathophysiological states; overproduction of NO from nNOS is associated with neurodegenerative disorders, such as Parkinson's, Alzheimer's and Huntignton's disease [51], while inhibition of eNOS causes white blood cell and platelate activation, hypertension and increases atherogenesis [52].

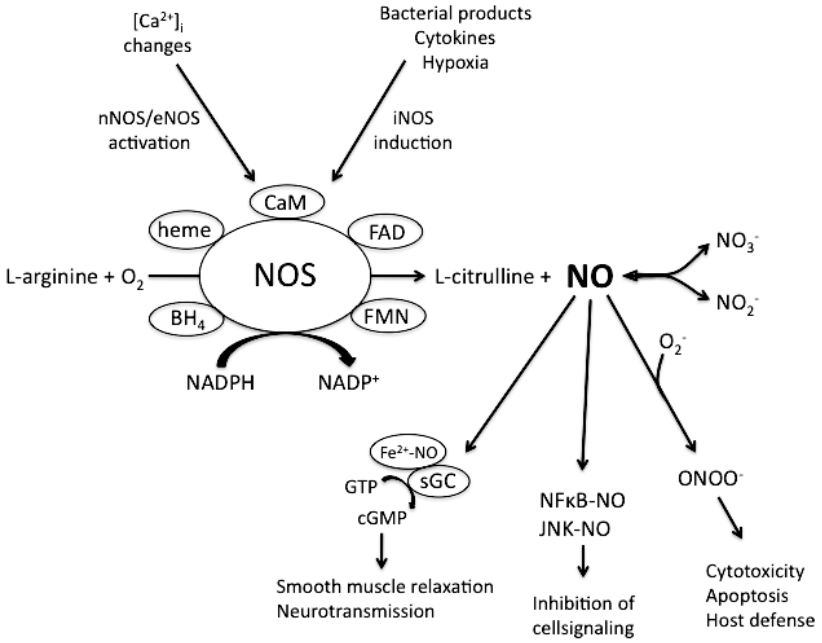

Fig 1. *NO production and signaling.*

iNOS (NOS II) was first described in macrophages [36, 53] and can be expressed in most cell types [22]. This isoenzyme binds CaM at physiological $[Ca^{2+}]_i$ [54], making it independent of Ca^{2+}-flux. Furthermore, iNOS is usually not expressed constitutively. Instead, it is transcriptionally induced during inflammation and immune activation by different cytokines, microbes and/or bacterial products [55, 56]. For example, the human NOS II gene promoter has binding sites for interferon (IFN)-γ, interleukin (IL)-6, and the cytokine induced transcription factor NFκB [57]. This means that there is a time lag of several hours between the stimulation and the produced, active enzyme [58]. However, when present it gives rise to high (micromolar) sustained concentrations of NO by remaining active for as long as 5 days [59]. Overexpression of iNOS, leading to overproduction of NO, is known to parallel with many chronic inflammatory settings, such as arthritis, diabetes, asthma and inflammatory bowel disease (IBD) [51, 60].

Several of NO's physiological functions were discovered by utilizing the endogenous arginine analogue N^G-monomethyl-L-arginine (L-NMMA), a competitive, non-selective NOS inhibitor [48, 61]. Further effects of NO, such as its role in leukocyte adhesion [62], were established with the synthetic NOS inhibitors L-N^{ω}-nitroarginine (L-NNA) and its methyl ester (L-NAME). Since upregulated nNOS and iNOS are known to be involved in many disease states, selective isoform inhibitors have been developed. Indeed, the selective iNOS inhibitors 1400W, L-NIL and GW274150 have all been shown to give promising results in different animal models of inflammation. However, for the claimed nNOS inhibitors L-NNA, 7-NI and L-NIO, the selectivity issue is still unresolved [63].

NOS-independent generation

Apart from being part of the circulating NO pool, NO_2^- and NO_3^- are absorbed from vegetables in the diet [64]. Both of these molecules can be reduced to NO in the NO_3^--NO_2^--NO pathway: NO_3^- goes through an enterosalivary circulation, in which it is excreted in saliva and reduced to NO_2^- by commensal bacteria in the oral cavity [65]. Swallowed NO_2^- is then protonated in the acidic environment of the stomach into nitrous acid, which in turn decomposes into NO and other nitrogen oxides [32, 66]. This gastric NO plays an important role in the defense system against swallowed microorganisms [67]. Furthermore, NO is also produced from NO_2^- under hypoxic conditions in the blood stream and tissues [31], forming a auxiliary pathway for NO generation when NOS activity is compromised [68].

1.1.3 NO in the gastrointestinal tract

The main function of the gastrointestinal (GI) tract is to transport the food we eat so that its nutrients can be absorbed in the intestine into the circulation and then eliminate waste products. In order to achieve this, numerous physiological processes must be temporally and functionally coordinated within the GI tract. These include peristalsis, secretion, absorption and immunological defense against pathogens, which are all modulated by NO (Fig 2).

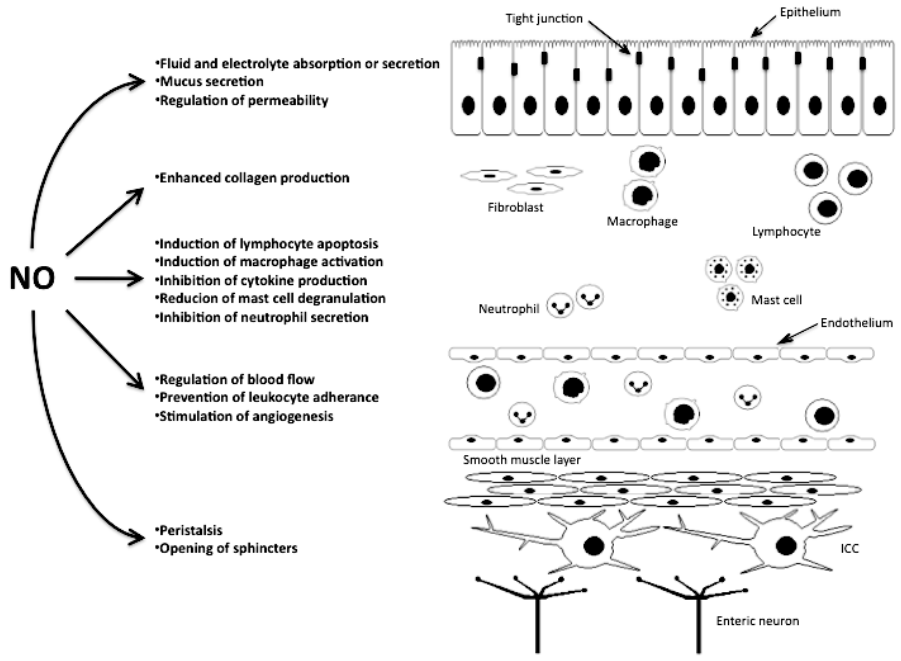

Fig 2. *NO effects in the GI tract.*

Intestinal motility

Intestinal motility and peristalsis are coordinated through the enteric nervous system (ENS). About 50% of ENS neurons express nNOS, and these are mainly located in the myenteric plexus and within muscle fibers [69]. In this system, NO acts as the principal inhibitory neurotransmitter that mediates non-adrenergic, non-cholinergic relaxation of smooth muscle cells and interstitial cells of Cajal (ICC) [70, 71]. Through these innervations, NO is involved in the accommodative relaxation and opening of sphincters. Indeed, altered nNOS activity has been implied in the GI disorders achalasia [72], gastroparesis [73] and hypertrophic pyloric stenosis [74].

Intestinal absorption and secretion

The L-arginine concentration is crucial for intestinal water and electrolyte transport, where low levels stimulate absorption and higher levels reverse the transport into secretion into the lumen [75]. This action of NO on electrolyte and water transport is thought to occur through a direct effect on epithelium and blood flow, or an indirect effect on neuronal reflexes [69]. The secretagogue effect of NO is dependent on the stimulation of prostaglandin E2 and the combined effect of these two to stimulate opening of chloride channels [76, 77]. On the other hand, the absorptive effect of NO on intestinal fluid is thought to occur due to suppression of prostaglandin synthesis and opening of basolateral potassium channels on enterocytes [78, 79]. Thus, depending on the concentration and local circumstances, NO might either act to promote absorption or secretion of intestinal fluid.

Mucosal defense

Several mechanisms exist in the GI tract to protect the mucosa from harmful ingested products, such as mucus and lysozyme secretion, as well as rapid turnover of the epithelial cell layer, which forms a barrier by being connected through tight-junctions. NO aids in protection of the mucosa by modulating several of these factors. Indeed, mucus secretion in the stomach is regulated by NO's activation of sGC in epithelial cells [80]. Also, NO donors promote derangement of the cytoskeleton and increases permeability of epithelial cells [81, 82]. However, this permeability effect is known to be caused by $ONOO^-$ [83]. Furthermore, NO aids in dilution and removal of toxins by its regulation of mucosal blood flow [84], affects the adherence of leukocytes to endothelium [62], stimulates angiogenesis and enhances collagen production by fibroblasts [85, 86], factors that are all important in mucosal healing.

Another mechanism vital to mucosal defense is that provided by tolerant immune cells in the lamina propria, which are also affected by NO. Residing mast cells are involved in the coordination of the inflammatory response and produce NO in response to inflammatory stimulators [87], which in turn downregulates further release of other pro-inflammatory mediators [88]. NO also inhibits the production of cytokines in macrophages and affects the action of macrophage-released cytokines on target cells [89, 90]. Moreover, macrophages are vital in the process of killing and removing pathogens that penetrate the epithelium, a mechanism that is dependent on their generation of NO [4]. Furthermore, NO is also known to downregulate neutrophil secretion and aggregation to endothelium [91].

1.1.4 NO and inflammation

NO's actions in inflammation are versatile, and include infection control, regulation of signaling cascades and transcription factors, regulation of vascular responses and leukocyte rolling, migration, cytokine production, proliferation and apoptosis [7, 92-94]. Due to the vastness of regulatory functions, NO has been claimed to be both pro- and anti-inflammatory [95, 96], as well as immunosuppressive (through its inhibitory or apoptotic effects in immune cells) [97]. Its generation during inflammation stems from both immune cells (e.g., macrophages, neutrophils, lymphocytes, mast cells, dendritic cells, eosinophils and natural killer cells) and other cells involved (e.g., epithelial, endothelial, smooth muscle and fibroblasts) [98]. Even though all isoforms of NOS are involved in the immune response, the bulk of activity has been assigned to iNOS, since its expression is increased in inflamed tissue and correlates with disease activity. Furthermore, the effect of NO can differ as inflammation progresses. That is to say, the response to NO will depend on the type of cell affected and the concentration of NO, as well as the redox state of the microenvironment [94].

1.2 INFLAMMATORY BOWEL DISEASE

Crohn's disease (CD) and ulcerative colitis (UC) are two chronic inflammatory disorders that cause tissue damage and loss of normal function in the GI tract. For example, both of these disorders are associated with disturbances in colonic contractility and motility [99, 100]. Over time, these conditions are characterized by cycles between quiescence and spontaneously relapsing disease. UC, first described in 1859 [101], exhibits a superficial inflammation restricted to the colonic mucosa, starting at the anorectal verge and extending proximally with severness of disease. Contrary to this, CD was first reported in 1932 as regional ileitis [102] and causes a transmural and multifocal inflammation that can affect the entire GI tract. However, disease is most commonly seen in terminal ileum, caecum and colon [103]. Due to the similarity in clinical symptoms, such as abdominal pain, weight loss, and diarrhea accompanied with blood, mucus and/or pus, these two disorders are commonly named IBD. In severe cases, signs of systemic inflammation may occur, such as fever, malaise and anorexia. Moreover, 30-40% of IBD patients also develop extraintestinal manifestations, most commonly seen in joints, skin, eyes or mouth [104]. Diagnosis is usually based on the combined picture of clinical symptoms and endoscopic, radiological, histological and laboratory findings (Table I). However, if inflammation only occurs in the rectum and colon (approximately 10% of IBD patients), discrimination between CD and UC becomes difficult, leading to the diagnosis of indeterminate colitis [105]. Furthermore, patients who suffer from longstanding extensive disease are at increased risk of developing colorectal cancer [106].

IBD is classified as a disease of modern society due to the increased frequency in developed countries since the 1960s, and "The hygiene hypothesis" has been postulated as a cause [107]. Indeed, the prevalence and incidence are highest in Northern Europe and North America [108], with a prevalence in Sweden around 0.5-1% of the population. During the development of IBD, UC precedes CD by 10-15 years, causing higher UC incidence. However, in Scandinavia the UC incidence is decreasing at the same time as CD is increasing [109]. The highest age-specific incidences are 15-25 and 25-35 years of age for CD and UC, respectively [110]. There is also evidence of

gender-specific prevalence between the disorders with more females having CD, while more males have UC [109].

Table I. *Montreal classification for diagnosing IBD*

Crohn's Disease			***Ulcerative Colitis***		
Classifiers	Categories	Definition	Classifiers	Categories	Definition
Age at diagnosis (A)	A1	<16 years	Extent (E)	E1	proctitis
	A2	17 – 40 years		E2	left sided (distal to splenic flexure)
	A3	>40 years		E3	pancolitis
Location (L)	L1	ileal	Severity (S)	S0	clinical remission
	L2	colonic		S1	mild (<4 stools/day, normal ESR)
	L3	ileocolonic		S2	moderate (>4 stools/day, minimal systemic signs)
	L4*	isolated upper GI		S3	severe (>6 bloody stools/day, systemic toxicity‡)
Behavior (B)	B1†	non-stricturing, non-penetrating			
	B2	stricturing			
	B3	penetrating			
	p*	perianal disease			

ESR = erythrocyte sedimentation rate. *Modifiers that can be added to the other categories in the same class. †Considered temporary during the first 5-10 years. ‡Systemic toxicity is defined as fever, tachycardia, anemia or elevated ESR.

1.2.1 Pathogenesis of IBD

Genetic factors

Although the exact etiology of IBD is unknown, genetic studies show involvement of the interactions between the host immune regulation and the microbiome in the pathogenesis. So far, 71 and 47 susceptibility loci have been identified in CD and UC, respectively [111, 112]. Of these, 28 are common to both disorders, suggesting a common mechanism in the pathophysiology [113]. Examples in CD are NOD2 [114, 115], ATG16L1 [116] and IRGM [117], which are involved in microbial recognition and autophagy. Examples in UC are IFN-γ, IL8RA and DAP that are involved in cytokine signaling and apoptosis [112]. Furthermore, IL23R, involved in Th17-signaling, IL-10, a cytokine involved in downregulation of inflammation, and TNFSF15, involved in tumor necrosis factor (TNF) signaling, have also been linked to both disorders [112, 118, 119]. These genetic factors can be interpreted to mean that the interplay between immune defense and gut microbiome (i.e., epithelial barrier function, bacterial recognition, autophagy, endoplasmic reticulum stress, and T cell differentiation) are connected to IBD pathophysiology. Furthermore, several of these genetic factors are known to interact with one another [120], potentially giving rise to a complex pangenetic change to the mucosal homeostasis evolving into an inflammatory response. Beyond these genetic changes, IBD patients also have a reduced diversity in their microbiome [121]. Although environmental factors such as geography, diet and lifestyle are known to influence the composition of the microbiome, recent studies implicate that several susceptibility loci can affect this composition [122]. Thus, in a genetically susceptible person, the dysregulation of the immune response to microbial

products are triggered by environmental factors [103]. At the time of printing this thesis, a new article showing a total of 163 susceptibility loci in IBD was published [123].

Leukocyte recruitment

One of the hallmarks of IBD is sustained leukocyte recruitment into the affected tissue, causing chronic inflammation and tissue damage [124, 125]. The inflammatory response is initiated by generation of specific cytokines and chemokines at the infected or injured tissue that activates and guides migration of leukocytes from the vasculature into the inflamed site [126]. This migration process is dependent on several classes of adhesion molecules (i.e. selectins, integrins and immunoglobulin-superfamily molecules) expressed on the immune cells and vascular endothelium [127] (Fig 3).

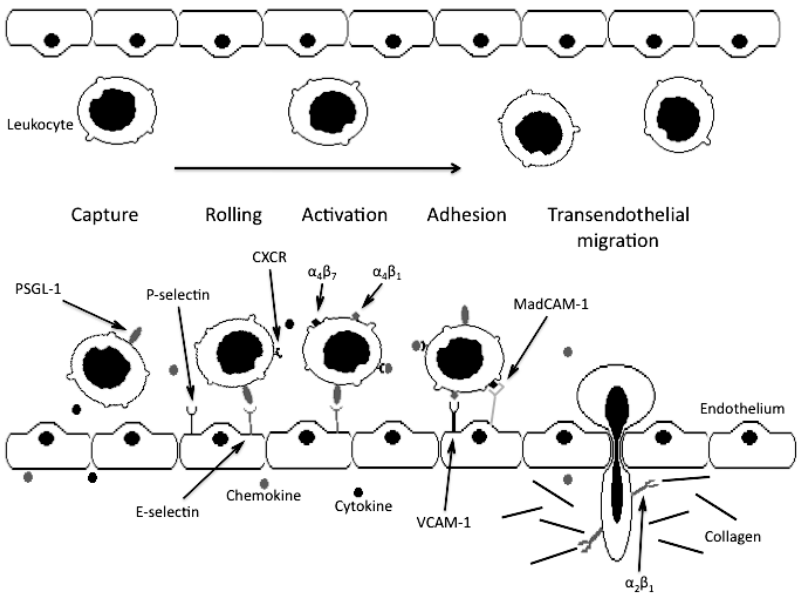

Fig 3. *Schematic drawing of leukocyte recruitment from the blood stream to the inflamed tissue. This process is composed of several phases: capture and rolling of leukocytes, regulated by selectins (P- and E-selectin) and their receptors (PSGL-1), activation by cytokines and chemokines, firm adhesion, mediated by integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$) and their immunoglobulin receptors (VCAM-1 and MadCAM-1, respectively), and finally transendothelial migration, regulated by integrins ($\alpha_2\beta_1$), their receptors (collagen), and chemokines.*

Integrins are transmembrane receptors that aid in the cell-cell and cell-extracellular matrix (ECM) interactions. Although expressed constitutively, ligand binding is required for activation [128]. These receptors are composed of two protein chains, one α-subunit determining ligand specificity and one β-subunit connected to the cell cytoskeleton and involved in intracellular signaling [129]. There are at least 24 different integrin receptors, made possible by pairing of different α- and β-subunits, yielding different specificities in adherence.

The $\alpha_2\beta_1$ integrin binds the ECM proteins collagen and laminin and is expressed on almost all cell types, although at very low levels on leukocytes [130]. However, when

activated by inflammatory signals, this integrin is upregulated on leukocytes (especially on neutrophils) to aid in the extravascular trafficking to the inflamed part of the tissue [131].

1.2.2 Treatments of IBD

Since there is no clear etiology for IBD, therapy aims at symptomatic amelioration by induction and maintenance of remission. Medical treatments in use modify the immune response in general and are thereby impeded by their limited specificity, with possible severe side effects (such as serious infections), and limited long-term benefits. Furthermore, treatment schemes depend on the location of inflammation, its activity and severity, as well as on complications of the disease.

Treatment of Crohn's disease

Patients with mild to moderate active CD are usually treated with glucocorticosteroids (GCS) to induce remission, and targeted formulations (such as budesonide) are especially used in patients with inflammation in the ileum or ascending colon [132]. However, GCS are not used for maintenance treatment due to their side effects (such as Cushing's syndrome, osteoporosis and diabetes) associated with long-term usage. Therefore, sulfasalazine or its better tolerated metabolite, 5-amonisalicylic acid (5-ASA), are indicated even though their efficacy is questionable [133]. Antibiotics (metronidazole and ciprofloxacin) are used for treatment of infectious complications, chronic fistulae, abscesses and perianal fissures when indicated. In patients with moderate disease activity, the immunomodulatory thiopurines (e.g., azathioprine or its active metabolite 6-mercaptopurine) are used for maintenance treatment. In case of relapse, these immunomodulators are usually used together with GCS. In those patients who develop intolerance to thiopurines, methotrexate can be used to induce remission and withdrawal of GCS, as well as for maintenance therapy. In patients with active moderate to severe CD that do not respond to GCS or immunomodulators, anti-TNF agents (infliximab or adalimumab) are indicated. Infliximab is also effective in closing fistulas. In those patients that become refractory to medication, surgical resection of the affected parts is warranted and as many as 50% of CD patients will require surgery within 10 years of diagnosis. However, this strategy is not curative, and the risk of recurrence is about 50% within 10 years after surgery [134].

Treatment of ulcerative colitis

The first-line treatment for mild to moderate UC is 5-ASA given rectally and/or orally for induction and maintenance of remission. As an alternative, probiotic therapy can either be used alone or in combination with 5-ASA [135]. GCS given as enema can also be used as induction therapy when 5-ASA on its own is ineffective. If this treatment fails, patients receive either oral GCS or thiopurines. If intolerance to thiopurines occurs, methotrexate can be used [136]. In patients with moderate to severe disease, cyclosporine or anti-TNF therapy is indicated. Furthermore, combination treatment of anti-TNF with azathioprine can be used to induce GCS-free remission. As with CD, if medical therapy fails, surgery is indicated. The most common procedure used is proctocolectomy with ileal-rectal or ileal pouch-anal anastomosis. However, as many as 40% of these patient evolve pouchitis [137].

Biological therapies in the pipeline

Several therapies targeting cytokines have been tested for efficacy in IBD. For example, anti-IL-12 p40 subunit antibodies have been tested for active CD inflammation mediated by Th1 and Th17 cells [138]. Fontolizumab, an antibody directed against IFN-γ, has been shown to be safe in the treatment of moderate to severe CD [139]. Also, two antibodies against the IL-2 receptor, daclizumab and basiliximab, have been tested in active UC [140, 141]. So far, no clear-cut efficacy of these antibodies is seen in CD and UC.

Another molecular target aims at interfering with adhesion molecules to block the extravasation of leukocytes into the inflamed tissue. Several antibodies have been tested and shown to have some efficacy. Natalizumab, targeting the α_4-subunit of the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, induces remission in patients with active CD [142]. On the other hand, vedolizumab that targets $\alpha_4\beta_7$ specifically is effective in treatment of active UC [143]. One alternative treatment is leukocyte apheresis, which removes certain populations of leukocytes from the blood to modulate the inflammatory response [144].

Several other new treatments are being tested for treating UC. One example is epidermal growth factor given as enema together with 5-ASA. This combination shows efficacy in induction of remission in UC [145] by stimulation of epithelial growth and barrier function. Another one being tested in UC is rosiglitazone [146], an agonist to the transcription factor peroxysome proliferator-activated receptor γ (PPARγ), known to be involved in anti-inflammatory effects.

1.2.3 Experimental colitis

Animal models are vital for studies elucidating pathogenesis of disease and novel drug targets. Although there is a continuous search for alternative methods, some parameters are impossible to study without the use of experimental animals, permitting *in vivo* and *in vitro* experiments that would not be possible in humans. Most of the current experimental colitis models utilize chemical induction (e.g., dextran sulfate sodium (DSS) and trinitrobenzenesulfonic acid (TNBS)), gene targeting (e.g. IL-10 deficiency) or immune cell transfer (e.g. $CD45RB^{Hi}$ transfer). These different types of models are used to study different aspects of IBD pathogenesis (e.g., defects in the epithelial barrier, innate immune cells or adaptive immune system) [147].

In the DSS model, acute or chronic colitis is induced by supplementing the drinking water with polymers of DSS for several days [148]. DSS is believed to be directly toxic to epithelial cells, causing barrier dysfunction and crypt distortion. The acute colitis produced is superficial and located to the left-sided colonic mucosa, with clinical signs of bloody diarrhea and weight loss. Histopathological examination shows ulcerations and infiltration of granulocytes in the colonic mucosa [149, 150]. These features show similarity to UC and this model has been extensively used to study UC pathogenesis. Furthermore, NO is involved in the inflammatory response in this model [151], and both NO donors and NOS inhibitors cause exaggerated inflammation.

1.2.4 IBD and NO

Since NO and NO-derived species are known to be involved in several processes in the GI tract, changes in NO concentration have been implicated in the pathogenesis of

several GI disorders. Roediger *el al* [152] reported already in 1986 that NO_2^- levels were increased in rectal dialysates from patients with UC. During the early 1990s, several reports demonstrated iNOS and NO to be involved in IBD [153-155]. Furthermore, rectal gas samples show increased NO concentrations in IBD patients [10, 156-159], with a higher increase of NO in UC patients and in active disease. Increased expression of iNOS is suggested to stem from colonic epithelial cells, as well as infiltrating immune cells in the lamina propria and circulating monocytes [160-162]. Furthermore, UC patients show decreased expression of nNOS in the muscularis mucosa and increased eNOS expression in the lamina propria [163]. As in other inflammatory conditions, NO's role in IBD remains obscure.

Numerous studies using NO donors and NOS inhibitors have been performed in experimental colitis to address the role of NO in intestinal inflammation [164] with several showing conflicting results. Timing in induction of disease in relation to treatment with NO donors/NOS inhibitors is vital; pre-treatment with NOS inhibitors aggravates the colitis, whereas delayed treatment ameliorates the colitis [165]. This suggests that early NOS inhibition affects constitutive NOSes, while delayed treatment mainly affect iNOS. Even taking timing into consideration, several studies have reported beneficial effects of NO in experimental colitis [166-170]. One further suggestion to this dilemma is that iNOS might be vital in the acute mucosal insult, while chronically upregulated production of NO is involved in detrimental tissue damage [164]. These discrepancies in results might also relate to the different species, strains and animal models used, as well as cell types affected and the local NO concentration.

1.3 GASTROINTESTINAL MOTILITY

1.3.1 Generation of contractility

The basic myoelectrical activity in smooth muscle cells of the GI tract is characterized by slow waves, which oscillate at different amplitude, frequency and duration in different parts of the GI tract. These slow waves are initiated by ICCs (pacemaker cells) [171], a distinct population of stellate cells located at the interface between circular muscles and the myenteric plexus [172]. ICCs make connections to each other and muscle cells by gap junctions, and are also in contact with nerve terminals. By this close proximity and the expression of a multitude of receptors, contractility in smooth muscle cell is regulated by myogenic, neural and hormonal stimuli [173].

Acetylcholine

One of the main stimulatory neurotransmitters in control of contractility is acetylcholine. This molecule acts on two different types of receptors, the nicotinic ligand gated ion channel and the muscarinic G protein-coupled receptor. The type 3 muscarinic receptor, expressed on smooth muscle, activates phospholipase C, yielding inositol 1,4,5-triphosphate, which in turn liberates sarcoplasmic reticulum Ca^{2+} into the cytosol to induce contraction. Muscarinic receptors are also present in the myenteric plexus, and are involved in both stimulation of contraction and secretion from glands in the GI tract [174]. The muscarinic antagonist atropine is known to inhibit spiking activity in smooth muscle cells [175].

Serotonin

Another important neurohumoral transmitter involved in induction of contractility is serotonin (5-hydroxytryptamine, 5-HT), which is mainly expressed in enterochromaffin cells in the mucosa of the GI tract, but also in neurons in the myenteric plexus [176]. Several receptor variants are involved in the response to 5-HT. Ondansetron is a selective antagonist acting on the 5-HT_3 receptor, which is involved in secretion and the initiation of periodic contraction activity in the small intestine [177].

1.3.2 Anatomy of the stomach and small intestine

In terms of gastric motility, the stomach can be divided into two parts: the proximal, including cardia and fundus, and the distal, made up by the distal corpus and antrum. These two parts exhibit different motility patterns. The proximal stomach is involved in generation of tonic contractions, while the distal stomach exhibits phasic myoelectrical activity. A pacemaker zone situated on the greater curvature of the corpus is involved in the generation of the slow wave activity (3 waves/min in man). The distal stomach is separated from the small intestine by the pyloric sphincter, a bundle of circular muscles that aids in the movement of gastric contents into the intestine.

The small intestine is divided into three parts: duodenum, jejunum and ileum. The major source of contractile activity in the small intestine stems from the muscularis externa, which consists of the outer longitudinal and the inner circular layers. The myenteric plexus, containing the ENS, resides between these two layers. A pacemaker zone of ICCs situated distal to the pylorus generates slow waves at a high frequency (about 10/min in man) in the circular layer. Together, these two layers generate peristalsis; the circular layer coordinates mixing and propulsion (known as segmentation), while the longitudinal layer shortens the gut length to accelerate transit. When measuring motility patterns in the small intestine by manometry or electrodes, the contractions registered originate from the circular muscle layer.

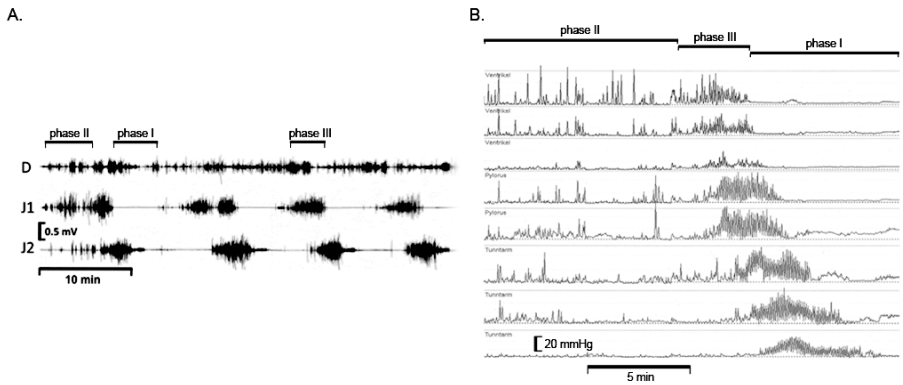

Fig 4. *Representative recordings of MMC cycle pattern in rat (A) and man (B).*

1.3.3 Migrating motor complex

The contractile frequency of smooth muscle cells in the distal stomach and small intestine forms a specific pattern of motility that changes with food intake. During the interdigestive state, a cyclic pattern occurs, the migrating motor complex (MMC). This

motility pattern was first described in dogs [178], and later in both rat and man [179, 180]. During the MMC, three distinct phases can be recognized [181] (Fig 4). The activity front (phase III) is a burst of slow wave activity, starting in the distal stomach and migrating aborally throughout the small intestine. This is followed by a quiescent period called phase I, in which no or few contractions occur. Next, sporadic spikes occur (phase II), which in turn is followed by the re-occurrence of phase III. Eating disrupts this motility pattern with one of irregular contractions for an extended period (6-7 hours in man) [182]. Furthermore, the MMC is dependent on the ENS, but still occurs in vagus-denervated dogs [183]. However, the central nervous system (CNS) is known to modulate the MMC.

The interdigestive pattern functions as a housekeeper mechanism that propels chyme, bacteria and cell debris down the GI tract. This protects the mucosa from damage and counters bacterial overgrowth in the small intestine. Transport occurs throughout phase II and III of the MMC [184]. The relevance of this mechanism in gut health is obviated by the fact that patients with neuromuscular disorders are at increased risk of developing bacterial overgrowth [185]. Furthermore, motility is also influenced by the immune system. For example, IBD patients show axonal degeneration and infiltration of several immune cells (e.g., lymphocytes, mast cells, eosinophils and macrophages) in the myenteric plexus, which is not restricted to the site of active inflammation in these patients [186, 187]. These changes are also accompanied by nerve dysregulation and changed concentrations of neurotransmitters [188-191], which are thought to be involved in the changed contractility pattern seen in these disorders.

1.4 NEUROPEPTIDE S

Neuropeptide S (NPS) was first described in a patent in 2002 [192] (Fig 5). Its receptor (NPSR1) is G protein-coupled and exists as two functional isoforms, NPSR1-A and NPSR1-B [193], which upon stimulation increases both $[Ca^{2+}]_i$ and intracellular cyclic adenosine monophosphate (cAMP) levels [194]. Since its discovery, the NPS/NPSR1-system has been implicated in a multitude of functions relating to its expression in the CNS, such as anxiety, arousal, locomotion and food intake [195-198]. Furthermore, the NPS/NPSR1-system is also expressed in the GI tract (e.g., epithelia, enteroendocrine cells, submucosal neurons and smooth muscle cells) [199, 200], and in leukocytes [201]. These findings suggest a role for NPS in both motility and inflammatory reactions. Indeed, NPSR1 has been linked to both asthma and IBD [193, 202], as well as to sensory and motor disturbances in the GI tract (i.e. hastening of colonic transit, gas, urgency and pain sensation) [203].

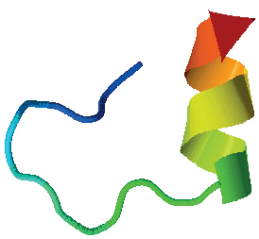

Fig 5. *Predicted structure of NPS as assessed with the I-TASSER server [204].*

2 AIMS OF THE THESIS

The overall aim of this thesis was to study the involvement of NO in inflammatory reactions as well as its regulatory role on motility in the GI tract. More specifically, the aims were:

- to determine the differential expression of NO-related genes in CD and UC in search of pathophysiological mechanisms involved in IBD

- to compare the therapeutic effect of blocking antibodies against α_2 (CD49b) and α_4 (CD49d) integrin subunits to conventional IBD drugs methotrexate, 5-ASA and azathioprine in the DSS colitis model and their effects on inflammatory markers, including iNOS

- to examine effects of NPS on the fasting motility pattern *in vivo* and on the contractility of circular muscle strips *ex vivo*, as well as potential effects on iNOS and other biomarkers of inflammation

- to investigate the role of NO on *in vivo* fasting motility in relation to muscarinic and 5-HT_3 receptor blockade in man

3 MATERIALS

3.1 STUDY SUBJECTS

Permission to perform the studies was obtained from the regional ethics committee at Karolinska Institutet and/or Uppsala University and all subjects gave informed consent prior to entering the study (ethics approval numbers: paper I: 03-718 and Ö 21-2012, paper III: 2010/157, and paper IV: 01-313 and 2012/569-32).

Paper I

Microarray analysis was performed on biopsies taken during routine colonoscopy from 20 patients with CD, 20 patients with UC and six control subjects. Inflamed tonsil tissue was resected from one tonsillitis patient and used as positive control in the immunohistochemical analysis.

Paper III

Organ bath studies were performed on normal tissue specimens from stomach (n = 5), small intestine (n = 5) and colon (n = 26) taken during surgery for stomach or colon resection.

Paper IV

Antroduodenaljejunal motility recordings were performed in healthy subjects given intravenous (i.v.) bolus injection of saline (n = 8), 10 mg/kg L-NMMA, or 1 mg atropine or 8 mg ondansetron followed by 10 mg/kg L-NMMA after 10 min (n = 6 in each group).

3.2 ANIMALS

The local ethics committee in northern Stockholm approved all experiments (ethics approval numbers: paper II: N446/09, and paper III: N248/09). All animals were obtained from Scanbur (Sollentuna, Sweden) and housed under standard conditions (temperature 19 – 24 °C, humidity 60% and regulated lighting in 12 h cycles) with food and drinking water available *ad libitum*.

Paper II

Sixty-five female balb/c mice at the age of eight weeks were used for evaluation of different treatment schemes in the DSS colitis model.

Paper III

Thirty male Sprague-Dawley rats, weighing 300-350 g, were implanted with electrodes to measure the effect of NPS on small bowel motility. An additional 12 rats had a catheter inserted into the external jugular vein for i.v. infusions to measure the effect of NPS on inflammatory markers' expression.

4 METHODOLOGY

4.1 PROCEDURES

4.1.1 DSS–induced colitis (paper II)

Colitis was induced in nine-week old mice by dissolving 2.5-3.0% DSS (molecular weight 45-49kDa; TdB Consultancy, Uppsala, Sweden) into the drinking water for 19 days. After establishing colitis at day 12, mice were divided into five different treatment groups and two control groups. All treatment groups received daily doses of active drug or vehicle by rectal administration from day 13 until the end of the experiment on day 19. One control group was given drinking water without any DSS (controls, n = 8), while the other had DSS but no pharmacological treatment (DSS alone; n = 10). All treatment groups were compared with the DSS alone group, thus enabling us to monitor the potential degree of clinical remission.

Pharmacological treatment

Active compound or vehicle (50 μL purified water, azathioprine group) was rectally installed through an injection catheter (X-ray Opaque feeding tube; Vygon, Ecouen, France; Fig 6) daily for a total of seven days. Drugs tested were as follows: Function-blocking monoclonal antibodies Ha1/29 (hamster anti-rat, n = 8; BD Pharmingen, San Diego, CA) and PS/2 (rat anti-mouse, n = 10; AbD Serotec, Oxford, UK) directed against the α_2 subunit and the α_4 subunit, respectively, in a dosage of 20 μg antibody in 50 μL purified water; Mesalazine (Pentasa, n = 9; Ferring Pharmaceuticals, Saint-Prex, Switzerland) in a dose of 15 mg/kg in 300 μL purified water and methotrexate (n = 9; MediGelium AB, Solna, Sweden) in a dose of 100 μmol/L in 100 μL purified water were all administered rectally each day. Azathioprine (Imurel, n = 8; Orion Cooperation, Espoo, Finland) in a dose of 10 mg/kg was administered daily in the drinking water together with DSS as biotransformation is needed for activation of this compound.

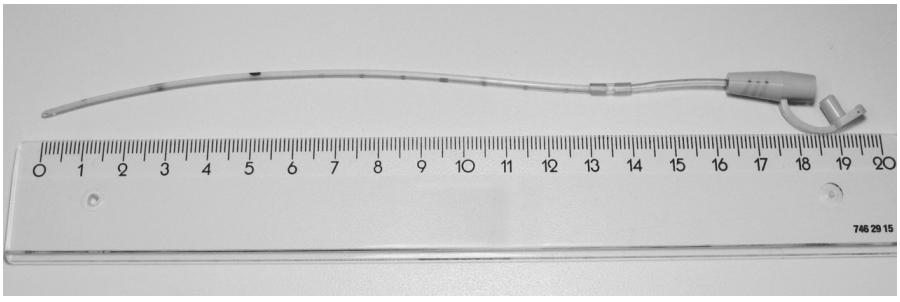

Fig 6. *Injection catheter used for rectal administration of drugs. Units are in cm.*

Evaluation of colitis

Daily assessment included measuring body weight, presence of fecal blood and diarrhea, which were used to calculate the disease activity index (DAI) [149] (see Table II). Water consumption was also measured. At the end of experiments, animals were anesthetized with pentobarbitone (50 mg/kg intraperitoneally (i.p.), Apoteket AB,

Stockholm, Sweden) and killed by cervical dislocation. The colon was resected between the ileocecal junction and the proximal rectum and its length measured. Additionally, biopsies from the most distal part of the colon were collected in phosphate-buffered formaldehyde (Apoteket AB), imbedded in paraffin, cut in 4 μm thick circular sections, mounted onto slides and stained with hematoxylin and eosin for histopathological grading. For this purpose, a grading system of four steps of acute inflammatory activity was adopted. The extent of inflammation and crypt damage was also graded according to Dieleman *et al* [150]. All grading steps (Table II) were performed in a blinded fashion.

Table II. *Grading systems of DSS colitis*

Disease Activity Index [†]			***Histopathological grading***		
Features	Score	Description	Features	Score	Description
Weight loss	0	None	Inflammation	0	normal mucosa
	1	1 - 5%		1	sporadic presence of granulocytes in lamina propria
	2	5 - 10%		2	smaller foci of granulocytes
	3	11 - 20%		3	larger quantities of granulocytes in lamina propria/granulocytes in crypts
	4	>20%	Extent[‡]	0	none
Stool texture	0	normal		1	mucosa
	2	loose		2	mucosa and submucosa
	4	diarrhea		3	transmural
Occult blood	0	normal	Crypt damage[‡]	0	none
	2	small amount on feces		1	damage of basal 1/3
	4	gross bleeding		2	damage of basal 2/3
				3	only surface epithelium intact
				4	loss of crypt and epithelium
			Percent involvement[‡#]	1	1 - 25%
				2	26 – 50%
				3	51 – 75%
				4	76 – 100%

[†]According to Cooper *et al* [149]. The mean of the three features was calculated for each animal and compared on a group basis. [‡]According to Dieleman *et al* [150]. [#]All of the histopathological features were scored as to the percent involvement and the final score for each feature in each animal calculated as the product of the feature score and its percent involvement score. The total histopathological score for each animal was calculated as the sum of all the features. All scores were compared on a group basis.

4.1.2 Surgery in rats (paper III)

Surgery was performed under anesthesia (a mixture of midazolam (5 mg/mL, Aktavis AB, Stockholm, Sweden) and Hypnorm (fentanylcitrate, 0.315 mg/kg plus fluanisone 10 mg/kg; Janssen-Cilag, Oxford, USA.) given subcutaneously (s.c.) at a dose of 1.5-2.0 mL/kg). Buprenorfin (Temgesic® 0.05 mg/kg; Schering-Plough, Stockholm, Sweden) was given s.c. after surgery to avoid post-operative pain.

Gastrointestinal motility in vivo in rats

The abdomen was opened via a midline incision and three bipolar insulated stainless steel electrodes (SS-5T; Clark Electromedical Instr., Reading, UK) implanted into the muscular wall of the small intestine at 5 (J1), 10 (J2) and 15 (J3) cm distal to the pylorus. All animals received an indwelling silastic catheter (Dow Corning Co., Midland, MI, USA) inserted into the external jugular vein for i.v. administration of NPS or vehicle (saline). The electrodes were pierced through the abdominal muscle wall and together with the vein catheter tunneled to the back of the animal's neck. After surgery, the animals were housed individually and allowed to recover for at least 7 days before experiments were undertaken. All animals were monitored daily.

Experiments were carried out in conscious animals after an overnight fasting in wire-bottomed cages with free access to water. The rats were placed in Bollman cages during the experiments, and the electrodes were connected to electroencephalography preamplifiers (7P5B) with output to a Grass Polygraph 7 B (Grass Instr., Quincy, MA, USA). All experiments started with a control recording of basal myoelectrical activity performed over a period of about 60 min (with four regular MMCs propagating over all three recording sites), during which a continues i.v. infusion of saline solution (sodium chloride 9 g/L; 300 mosm/kg H_2O, Fresenius Kabi, Halden, Norway) was given using a microinjection pump (CMA 100; Carnegie Medicine, Stockholm, Sweden). As the fifth activity front vanished from the first electrode site, an i.v. infusion of NPS (0.1, 0.3, 1, 2 or 4 nmol/kg/min; each dose n = 6, NeoMPS, Strasbourg, France) was started through the microinjection pump and continued for 60 min. Further recordings continued until the basal MMC pattern returned (within a total experiment time of 6 h). The occurrence and timing of the small intestinal phase I-III activity was analyzed at the J2 recording site. The activity front of the MMC was identified as a period of clearly distinguishable intense spiking activity with an amplitude at least twice that of the preceding baseline and a frequency of at least 40 spikes/min, propagating aborally through the whole recording segment and followed by a period of quiescence. The MMC cycle length, duration and propagation velocity of the activity fronts were calculated as a mean of the study period. For characteristics of the MMC in rat, see Table III.

Table III. *Characteristics of the MMC in the small intestine*

	Rat		***Man***	
Characteristic	Duration (min)[†]	Frequency (spikes)	Duration (min)[‡]	Frequency (pressure waves)
MMC cycle	12 - 19	NA	34 - 231	NA
Phase I	4 - 9	≤3 / min	1.6 - 30	≤3 / 10 min
Phase II	2 - 5	>3 / min, <40 /min	16 - 180	>3 / 10 min, <10 / min
Phase III	4 - 7	≥40 / min	2 - 9	≥10 / min

NA = not applicable. [†]95% confidence intervals according to Bränström *et al* [205]. [‡]Minimum to maximum duration for all pre-infusion values from paper IV.

4.1.3 Gastrointestinal motility *in vivo* in humans (paper IV)

Subjects were studied after overnight fasting in a comfortable sitting position. Intraluminal pressure was recorded in all subjects using a pneumohydraulic water-perfused manometry system. A manometry eight-lumen polyethylene tube of 4.8 mm diameter (Cook, Copenhagen, Denmark) was introduced through an anesthetized nostril and, under fluoroscopic guidance, passed into the upper jejunum. The four most

proximal measuring points (30 mm apart) were placed into the antropyloric region. The four aborad measuring points were placed in the horizontal duodenum and at the ligament of Treitz, respectively, spaced 100 mm apart between each measuring point. Water was perfused through the catheter at a constant rate of 0.1 ml/min by means of a pneumohydraulic pump (Arndorfer Medical Specialities Inc., Greendale, WI, USA). Pressure changes were measured by applying a transducer (480-AME; Sensonor, Horten, Norway), and the signal amplified with a PC polygraph (Synmed AB, Stockholm, Sweden). All signals were digitized and stored on a computer using the Polygram NET software (Synmed). An i.v. cannula was placed in the left arm of each study subject for infusions. Basal antroduodenojejunal motility was registered for 4 h. Subjects were then given a bolus injection i.v. of either: saline, 10 mg/kg L-NMMA (Clinalfa, Bachem GmbH, Weil am Rhein, Germany), 1 mg atropine (Atropin Mylan, Mylan AB, Stockholm, Sweden) followed by 10 mg/kg L-NMMA after 10 min or 8 mg ondansetron (Zofran, GlaxoSmithKline, Brentford, UK) followed by 10 mg/kg L-NMMA after 10 min. Postinfusion antroduodenojejunal motility was registered during the next 4 h. Blood pressure was measured every 60 min throughout the protocol.

All pressure wave activity was inspected manually and only pressure waves greater than 10 mmHg were included in the analysis. The occurrence and timing of the small intestinal phase I-III activity was analyzed in one of the two most distal recording sites. The MMC cycle length was calculated from the ending of phase III activity. For each variable of the MMC cycle, the mean pre-infusion value was calculated for each subject based on all measurements except for the 60 min period subsequent to L-NMMA infusion to decrease the variability of these variables, i.e. the duration of a systemic blood pressure effect of L-NMMA. Two different MMC cycle lengths were investigated: the one directly affected by infusion of L-NMMA and the first post-infusion. For characteristics of the MMC in man, see Table III.

4.1.4 Organ bath (paper III)

Excised human tissue segments were placed in ice-cold Krebs solution (in mM: 121.5 NaCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 4.7 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 5.6 D-glucose, equilibrated with 5% CO_2 and 95% O_2) within 5-10 min after resection and transported to the laboratory. The mucosa was removed and strips (2–3 mm wide, each 12–14 mm long) cut along the circular axis in freshly made, oxygenated cold Krebs solution. The strips (n = 4, from each patient) were mounted between two platinum ring electrodes in organ bath chambers (5 mL, Panlab, ADInstruments, Sydney, Australia, Fig 7) containing Krebs solution, bubbled continuously with 5% CO_2 and 95 % O_2 and maintained at 37 °C and pH 7.4. Tension was measured using isometric force transducers (MLT0201, Panlab, ADInstruments). Data acquisition was performed using Powerlab hardware and LabChart 7 software (ADInstruments). Tissues were equilibrated to a 2 g tension baseline for at least 60 min during which time the bathing medium was replaced every 15 min. After equilibration, muscle strips were stimulated with acetylcholine (ACh, 1 µM, Sigma-Aldrich, St. Louis, MO) for 3-5 min to test muscle viability and Neurokinin A (NKA, 2 nM, Apoteket AB, Stockholm, Sweden) for 10 min as a control of contractile response to neuronal transduction. These dosages of ACh and NKA showed submaximal effects corresponding to the C_{50} value on the tissue. The effects of NPS (concentrations used: 1, 5, 10, 15, 20, 114 or 200 nM, Bachem, Bubendorf, Switzerland) were studied on baseline muscle tension. To test for

possible prejunctional effects of NPS, muscle contraction was evoked by electrical field stimulation (EFS) using biphasic square wave pulses of 0.6 ms duration (10 Hz, 50 V, 0.6 train/min) with a GRASS S88 stimulator (Grass Technologies, Astro-Med Inc., West Warwick, RI). An initial recording was made without NPS, followed by addition of NPS a few seconds before a second EFS recording. This was followed by a washout and a third EFS recording.

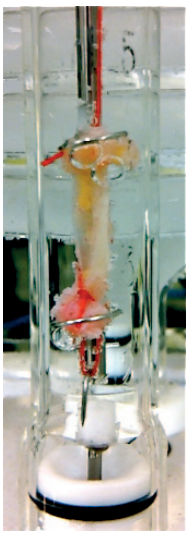

Fig 7. *Muscle strip mounted in an organ bath chamber.*

4.2 GENE EXPRESSION (PAPER I-III)

4.2.1 Tissue collection

All tissue specimens were immediately submerged in RNA stabilization reagent (RNAlater, Ambion, Applied Biosystems, Austin, TX), stored overnight at 4 °C to allow the solution to penetrate the tissue and then stored at -20 °C until RNA extraction for good RNA recovery. In *paper I*, biopsies were taken from inflamed parts of the colon from patients with CD and UC, while control biopsies from non-inflamed tissue were taken from subjects undergoing surveillance screening for colorectal cancer. In *paper II*, biopsies were collected from the most distal part of the colon in a subset in each group of animals (n = 3-5). In *paper III*, tissue segments were collected from the corpus of animals treated with i.v. infusion of either NPS (4 nmol/kg/min, n = 6) or saline solution (n = 6) given during 60 min.

4.2.2 RNA extraction

In *paper I* and *III*, total RNA was extracted with RNeasy Mini kit (Qiagen, Hilden, Germany) after homogenization with a rotor-stator knife (*paper I*, Ultra-Turrax T8, IKA®-Werke, Staufen, Germany) or with means of a blunt needle and syringe (*paper III*). An enzymatic digestion step was included to remove traces of DNA (DNase I (Promega (*paper I*), Madison, WI; or Qiagen (*paper III*)). In *paper II*, biopsies were subjected to a protocol in which both RNA and protein were extracted and purified at the same time. Biopsies were homogenized in Ultraspec with a rotor-stator knife, RNA

extracted with chloroform and purified with RNA Track resin according to the Ultraspec-II RNA isolation system (Biotecx laboratories, Houston, TX). RNA quality and quantity was checked on GelRed-stained agarose gels and by spectrophotometric measurement resulting in A_{260}:A_{280} ratios of 1.8-2.0.

4.2.3 Microarray (paper I)

Three μg of each RNA sample was labeled with ^{32}P-dCTP (GE Healthcare, Waukesha, WI) and hybridized to a human NO gene array (GEArray Q; SuperArray Inc., Frederick, MD) in accordance with the assay protocol. This array consists of 96 cDNA fragments specific for human mRNAs encoding genes involved in the NO pathway, as well as housekeeping transcripts. Each sample was hybridized to one membrane, while controls were pooled and measured in quadruplicate. After washing, the array image was visualized using a phosphor imager (BAS-1500, Fujifilm Corp., Tokyo, Japan) and TIFF documents created (Image gauge software 3.45, Fujifilm Corp., Fig 8). Raw data were analyzed with GEArray Expression Analysis Suite software (version 2.0, SuperArray Inc.), with parameters set to measure total intensity of each spot. All hybridization signals were corrected for local background and normalized according to the quantile normalization method using preprocessCore package in the R environment software [206]. Only mRNAs regarded as present was included in the analysis, i.e. if recovered in ≥ 50% of the samples in each group with intensity signals exceeding the lower 75th percentile of local background.

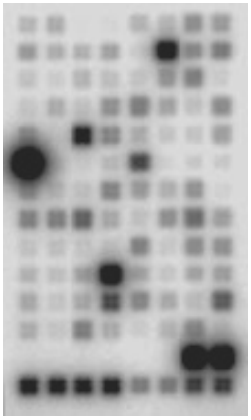

Fig 8. *Representative picture of sample hybridization to a NO gene array membrane.*

Functional gene interactions

Cluster 3.0 [207] and TreeView [208] were applied to reveal underlying patterns in gene expression data. Significant mRNAs were clustered using average linkage and Spearman rank correlation as measures of similarity. Normalized gene expression data was median centred for genes and hierarchical clusters were generated using self-organizing maps. Clustered mRNAs in each disease subgroup were subjected to a PubMed search to identify functional relationships. Gene annotation analysis was performed with Onto-express [209, 210] to discern functional categories over-represented in the differentially expressed gene groups of each disease. Pathway-express [211] was utilized to find gene interactions between differentially expressed

genes. Analysis for Gene-Ontology annotations and involved pathways were based on genes represented on the human NO gene array.

4.2.4 Quantitative PCR (paper II, III)

Each sample was reverse-transcribed into complementary DNA (cDNA) with the Superscript 1st strand system (Invitrogen, Life technologies Corp., Carlsbad, CA) and the quantitative expression of mRNA transcripts of interest (specified in each paper) evaluated with real-time quantitative polymerase chain reaction (qPCR) using the 7300 Real Time PCR System (*paper II* and *III*, Applied Biosystems, Foster City, CA) or Bio-Rad Labs real-time detection system (iCycler iQ (*paper III*), Hercules, CA, USA). For TNFα in *paper III*, primers were designed according to the literature [212] and manufactured by CyberGene® (Stockholm, Sweden). For all other transcripts, ready-made gene-specific primers and TaqMan probe mixes (Applied Biosystems) were used. All samples were analyzed in triplicate for each transcript together with serial dilutions of cDNA mixes to generate standard curves for the threshold cycles (C_t) of each mRNA transcript, as well as to determine the levels of expression in the samples by comparison. In *paper II*, all expression levels were normalized to the level of expression of the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample and expressed as relative expression ratio according to the relative standard curve method [213]. In *paper III*, the expression of each gene was normalized to the mRNA content of the endogenous control 18S and the level of expression recalculated with the REST software [214] to obtain the relative expression of mRNA relative to the control.

4.3 PROTEIN EXPRESSION

4.3.1 Immunohistochemistry (paper I)

Immunohistochemical analysis was performed on paraffin-embedded sections of biopsies collected from patients and controls during colonoscopy. Tissue from a tonsillitis resection was included as positive control. Analyses were performed utilizing the avidin-biotin-immunoperoxidase complex method as previously described [215]. In brief, 4 μm thick sections were mounted on slides and deparaffinised with xylene. Antigen retrieval was achieved, either by heating in 0.1% unmasking solution (Vector Labs, Burlingame, CA) five times for 5 min at 450W in a microwave oven, or by heating in TE-buffer for 20 min at 98 ºC. Sections were incubated overnight at 4 ºC with a primary antibody: monoclonal mouse anti-iNOS (1:200 dilution; Sigma-Aldrich), monoclonal mouse anti-IL-1β (1:50; ImmunoKontact, Abingdon, Oxfordshire, UK), monoclonal mouse anti-MMP1 (2.5 mg/L; Calbiochem, Merck, Darmstadt, Germany) or polyclonal rabbit anti-NFκB p65 (pSer536) (1:50; AbD Serotec, Kidlington, Oxford, UK). As secondary antibody, biotinylated horse anti-mouse IgG or biotinylated goat anti-rabbit IgG (1:400; Vector Labs) was used. The primary antibody was replaced by isotype-specific control antibodies (mouse IgG1 or rabbit immunoglobulin fraction; DakoCytomation, Glostrup, Denmark) in parallel analysis of each sample to demonstrate specific staining. For each antibody staining; 5 CD, 5 UC and 2 control slides as well as one tonsil slide, were evaluated. Consecutive images were captured for each section with a digital camera (DXM1200; Nikon Ltd, Tokyo, Japan) coupled to a light microscope (Eclipse E800; Nikon Ltd) at 20X and

TIFF documents were created (ACT-1 software; Nikon Ltd). Images for each section were measured for the amount of positive staining as percent of total area with Photoshop CS4 (Adobe Systems Inc, San Jose, CA). An investigator blinded to staining and clinical data of the patients performed all image evaluations.

4.3.2 Western blot (paper II)

Proteins left in homogenates after RNA extraction were extracted and purified according to the Trizol reagent protocol (Invitrogen) and redissolved in lysis buffer containing 0.5% sodium dodecyl sulfate, 4 M urea in Tris-HCl (pH 8.0) and proteinase (Complete mini; Roche, Basel, Switzerland) and phosphatase (Phosphatase inhibitor cocktail 2; Sigma-Aldrich, St. Louis, MO) inhibitors. Extracted protein content was assessed with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and samples loaded in triplicate onto 1 mm thick 4% to 8% sodium dodecyl sulfate-polyacrylamide gels (Pierce) according to the manufacturer instructions, transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA) and blocked in 1% bovine serum albumin (Sigma-Aldrich). Membranes were probed overnight with rabbit anti-mouse polyclonal iNOS antibody (1:5000, Cat.No: 610332, BD Biosciences, Franklin Lakes, NJ) or mouse anti-rabbit IgG1 GAPDH antibody (1:40000, clone 6C5, Abcam, Cambridge, UK). Subsequently, membranes were incubated in horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Pierce), developed with SuperSignal West femto (iNOS) or pico (GAPDH) substrate kit (Pierce) and captured on hyperfilm ECL (GE Healthcare, Buckinghamshire, UK). Lipopolysaccharide and IFN-γ stimulated mouse macrophages served as positive control for iNOS. Films of immunoblots were scanned with a flatbed scanner and the relative iNOS protein expression on the digital images was semi-quantitatively analyzed with ImageJ [216] according to the program's gel analysis method. All expression values were relative to a common standard sample used on all gels, scaled to the corresponding GAPDH expression in each sample and expressed as relative expression ratio.

4.3.3 Enzyme-linked immunosorbent assay (paper II)

In *paper II*, interfering detergents (such as sodium dodecyl sulfate) were removed from protein samples (Pierce detergent removal spin columns) and protein content assessed with Coomassie plus Bradford kit (Pierce). Ten μg of each sample was analyzed in duplicate on Quantikine mouse immunoassays (R&D Systems, Minneapolis, MN) for CXCL2 and IL-1β content according to the manufacturer's instructions.

4.3.4 Electrochemiluminescence (paper III)

Tissue specimens from stomach, duodenum, jejunum and colon were immediately frozen at -80 °C for protein analysis of NPS effect on inflammatory markers. All samples were homogenised on ice in lysis buffer (200 mM NaCl, 5 mM EDTA, 100 mM Tris (pH 7.4), 10% glycerol, 2x SigmaFAST protease inhibitor (Sigma, Saint Louis, MI)) using a 1 mL glass Dounce tissue grinder (Wheaton, Millville, NJ) with 30 strokes for each of the loose and tight pestles. Homogenates were incubated 30 min on ice, then centrifuged at 10 000xg for 10 min at 4 °C to pellet remaining debris. The protein concentration was measured in the supernatants using Bradford reagent (Bio-Rad labs). Fifty μg of each sample was assayed in duplicate for the content of seven

inflammatory cytokines and chemokines (IFN-γ, IL-1β, IL-4, IL-5, IL-13, CXCL1, and TNFα) on a multispot array using an electrochemiluminescence sandwich immunoassay method (Meso Scale Diagnostics, Gaithersburg, MA) according to the manufacturer's protocol.

4.4 SAMPLING OF RECTAL AND EXHALED NO (PAPER IV)

Exhaled and rectal NO was measured before and at 60, 120 and 180 min after administration of L-NMMA using chemiluminescence techniques (exhaled: NIOX, Aerocrine AB, Stockholm, Sweden; rectal: CLD 700, EcoPhysics, Dürnten, Switzerland), calibrated with known concentrations of NO (100-10 000 ppb, AGA, Lidingö, Sweden) and having a lower detection limit of 1 ppb. In brief, test subjects were asked to exhale slowly into a mouthpiece with an internal restrictor, connected with the instrument through a side-arm sampling tube, and NO concentrations measured at an expiratory flow rate of 50 mL/s. Rectal NO levels were sampled with all-silicone catheters (Argyle, Sherwood Medical, Tullamore, Ireland; Fig 9). The catheter tip was positioned 10 cm above the anal sphincter using lubrication gel, the balloon of the catheter inflated with 10 mL of ambient air through a syringe and left for 10 min to equilibrate with the intra-luminal gases. Subsequently, the gas sample was withdrawn from the balloon, diluted to a final volume of 50 mL with ambient air and the NO concentration measured immediately with correction for the dilution factor.

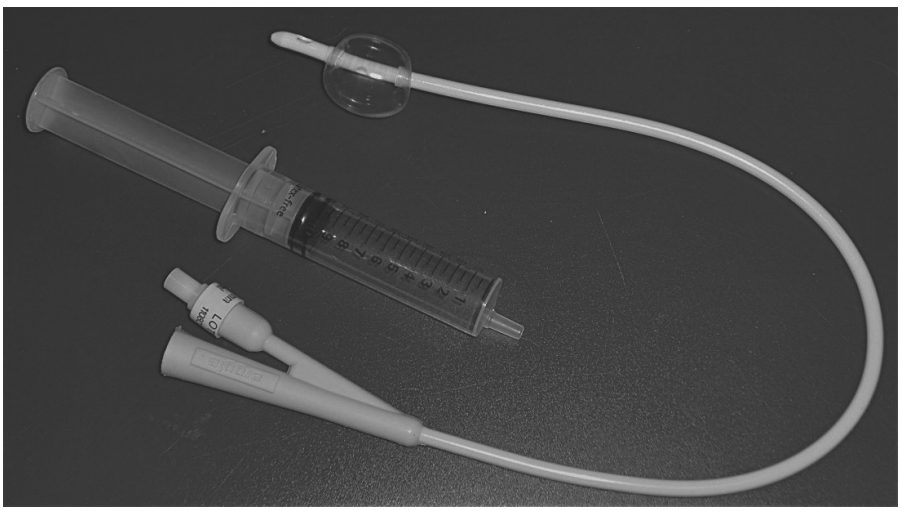

Fig 9. *Balloon catheter used for sampling rectal NO.*

4.5 STATISTICS

Values are expressed as mean ± SEM unless otherwise specified. $P < 0.05$ was considered statistically significant. The Prism software package 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical comparisons.

Paper I

Differences in gene expression were examined by the one-sample Wilcoxon signed rank test in combination with Q-value analysis [217]. Age and gender differences

between groups were analyzed with the Kruskal-Wallis test with Dunn's multiple comparison and χ^2 test, respectively. Unpaired *t* test was utilized to compare the duration of disease. Spearman rank correlation was used to compare the expression pattern between microarray analysis and image evaluation of the immunohistochemical staining. Gene annotation and gene interaction analyses were calculated with hypergeometrical distributions and P-values corrected for the false discovery rate.

Paper II

Differences in body weight before the onset of DSS treatment (day 0), before the onset of treatment (day 13) and during the treatment period (day 13-19) as well as colon length was tested with one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Acute inflammation was tested with Kruskal-Wallis test with Dunn's multiple comparison test. End-point differences in DAI, extent of inflammation, crypt and colitis scores were all tested with Mann Whitney test. mRNA and protein expression of MIP-2 differences were tested with one-way ANOVA with Dunnett's multiple comparison test, while protein expression differences in iNOS and IL-1β were tested with Kruskal-Wallis test with Dunn's multiple comparison test. Correlations between mRNA and protein expression were assessed with Pearson correlation coefficient, *r*.

Paper III

Paired *t* test were used when comparing the MMC cycle length interval, phase III duration and velocity. Unpaired Student's *t* test were used when comparing the protein expression of cytokines and chemokines. Repeated measures ANOVA was used for comparing contractility changes to EFS-stimulated colon. The REST software was used to test differences in mRNA expression.

Paper IV

Differences in MMC parameters were tested with paired *t* test in the same group and with one-way ANOVA with Bonferroni's multiple comparison test between groups for the following parameters: duration of phase I, phase III, propagation velocity, amplitude and contraction frequency during phase III. Kruskal-Wallis test with Dunn's multiple comparison test was used to evaluate differences in MMC, phase II duration and time to effect of L-NMMA. Changes in blood pressure were tested with paired *t* test, while differences in NO production were evaluated with Wilcoxon signed rank test.

5 RESULTS

5.1 NO-RELATED GENE EXPRESSION IN IBD (PAPER I)

By utilizing a specific microarray, we studied the gene expression of genes involved in NO signaling. In our CD and UC patient cohort, 19 and 23 genes respectively were found to be differentially expressed, with a false discovery rate of about 1%. Most of these genes were upregulated in both groups, with only four and six being downregulated in CD and UC, respectively. Furthermore, 14 out of 28 genes found differentially expressed were common to both diseases. These results correlated with changes in protein levels of a selection of these genes (iNOS, MMP1, IL-1β and NFκB; Spearman $r = 0.47$ (0.18-0.69, 95% CI); $P = 0.002$).

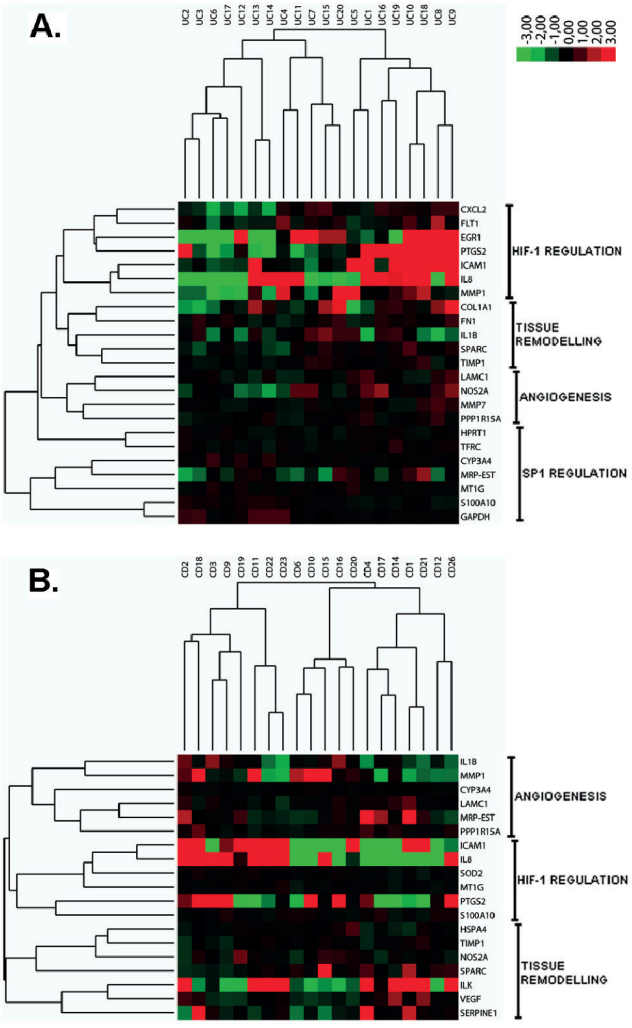

Fig 10. *Heatmaps illustrating hierarchical clustering of significant transcripts expressed in ulcerative colitis (UC, A) and Crohn's colitis (CD, B).*

Hierarchical clustering together with a PubMed search revealed gene clusters of co-regulation or co-expression common to both disorders (Fig 10), i.e. angiogenesis, inflammation response mediated by the transcription factor hypoxia-inducible factor 1 (HIF-1) and tissue fibrosis. A fourth cluster was also found in UC with transcripts regulated by the transcription factor Sp1. Moreover, gene interaction analysis found two pathways involved in both CD and UC: epithelial cell signaling in Helicobacter pylori infection and leukocyte transendothelial migration, based on the expression of IL-8 and ICAM-1 in these disorders, respectively.

A few of the genes we found differentially expressed are novel in the context of IBD. For example: in CD, ILK was upregulated, while HSPA4 was downregulated. These two transcripts are involved in intracellular linkage of integrins to cytoskeleton and regulation of tight junction assembly in epithelial cells, respectively. In UC, GAPDH and TFRC were downregulated. GAPDH is part of an inhibitory translation complex involved in resolution of inflammation, while M1 macrophages downregulates the expression of TFRC in an attempt to withhold iron from invading pathogens. These changes of expression of disorder-specific transcripts implicate them as part of the mechanisms involved in withholding a pronounced inflammatory response in CD and UC, respectively.

5.2 INTEGRIN ANTIBODY TREATMENT IN DSS COLITIS (PAPER II)

DSS treated groups receiving rectal enema with function blocking anti-α_2 integrin antibody and methotrexate showed signs of alleviated colitis as assessed by several parameters. Both treatment groups had less body weight loss compared to the DSS alone group (2.1 ± 0.7%, 1.4 ± 0.6% and 5.2 ± 1.2%, respectively; $P = 0.003$). The anti-α_2 group had no signs of fecal blood, while the methotrexate group had a reduced DAI score at the end of treatment (0.78 ± 0.2 compared to the DSS alone groups 1.6 ± 0.3, $P = 0.03$). In histopathological grading of colonic sections, all treatment groups, except the anti-α_4 treated, had decreased acute inflammation, while the extent of inflammation also was reduced in the anti-α_2 treated. Moreover, the methotrexate group had decreased crypt damage and total colitis score, firm signs of mucosal healing (Fig 11).

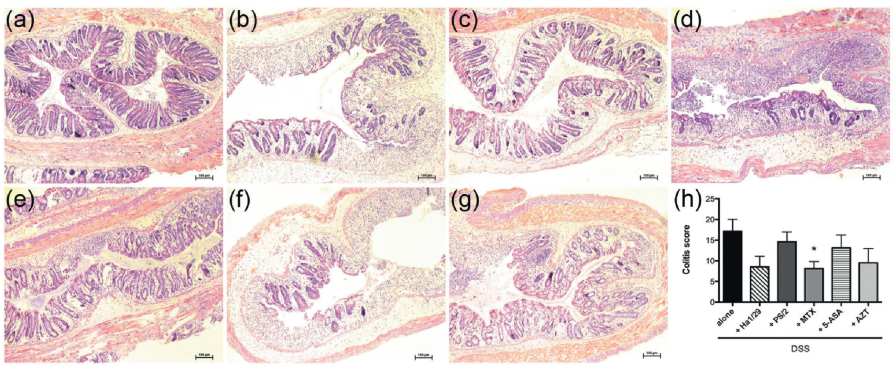

Fig 11. *Micrographs showing hematoxylin-eosin staining of circular sections of distal colon and colitis score. (a) healthy control, (b) DSS alone, (c) anti-α2 antibody, (d) anti-α4 antibody, (e) methotrexate, (f) 5-ASA, (g) azathioprine, and (h) colitis score. *P < 0.05 between DSS alone and methotrexate groups.*

Signs of ameliorated inflammation were also discernable in the expression of inflammatory markers. The methotrexate group had decreased mRNA expression of IL-1β compared to the positive control group with DSS alone (8.9 ± 3.4 and 33.3 ± 10.2 fold change, respectively, $P < 0.05$). However, the methotrexate group also had increased expression of iNOS at both the mRNA and protein level compared to the control group (48.2 ± 14.3 fold change and 1906 ± 1130 fold change, respectively). In the anti-α_2 treated group both iNOS and IL-1β were decreased at the protein level compared to the DSS positive control group (iNOS: 11.1 ± 7.5 fold change compared to DSS alone group 986 ± 539 fold change ($P = 0.004$) and IL-1β: 0.78 ± 0.4 pg/mL compared to DSS alone group 20.7 ± 6.5 pg/mL ($P < 0.05$)), while MIP-2 had a trend of decreased expression (0.39 ± 0.4 pg/mL compared to DSS alone group 17.3 ± 4.9 pg/mL ($P = 0.05$)).

5.3 NPS EFFECTS ON MOTILITY AND INFLAMMATION (PAPER III)

NPS infusion in the rat prolonged both the MMC cycle length and phase III duration in a dose-dependent manner (from 10.5 ± 0.8 min to 15.8 ± 1.5 min ($P = 0.02$) and from 4.4 ± 0.2 min to 11.3 ± 0.4 min ($P < 0.0001$), respectively, with 2 nmol/kg/min. Fig 12). Furthermore, in contractility studies of human small intestine, NPS decreased the baseline contraction amplitude, possibly by acting directly on smooth muscle cells. In human colon, a similar effect was seen sporadically and not with all concentrations tested. However, if 1 nM of NPS was given prior to EFS, a majority of the colon preparations showed an inhibitory response (spike amplitude decreasing from 51.3 ± 13.3 mN to 32.1 ± 10.5 mN, $P = 0.04$), indicating the presence of a prejunctionally driven relaxation by NPS in this part of the GI tract.

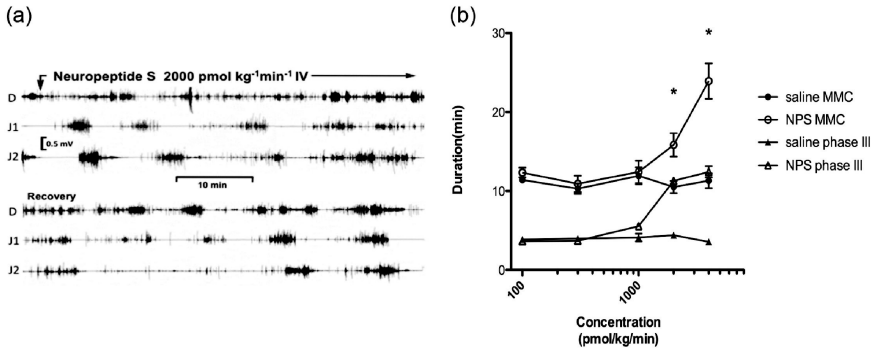

Fig 12. *Effect of NPS infusion on MMC small bowel motility in rat. (a) electromyographic recording of infusion with 2 nmol/kg/min NPS. (b) effect of increasing concentrations of NPS on MMC cycle length and phase III duration. *P < 0.05 between saline and NPS infusion.*

Infusion with NPS (4 nmol/kg/min) also increased the expression of inflammatory markers. The iNOS mRNA and CXCL1 protein expression were increased in stomach 5.1-fold ($P = 0.03$) and 1.7-fold ($P = 0.02$), respectively, while IL-1β protein expression was increased in duodenum 2.7-fold ($P = 0.04$).

5.4 NO EFFECTS ON MMC IN MAN (PAPER IV)

Specific effects of L-NMMA on NO production were verified by decreased concentrations of NO in exhaled breath and rectal gas samples (from 10.4 ± 2.5 ppb to 4.2 ± 0.8 ppb (P=0.002) and from 49.8 ± 19.0 ppb to 40.8 ± 15.1 ppb (P = 0.01), respectively), as well as by increased blood pressure (mean arterial pressure increased from 91.7 ± 2.5 to 100.5 ± 2.5, P = 0.0007. Fig 13a).

L-NMMA counteracted the inhibitory effect of NO on the MMC by eliciting a premature phase III within 4.2 ± 0.6 min in all but one subject given atropine. This effect was mainly seen in the duodenaljejunal segment, with only five out of 18 subjects showing an effect in both antrum and small intestine. Furthermore, the premature phase III caused a decrease of the MMC cycle length (from 114.0 ± 16.8 min to 50.6 ± 16.8 min, P = 0.01), mainly due to a shortening of the phase II duration (from 99.7 ± 19.1 to 40.0 ± 15.0, P = 0.02).

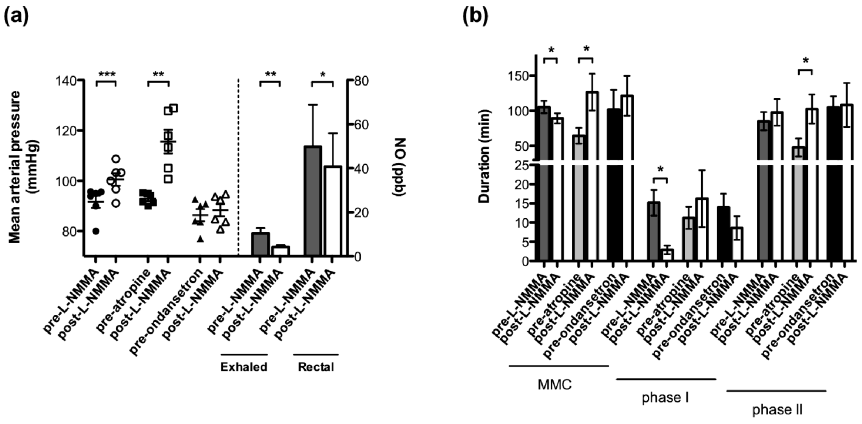

Fig 13. *Effects of infusion of L-NMMA, atropine and ondansetron on (a) physiological NO production (measured as mean arterial pressure, as well as exhaled and rectal NO gas) and (b) MMC characteristics (MMC cycle length, phase I and phase II duration). *P < 0.05 between pre- and post-infusion.*

Additionally, the subsequent MMC cycle length was also shortened in subjects given L-NMMA due to a strong suppression of the phase I duration (MMC: from 105.4 ± 8.7min to 89.3±7.2 min (P = 0.03) and phase I: from 15.2 ± 3.3 min to 2.9 ± 1.1 min (P = 0.02). Fig 13b). In contrast, the subsequent MMC cycle was prolonged in subjects pre-treated with atropine due to an extended phase II duration (MMC: from 64.5 ± 11.2 min to 126.6 ± 26.1 min (P = 0.04) and phase II: from 48.0 ± 12.9 to 102.6 ± 20.8 (P = 0.03). Fig 13b). However, pre-treatment with ondansetron attenuated the effects seen with L-NMMA, showing no differences between any of the pre-infusion values studied.

6 GENERAL DISCUSSION

Apart from being involved in a multitude of functions to orchestrate uptake of nutrients, the GI tract also forms a barrier between the body and the outside world. As part of the protection against harmful microorganisms, the resident flora in the colon lives a symbiotic relationship with the mucosal immune system. However, if this homeostatic balance is perturbed by pathogenic microorganisms expanding their habitat (i.e., invading the intestinal border), the mucosal immune system will respond. If the immune response is activated for an extended time period, this results in detrimental chronic inflammation. Indeed, a dysregulated immune response to microbial components is thought to be one of the main causative mechanisms in the pathophysiology of IBD [103, 218].

NO's involvement in this immune response is still not fully understood, with disputing studies showing this second messenger to be pro-inflammatory, anti-inflammatory, as well as immunosuppressive [95-97]. Its importance in IBD has been established by a multitude of studies showing iNOS to be upregulated in active disease [153-155, 219], as well as studies showing accompanying increased rectal concentrations in the colonic lumen [10, 156-159]. Furthermore, changes in NOS expression in the colon of IBD patients [163] could be involved in the dysmotility response seen in these disorders.

In this thesis, the involvement of NO in inflammatory reactions as well as its regulatory role on motility in the GI tract was elucidated by studying the differential expression of genes regulated by NO in colonic biopsies from patients with IBD (paper I), the therapeutic effect of integrin-blocking antibodies as compared to conventional IBD drugs in an experimental colitis model and their effects on inflammatory markers, with emphasis on iNOS (paper II), NPS effects on motility, contractility and inflammatory biomarker expression, including iNOS (paper III), and finally the NOS inhibitor L-NMMA effects on the MMC in relation to muscarinic and 5-HT_3 receptor blockade in man (paper IV).

In paper I, we found several NO-related genes to be differentially expressed in IBD. These results together with cluster and interaction analyses gave further knowledge on which pathways are involved in perpetuating inflammation in CD and UC. As a sign of an ongoing inflammatory response in our patient cohort, genes found to have significantly changed expression are predominately involved in inflammatory signaling, tissue remodeling or apoptosis/oxidative stress. Furthermore, NO's involvement during the inflammatory response in IBD was confirmed by the upregulated expression of iNOS in both CD and UC patients (1.88 fold change and 2.36 fold change compared to controls, respectively). Although several of these differentially expressed genes have been previously implicated in IBD [220-228], a few of them are novel (i.e., common to both disorders: *MRP-EST* and *PPP1R15A,* specific for CD: *ILK, SOD2* and *HSPA4,* and specific for UC: *GAPDH, HPRT1* and *TFRC*). These disorder-specific genes point to mechanisms involved in each disease modality. ILK, SOD2 and HSPA4 indicate the importance of leukocyte infiltration [229], T cell evasion of apoptosis [230] and epithelial barrier dysfunction [231] in CD. Similarly, downregulation of GAPDH and TFRC, involved in resolution of inflammation [232]

and withholding iron from invading pathogens [233, 234], respectively, speaks in favor of a dysregulated immune response and the microbiome being involved in the development of UC. These possible disease mechanisms are in-line with current knowledge of IBD pathophysiology.

The cluster analysis added further information as to how the differentially expressed genes are co-regulated and co-expressed in CD and UC. By performing a PubMed search on these clusters, important processes mediating the inflammation were pinpointed; HIF-1 mediated inflammation, angiogenesis and tissue fibrosis were found in both disorders. These common processes involved in the inflammatory response, as well as the notion that half of the differentially expressed genes were common to both CD and UC, are in accordance with the concept of that, apart from disorder-specific mechanisms involved in the development of these diseases, a common mechanism exists in the pathophysiology of IBD [224, 225]. The gene clusters implicate HIF-1 as a central molecule involved in the inflammatory response. This transcription factor is activated by pro-inflammatory cytokines and hypoxia, two mechanisms involved in the early immune response. By subsequent activation of NFκB, HIF-1 influence the downstream reactions to induce remodeling of the stromal microenvironment and increased infiltration of inflammatory cells [235, 236], causing the enhanced tissue fibrosis and angiogenesis seen in IBD. Furthermore, the gene interaction analysis revealed two common pathways involved in CD and UC due to the highly increased expression of IL-8 and ICAM-1 in both disorders: epithelial cell signaling in Helicobacter pylori infection and leukocyte transendothelial migration. These two transcripts' involvement in recruitment and trafficking of leukocytes to the inflamed tissue [237] highlights the importance of this pathway in the perpetuating inflammatory response seen in IBD.

In paper II, we further evaluated the concept of blocking the infiltration of leukocytes into the inflamed colon tissue by comparing two integrin-blocking antibodies, acting on the α_2 and α_4 subunits of the $\alpha_2\beta_1$, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, to the conventional IBD treatments 5-ASA, methotrexate and azathioprine in the DSS colitis model. This model was used due to its resemblance to human IBD, especially UC, in both clinical and histological features, as well as its usage for investigating the involvement of leukocytes in intestinal inflammation [238]. In this study, therapies were administered by rectal enemas to increase the drug concentration at the mucosal inflammatory site, as well as to reduce side effects evoked by systemic administration of immunomodulators. However, since azathioprine needs bioactivation in the liver for its therapeutic effect, this drug was administered in the drinking water.

Our results show that treatment with anti-α_2 antibodies alleviates clinical and histopathological signs of acute colitis. Apart from reducing body weight loss and the acute inflammation and its extent, the antibodies also ameliorated rectal bleeding, showing potential use for this antibody to stimulate epithelial restitution [239]. In further support of an ameliorated colitis, this treatment also yielded a reduction in the expression of the inflammatory biomarkers iNOS and IL-1β, as well as a trend to reduce the leukocyte chemokine CXCL2. These results are in agreement with a previous study in our group, showing that this antibody also ameliorated DSS induced inflammation in a similar way when treatment was started at the same time as colitis induction [240]. That study showed that the alleviating effect of the anti-α_2 antibody

predominately occurred due to a dampening of the neutrophil accumulation into the inflamed tissue. This is also true for the mouse air pouch model of acute inflammation [131]. Furthermore, these antibodies seem to exert their main effect extravascularly as opposed to intravascularly; due to the size of the antibody molecules, intravascular uptake is unlikely when administered topically. This further supports the use of this antibody for local administration with rectal installation directly to the inflamed mucosa and shows the potential for anti-α_2 antibodies as a treatment in IBD.

Treatment with methotrexate also induced multiple anti-inflammatory effects. However, these effects were not accompanied by a broad reduction in inflammatory markers. Especially iNOS was highly increased at both the mRNA and protein level. This result raises the question as to the involvement of iNOS as only a pro-inflammatory marker in colitis, as this treatment group had one of the best outcomes with reduced crypt and colitis scores. The beneficial effects seen with methotrexate in this disease model are of special interest since the compound used in this study was especially developed for topical administration in the lower GI tract. The lack of response with the anti-α_4 antibody in this study was surprising when compared to the beneficial effects seen with this type of antibody in human IBD [143, 241]. However, the α_4 integrin is mainly involved in the arrest of leukocytes on the endothelium [242], making it plausible that rectal administration of this antibody does not reach the therapeutic concentrations needed for an intravascular effect. Both 5-ASA and azathioprine reduced the acute inflammation score in our disease model. However, this was not accompanied by symptom improvements.

As for paper III, the findings of D'Amato *et al* [202] and Camilleri *et al* [203] showing polymorphisms in NPSR1 to be involved with IBD and colonic transit, respectively, led us to investigate what effect NPS has on motility in the normal setting. Our study showed that NPS extended the MMC cycle length and phase III duration in upper small intestine, indicative of decreased motility with NPS during fasting. These results are very interesting as compared to IBD, since decreased motility is seen in both CD [243, 244] and UC [245, 246]. Although our results are at odds with a study performed by Petrella *et al* [247], who found no effect of NPS on gastrointestinal transit, they did not measure motility in terms of contractility. Our *ex vivo* contractility study of NPS on normal human muscle strips is also in agreement with the reduced amplitude seen in inflamed muscle both at rest, after EFS stimulation [248] and after stimulatory modulators [100, 249, 250]. Even though Han *et al* [251] showed central administration of NPS to inhibit colonic transit, indicative of a relaxatory effect of NPS, they saw no effect of NPS *ex vivo* on colonic muscle strips. Moreover, the relaxatory response to NPS in our study differed between muscle strips from small intestine and colon, suggesting postjunctional receptors expressed directly on smooth muscle in small intestine, whereas the colonic control seems to be at a prejunctional site. This difference in effect could be due to the variable expression of the NPS/NPSR1 system throughout the GI tract, in which higher expression of NPS and NPSR1 is seen in the upper part as opposed to the lower part of the GI tract [195].

We also found increased expression of biomarkers iNOS, IL-1β and CXCL1 in the infusion study portion of paper III, showing an induced inflammatory response to exogenously applied NPS. This combination of inflammatory markers suggests that

NPS stimulates neutrophil infiltration into the upper GI tract, since CXCL1 is a strong neutrophil attractant [252] and these inflammatory cells are known to produce both iNOS and IL-1β [253, 254]. In this context, it has been shown that activated neutrophils can reduce the contractile response in colonic circular muscle [255], also suggesting that the inflammatory response to NPS could be involved in the changed motility pattern. Whether the motility response as showed in this study is due to NPS working directly on its receptor, a NPS-stimulated neutrophil infiltration, or both, is at this point unknown.

The results of paper IV regarding L-NMMA's effect on human MMC are in agreement with what others have shown [256, 257]. The specific effect on NO production was verified by the predicted changes seen with blood pressure, as well as breath and rectal gas concentrations. However, when atropine or ondansetron were given prior to L-NMMA, some of the effects of L-NMMA on the MMC were overridden; only the induction of phase III motility remained in all different treatment groups, showing the importance of NO in the initiation of the activity front. Transition from phase I to phase II on the other hand seems to be under a balanced control between inhibitory nitrergic and excitatory cholinergic and serotonergic pathways. This concept is confirmed by the finding that MKC-733, a 5-HT_3 receptor agonist, reduces phase I duration [258]. Further physiological knowledge on how NO regulates the MMC and motility in health is important for establishing the proposed usage of NOS inhibitors in motility disorders such as small intestinal bacterial overgrowth and gastroparesis to stimulate phase III motility.

NO's involvement in the inflammatory response is complex (Fig 14). In the physiological setting, iNOS expression is tightly regulated by both nNOS and eNOS through their production of NO, acting to suppress NFκB activity [259, 260]. However, in the early inflammatory response, the demand of NO production changes, leading to activation of NFκB and subsequently increased levels of NO. In this process nNOS seems to be downregulated in rat models of IBD [261-264], possibly as a mechanism to induce motility for eviction of the intestinal bacterial load. On the other hand, eNOS is at first activated in order to co-stimulate the iNOS induction to get high-output NO production [265]. In turn, the higher concentration of NO subsequently inhibits eNOS activity and expression [266, 267] in order to facilitate increased expression of adhesion molecules and infiltration of leukocytes to the inflamed tissue. When these immune cells are present at the inflammatory site, large quantities of NO are required in order to kill invading pathogens. These high concentrations of NO could also be the cause of the hypomotility response seen during active IBD.

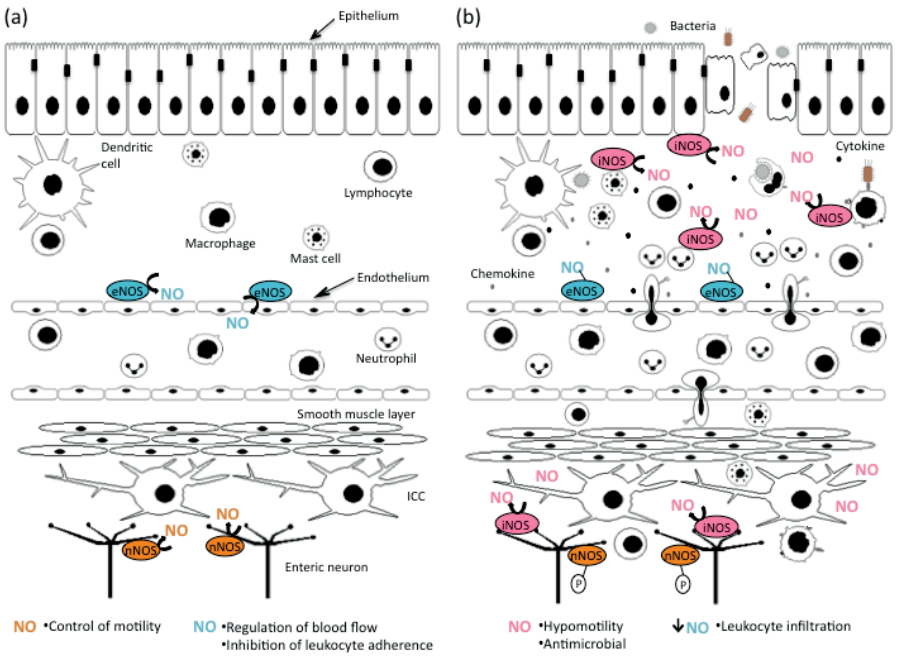

Fig 14. *Schematic picture of NO production in the GI tract in (a) the physiological setting and (b) during an inflammatory response.*

Many animal studies have implicated that iNOS is beneficial in the acute inflammatory response [170, 268, 269], but that sustained upregulation of NO production by iNOS is detrimental to the tissue [269, 270], implicating NO as part of the dysregulated immune response seen in chronic inflammation. This concept is complicated by the fact that iNOS-produced NO is involved in both cytotoxic and immunoregulatory functions (e.g. inhibition of leukocyte adherence and mast cell activation, as well as apoptosis of regulatory T cells). Apart from the fact that these effects depend on the cell type expressing iNOS; the amount of NO produced also seems vital. This concentration-dependent effect is mainly explained by NO's biphasic control on NFκB activity [271]; lower amounts of NO activates this pro-inflammatory transcription factor, leading to increased expression of cytokines and chemokines, whereas higher amounts suppresses its activity in order to resolve inflammation. This implies that the amount of NO produced in an immunoregulatory cell will determine if the main effect will be pro- or anti-inflammatory. This concept also seems to have bearing on the clinical outcome in IBD. Our group has previously shown that high rectal NO levels (above 2000 ppb) before start of treatment in relapsing IBD patients can be used as a marker for discrimination between response to steroid treatment as well as requirement of surgery. These studies show that high levels of NO correlate with patients responding to treatment and those who did not need surgery [215, 272]. This suggests that too low amounts of NO during an inflammatory response causes a more severe chronic inflammation due to constant activation of NFκB, without induction of the anti-inflammatory mechanisms involved in immune resolution. Whether or not a dysregulated eNOS expression is also involved in this setting needs to be further

evaluated, especially since UC patients seems to have increased eNOS, whereas CD patients have reduced eNOS expression [273].

Moreover, this concept suggests that high iNOS expression could be used as a marker of "healing in progress". Indeed, L-NAME given after termination of DSS treatment impairs the healing of induced lesions [274], while L-arginine supplementation after induction of DSS colitis alleviates the inflammatory reaction [275]. Furthermore, our group has also shown that rats pre-treated with a gastrin receptor antagonist or proton pump inhibitor to suppress diclofenac-induced ulcers show increased expression of iNOS, whereas diclofenac in itself did not change the iNOS expression. This demonstrated that iNOS associates with prevention of stomach ulcers, and could possibly favor healing of existing wounds (unpublished data [276]). This is supported by our finding in paper II that methotrexate treated animals had amongst the highest expression of iNOS together with a low histopathological colitis score. However, the use of increased expression of iNOS as a marker of healing would only be valid during the early resolution of inflammation, since it is also known that IBD patients as a sound response to treatment shows reduced iNOS expression together with a reduction in pro-inflammatory cytokines [215], suggesting an anti-inflammatory downregulation of NFκB.

To conclude, during an acute inflammatory reaction in the GI tract, induction of iNOS expression aids in the immune response to invading pathogens. However, in the chronic inflammatory setting iNOS expression becomes dysregulated. Indeed, IBD patients and experimental colitis models show increased iNOS expression during active colitis, with the simultaneously produced NO being involved in a multitude of effects ranging from pro-inflammatory to anti-inflammatory and immunosuppressive actions depending on the effector cell and the amount of NO. The importance of the NO signaling in inflammatory reactions in the gut is further recognized by the regulatory role changes in NOS expression has on vital immune functions, such as motility and leukocyte infiltration. Treatment regimens that aims to resolve the dysregulated NOS expression holds great promise in also resolving the chronic inflammatory reactions in the GI tract occurring due to aberrant NO production.

7 CONCLUSIONS

NO is involved in the dysregulated immune response occurring in IBD. Several NO-related genes show differential expression in CD and UC, with half of them being common to these disorders. High expression of IL-8 and ICAM-1 highlights the importance of leukocyte trafficking as a mechanism involved in perpetuating disease. Cluster analysis further pinpointed NFκB and HIF-1 as vital signaling pathways involved in the aberrant inflammatory response evolving to tissue fibrosis. Disorder-specific differentially expressed genes holds promise as basic mechanisms involved with the etiology of these diseases.

Topical treatment regimens in disease states where the inflammation is limited to the colon holds the potential of reducing severe side effects experienced with conventional IBD drugs. Local administration of the function-blocking anti-α_2 integrin antibody alleviates symptoms and histopathological signs of colitis in the acute DSS model, including downregulation of iNOS expression, showing on the importance of the $\alpha_2\beta_1$ integrin in mediating the extravascular trafficking of leukocytes in experimental colitis and provides evidence for the use of this antibody as a potential novel drug target for treatment of IBD. Topical treatment with methotrexate also showed promising results in this disease model as a new way of administering a known immunomodulator locally to the inflamed tissue.

NPS elicits a motility response in the GI tract that is similar to that seen in inflammatory settings. However, in the upper GI tract, NPS seems to act directly on smooth muscle cells, while the effect in colon seems to be regulated at a prejunctional site. Although NPS can induce the expression of some inflammatory markers, including iNOS, this study did not show on an invariable inflammatory reaction. These results suggest that an extended activation of the NPS/NPSR1 system may be involved in the induction of an inflammatory response in the gut.

NO acts as a regulatory inhibitor throughout the MMC, predominately suppressing induction of phase III activity and extending phase I duration. NOS inhibitors have the potential to be used as drugs for dysmotility disorders in order to initiate phase III activity. The inhibitory effect of NO on phase III activity was shown to be independent of muscarinic and 5-HT_3 receptor blockade, whereas the length of phase I is possibly regulated as a balance between inhibitory nitrergic and excitatory cholinergic and serotonergic pathways. Moreover, the transition from phase II to phase III activity seems dependent on muscarinic mechanisms. Further studies are needed to establish NO's regulation of the MMC in relation to other important neurotransmitters.

This thesis establishes that NO production is induced during inflammatory reactions in the GI tract, as shown by increased expression of iNOS in IBD patients, the DSS colitis model, as well as with the potentially inflammatory molecule NPS. This increase in NO is shown to be involved in the vast leukocyte infiltration to the inflamed tissue and the hypomotility response occurring in GI disorders, supporting the use of iNOS expression as an inflammatory marker of an aberrant NO production.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kväveoxid (NO) är en biologiskt verkande molekyl involverad i regleringen av flertalet fysiologiska mekanismer i kroppen såsom nervsignalering, kontraktion av glatt muskulatur samt avdödande av mikroorganismer och nedreglering av immunförsvaret. Inflammatorisk tarmsjukdom (IBD), bestående av Crohn's sjukdom (CD) och ulcerös kolit (UC), ger upphov till en kronisk inflammation i magtarmkanalen med okänd bakomliggande orsak. Dessa sjukdomar karakteriseras av en ökad mängd NO inuti tarmen, en extrem ökning av immunförsvarsceller i den inflammerade vävnaden samt förändrad tarmmotorik. Vidare regleras tarmmotoriken av flertalet signalsubstanser, såsom acetylkolin och serotonin (5-HT), genom att dessa molekyler aktiverar sina respektive receptorer (bland annat muskarina- och 5-HT_3-receptorer) som finns uttryckta i magtarmkanalen.

Denna avhandlings syfte var att undersöka hur NO är involverad i inflammatoriska reaktioner i magtarmkanalen samt hur den reglerar magtarmkanalens motorik genom att studera: (1) förändrat genuttryck av gener relaterade till NO vid IBD, (2) effektiviteten av ett potentiellt nytt läkemedel, α_2-integrin antikropp, i förhållande till konventionella IBD läkemedel i en experimentell kolitmodell, (3) huruvida neuropeptid S (NPS) har fysiologiska effekter på tarmmotorik och muskelkontraktilitet samt på uppkomsten av inflammation och (4) NOs effekter på det migrerande motorkomplexet (MMC) i samband med blockering av signaleringen via muskarina- och 5-HT_3-receptorer.

Klusteranalys av det NO-relaterade genuttrycket i CD och UC visade på att genen hypoxi-inducerbar faktor 1 (HIF-1) utgör ett nav i den process som förvärrar inflammationen i dessa sjukdomar genom att reglera både inflammationen, nybildningen av blodkärl samt bildningen av fibrös vävnad. Vidare visade interaktionsanalys på att ökat uttryck av de inflammationsstimulerande molekylerna IL-8 och ICAM-1 är av betydelse för det kraftigt ökade inflödet av immunförsvarsceller i både CD och UC.

Rektalbehandling med den funktionsblockerande antikroppen anti-α_2 visade sig lindra inflammation i tarmen genom att minska kroppsviktsnedgången, rektal blödning, inflammationsskattningen samt uttrycket av de inflammatoriska biomarkörerna inducerbart NO syntas (iNOS) och IL-1β. Behandling med det etablerade läkemedlet metotrexat gav också upphov till lindring av inflammationen i kolon, dock utan en samtidig bred minskning i uttrycket av inflammationsmarkörer. Denna studie visar på fördelarna med lokal administrering av läkemedel till den inflammerade vävnaden samt att preparat riktade mot integrin $\alpha_2\beta_1$ potentiellt kan användas som en ny behandlingsform av IBD.

Studier med NPS visar att denna molekyl förlänger längden på MMC-cykeln och fas III-aktiviteten i den övre delen av tunntarmen samt att kontraktilitetsamplituden i muskelvävnadsbitar minskas. Vidare visar resultaten på att effekten av NPS varierar längs med magtarmkanalen. NPS ger upphov till en direkt effekt på den glatta

muskulaturen i tunntarmen medan effekten i kolon framförallt verkar vara kontrollerad av en neuronal mekanism. Dessa effekter av NPS på tarmmotorik och kontraktilitet överensstämmer med förändringarna som uppstår vid inflammatoriska reaktioner i magtarmkanalen. NPS gav även upphov till en ökning i uttrycket av de inflammatoriska markörerna iNOS, IL-1β och CXCL1, vilket ytterligare talar för att NPS kan inducera ett immunsvar i magtarmkanalen.

Behandling med NOS-inhibitorn L-NMMA visade på att NO reglerar initieringen av fas III-aktivitet i MMC-cykeln. Samtidig muskarin- och 5-HT_3-receptorblockad talar för att övergången från fas I- till fas II-aktivitet sker via balanserad reglering mellan hämmande NO och stimulerande acetylkolina samt serotonina mekanismer.

Dessa studier visar att uttrycket av iNOS är ökat vid inflammatoriska reaktioner i magtarmkanalen, vilket ger upphov till en ökad produktion av NO. Detta talar för en orsaksmekanism där hög mängd NO ger upphov till den minskade tarmmotoriken som uppstår vid inflammatoriska sjukdomar i magtarmkanalen.

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