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NITRIC OXIDE:

A SURROGATE MARKER OF BOWEL INFLAMMATION

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To my Family

A good set of bowels is worth more to a man than any quantity of brains

Josh Billings (Henry Weeler Shaw), AD 1818-1885

ABSTRACT

The gas nitric oxide (NO) is a pluripotent biological messenger involved in numerous physiological and pathological processes in the gastrointestinal (GI) tract. In the intestinal mucosa NO is synthesized from the amino acid L-arginine via a reaction catalyzed by NO synthase (NOS). During inflammation, mucosal NO generation is increased and NO gas is released in the gut lumen. We have developed a procedure for direct measurement of gaseous NO in the GI tract. Employing this procedure, the present thesis was designed to evaluate further the potential usefulness of measurements of rectal levels of NO in diagnosing inflammatory bowel disease (IBD) and monitoring the response of this disease to treatment.

Altogether, 89 patients with IBD, 39 with irritable bowel syndrome (IBS) and 28 with collagenous colitis (CC), were examined. Rectal NO levels were measured employing a tonometric method using a silicon catheter equipped with an inflatable balloon and compared to clinical indices of disease activity. We also characterized the effect of systemic inhibition of NOS (by L-NMMA) on rectal levels of NO in patients with collagenous colitis to further pinpoint the source of rectal NO. Finally, we investigated the possibility that commensal gut bacteria can produce NO.

In healthy subjects rectal levels of NO were low and varied little with time. Slightly and highly elevated levels were observed in patients with IBS and IBD respectively. This parameter demonstrated a sensitivity of 95% and specificity of 91% in discriminating between IBS and active IBD. Rectal levels of NO were correlated to disease activity in patients with IBD or CC and were reduced markedly in IBD patients who responded to treatment. Surprisingly, rectal levels of NO were not correlated to fecal calprotectin levels, another marker of IBD.

Intravenous administration of L-NMMA reduced rectal NO levels in only half of the patients with CC examined, despite clear evidence of effective systemic inhibition of NOS. This could indicate the existence of alternative, NOS-independent sources of intestinal NO in this disorder. Human feces and certain isolated strains of bacteria were capable of generating NO in the presence of nitrate and/or nitrite *in vitro*. In addition, NO generation was observed in the gut lumen of conventional rats, but not of germ-free rats or rats colonized by lactobacilli. Thus, bacteria can be a significant source of NO in the gut.

We conclude that measurements of rectal levels of NO could be clinically useful as a rapid and minimally invasive procedure for discriminating between active bowel disease and IBS, as well as a possible source of useful supplemental information when monitoring the treatment of patients with IBD. Future studies will reveal the biological significance of NO generation by GI bacteria with regards to the regulation of GI integrity and the clinical usefulness of fecal calprotectin and rectal NO as surrogate markers for bowel inflammation, as well as the exact role played by these substances in the pathogenesis of this disorder.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I. **Claudia I Reinders**, Max Herulf, Tryggve Ljung, Jakob Hollenberg, Eddie Weitzberg, Jon Lundberg, Per M Hellström. Rectal mucosal nitric oxide in differentiation of inflammatory bowel disease and irritable bowel syndrome. **Clinical Gastroenterology and Hepatology** 2005;3(8):777-783.

II. **Claudia I Reinders**, Per M Hellström, Jan Björk, Eddie Weitzberg, Jon Lundberg. The effect of intravenous L-NMMA on nitric oxide production in collagenous colitis. **Scandinavian Journal of Gastroenterology** 2004;39:32-36.

III. Tanja Sobko, **Claudia I Reinders**, Emmelie Å Jansson, Elisabeth Norin, Tore Midtvedt, Jon Lundberg. Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite. **Nitric Oxide: Biology and Chemistry** 2005: E-pub ahead of print. *The first two authors contributed equally to this study.*

IV. **Claudia I Reinders**, Daisy Jonkers, Emmelie Å Jansson, Reinhold W Stockbrügger, Ellen E Stobberingh, Per M Hellström, Jon Lundberg. Nitric oxide and calprotectin in inflammatory bowel disease. **Manuscript**.

CONTENTS

LIST OF ABBREVIATIONS

1400W	[N-(3-(Aminomethyl) benzyl) acetamidine]
CC	Collagenous colitis
CD	Crohn's disease
CDAI	Crohn's disease activity index
cNOS	Constitutive nitric oxide synthase
EC	Enteroendocrine cells
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
GC	Guanylyl cyclase
GI	Gastrointestinal
HBI	Harvey Bradshaw Index
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
iNOS	Inducible nitric oxide synthase
L-NAME	N^G-Nitro-L-arginine methylester
L-NIL	L-N^6-(1-Iminoethyl)-lysine
L-NMMA	N^G-Methyl-L-arginine
L-NNA	N^G-Nitro-L-arginine
N_2	Nitrogen
NF-κB	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_2^-	Nitrite
NO_2^*	Nitrogen dioxide in the excited state
NO_3^-	Nitrate
NOS	Nitric oxide synthase
O_2	Oxygen
O_2^-	Superoxide
O_3	Ozone
$ONOO^-$	Peroxynitrite
PI-IBS	Post-infectious IBS
ppb	Parts per billion
ppm	Parts per million
STAT	Signal transducer and activator of transcription
UC	Ulcerative colitis

INTRODUCTION

Nitric oxide (NO), one of the smallest and simplest biologically active molecules present in nature, and once considered to be merely a potentially toxic environmental pollutant, is now recognized to act as a universal messenger throughout the human body. For example, in the small and large intestines NO is involved in regulating numerous physiological events, including maintenance of a rate of blood flow adequate to match the demands required for digestion, tissue oxygenation and mucosal integrity. Furthermore, NO plays a role in the regulation of fluid and electrolyte transport, motility and immune responses in the gastrointestinal (GI) tract.

The groups of disorders referred to as inflammatory bowel disease (IBD) are characterized by chronic inflammation of the GI tract, with acute episodes interspersed with periods of spontaneous or treatment-induced remission. During inflammation the level of NO in the intestinal lumen is elevated. The investigations described in this thesis were designed to further evaluate the potential usefulness of NO measurement for diagnosis and therapeutic monitoring of inflammatory conditions in the intestine. In addition, we examined alternative sources of rectal NO.

NITRIC OXIDE

In 1980, the seminal discovery that endothelial cells lining blood vessels produce a factor (referred to as endothelium-derived relaxing factor, EDRF) required for relaxation of smooth muscle and vasodilatation was reported.[42] Seven years later, two research teams[61,106] demonstrated independently that EDRF is, in fact, NO. Since then, the number of scientific publications dealing with NO has increased explosively. In 1992, NO was declared to be "the molecule of the year" by the journal Science, and in 1998 Furchgott, Ignarro and Murad, the investigators responsible for the discovery that NO is a signaling molecule involved in cardiovascular function, were awarded the Nobel Prize in Physiology or Medicine. That is the story on how a tiny molecule became a big celebrity.

Formation

At high temperatures molecular nitrogen (N_2) and oxygen (O_2) can combine to form NO. This process occurs, for example, in connection with bolts of lightning and in the combustion chambers in cars. Of course, the human body produces NO in a different fashion.

Enzymatic synthesis

NO is derived enzymatically from the guanidine group of the amino acid L-arginine via an oxidation reaction catalyzed by a family of enzymes referred to as NO synthases (NOSs) (Fig. 1). This reaction occurs in virtually all mammalian cells. Of the three isoforms of NO synthase identified to date, two are constantly expressed in, e.g., neuronal (nNOS) or endothelial (eNOS) tissue and are therefore designated as

constitutive NOS (cNOS); while the third isoform is expressed in response to certain cytokines, microbes or bacterial products and is thus called inducible NOS (iNOS).[73,75]

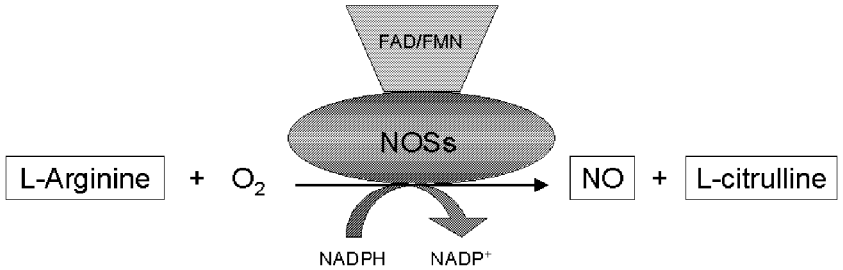


Figure 1: Enzymatic formation of NO and citrulline from the amino acid L-arginine and O_2 in a nicotinamide adenine ainucleotide phosphate (NADPH)-dependent reaction catalyzed by the enzyme NOS. Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are important co-factors in this reaction.

The production of NO by cNOS is at a low level (pico- to nanomolar quantities), short-lasting and under the transient and strict control of agents that mobilize calcium. In marked contrast, transcriptionally regulated iNOS synthesizes NO in high (micromolar) amounts.[29] Up-regulation of iNOS requires several hours following stimulation, but once this has occurred, the levels of this enzyme and of NO can remain elevated for as long as 5 days.[138]

Investigations on enzymatic production of NO and on subsequent inhibition of this synthesis by endogenous inhibitors have played a central role in helping us to understand the biological functions of NO. Most endogenous inhibitors of NOS are arginine analogues, e.g., L-NNA, L-NMMA and the more potent L-NAME, and exhibit no selectivity for any particular isoform.[124] Aminoguandine has been proposed to be a selective inhibitor of iNOS, but its mechanism of action is not yet fully understood.[102] L-NIL and 1400W are highly selective inhibitors of iNOS that have been developed relatively recently.[43,103] Furthermore, glucocorticosteroids are able to partially inhibit the expression of iNOS, probably through inhibition of nuclear factor kappa B (NF-κB).[71]

Non-enzymatic formation

In the human body NO can also be formed by reduction of nitrite in an acidic environment, for example in the stomach. Such formation was first described independently in 1994 by both Benjamin and co-workers and our research group.[9,88] Nitrate excreted in saliva is rapidly converted to nitrite by facultative anaerobic bacteria on the surface of the tongue.[33] At low gastric pH, this nitrite is converted to nitrous acid, which rapidly decomposes to produce NO (and other nitrogen oxides) at a level of parts-per-million (ppm).[9,88]

Bacterial production

Anaerobic denitrifying bacteria present in, e.g., soil and sediment, also generate NO, but this process involves pathways that differ from those occurring in eukaryotic cells. In such bacteria, NO is an intermediate in the nitrogen cycle, which reduces nitrate (NO_3^-) in a step-wise fashion to N_2.[87] The enzyme in this pathway that is responsible for generation of NO is a nitrite (NO_2^-) reductase. Further reduction of NO to nitrous oxide by NO-reductase prevents the accumulation of potentially toxic levels of NO in the vicinity of the bacteria.[160] The human GI tract is colonized by a massive number of different bacteria, but little is presently known concerning the ability of these commensal bacteria to produce NO.

The chemistry of NO

The chemistry of NO is complex. With a molecular weight of only 30 Dalton, NO is one of the smallest biological messengers. It is a colorless gas at room temperature and atmospheric pressure and diffuses easily across biological membranes because of its uncharged and lipophilic nature. The solubility of NO in lipids is 9-fold greater than in aqueous solutions indicating that this molecule may accumulate preferentially in lipid-rich environments such as the bilayer of cellular membranes.[21,92] When bubbled into a physiological buffer, most of the NO appears immediately in the gas phase above this solution.[21] The presence of an unpaired electron makes NO a free radical that is reactive and unstable and has a half-life of only 1-5 seconds in most biological systems.[84,142] Despite this reactivity, under normal physiological conditions NO reacts almost exclusively with other radicals, oxygen-driven species such as molecular O_2 and superoxide (O_2^-), transition metals and metal-containing proteins.

It is generally accepted that inactivation of NO occurs via oxidation. At high concentrations, gaseous NO can react with O_2 to form the poisonous gas nitrogen dioxide (NO_2), called 'brown gas', i.e., NO is unstable in air. However, these reactions involve second-order kinetics, so, the rate of NO oxidation is proportional to its concentration squared, rendering this oxidation much slower at low concentrations of NO. Consequently, gaseous NO at biologically relevant concentrations (10-10000 parts per billion (ppb)) is relatively stable in ambient air containing 21% O_2.[13]

In aqueous solutions NO is less stable, its half-life in such environment being dependent on the partial pressure of O_2. In this case NO is converted primarily to NO_2^-, which may be subsequently oxidized further to form NO_3^-.[60] In the human body the half-life of NO is reduced further by scavenger proteins and by reaction with transition metals, e.g., the iron in oxyhemoglobin to form NO_3^- and methaemoglobin.[32,47] Considering the large amounts of oxyhemoglobin present in the body, this reaction may be the primary determinant of the movement and concentrations of NO *in vivo*.[60]

O_2^- formed via one-electron reduction of O_2 as a by-product of normal aerobic metabolism, can react with NO to form peroxynitrite ($ONOO^-$). $ONOO^-$ is much more reactive than O_2^- or NO and can decompose to highly reactive radicals capable of oxidation and degradation of various cellular components.[7] Fortunately, eukaryotic cells contain high levels of superoxide dismutase, which maintains the steady-state concentration of O_2^- remarkably low.[8]

NO in the GI tract

Expressed simplistically, the major function of the GI tract is to absorb nutrients and eliminate waste. In order to do this, the GI tract must translocate ingested food from the mouth all the way to the rectum, secrete fluids, digest the food to release and absorb nutrients, and eliminate the rest-products. NO may play critical roles in all these processes (Fig. 2).[29]

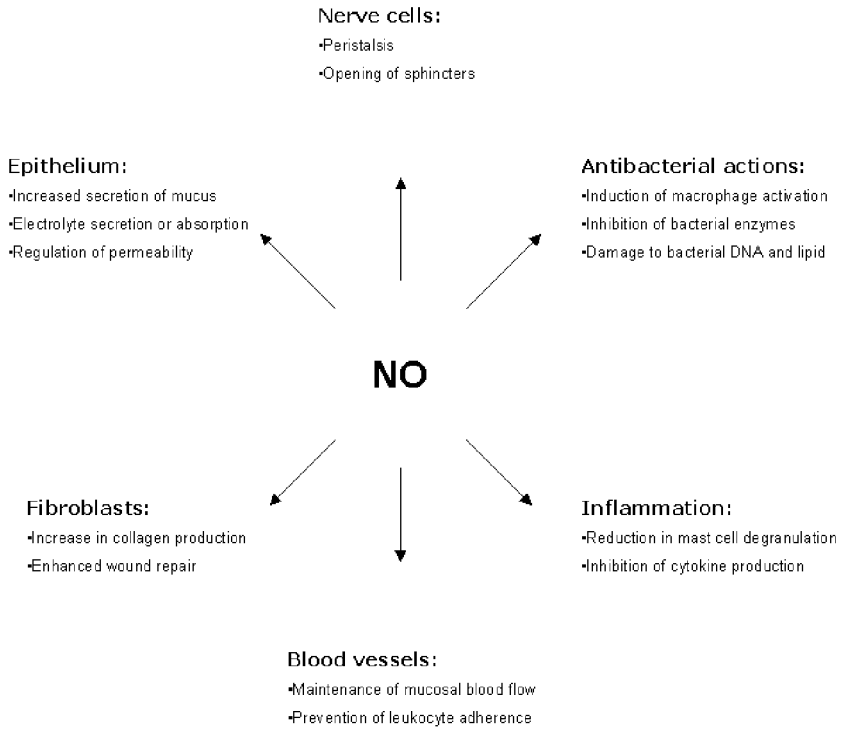


Figure 2: Some functions of nitric oxide in the gastrointestinal tract

NO exerts many of its physiological effects by binding to a specialized heme group in receptors coupled to guanylyl cyclase (GC), thereby triggering conformational changes in GC and activating this enzyme. GC converts GTP to cyclic GMP, which subsequently cause modifications in various cell functions by influencing a number of downstream targets, including protein kinases, phosphodiesterases and ion channels.[12]

Movement of foodstuffs and elimination of waste

Neurons in the central and peripheral nervous systems have the capacity to produce significant quantities of NO via nNOS. Moreover, the motility of the GI tract is controlled directly by enteric inhibitory and excitatory motor neurons that innervate the smooth muscle layers. NO is now known to be the principle inhibitory neurotransmitter that mediates non-adrenergic non-cholinergic relaxation of smooth muscles in the gut.[18] The role of NO synthesized by nNOS in regulating peristalsis and sphincter function of the intestine is apparent from characterization of knock-out mouse strains lacking

nNOS. Also, it is supported by the observation that impaired NO release is associated with disease characterized by lack of relaxation of the sphincter or bowel segments.[29,65]

Absorption and secretion

NO participated in intestinal transport of water either directly by influencing the epithelium and blood flow or indirectly by stimulating neuronal reflexes. The elevated levels of cGMP generated as a consequence of stimulation of GC by NO is a potent activator of intestinal secretion.[16] In addition, NO up-regulates the level of expression of vasoactive intestinal polypeptide (an important neurotransmitter in secretomotor neurons) and increases the production of prostaglandin E2, a known secretory molecule.[3,156] Furthermore, NO also exerts a direct secretory effect by opening chloride channels.[141]

NO is also able to promote absorption, but the mechanisms underlying this action have not yet been fully elucidated.[129] Thus, depending on its concentration and the site of delivery and prevailing conditions, NO can apparently regulate both secretion and absorption in the GI tract.

The gastrointestinal barrier function

The GI mucosa forms a barrier that protects against damage. This barrier is composed of a layer of intestinal epithelial cells that turn-over rapidly, non-specific agents such as lysozyme (an enzyme that can destroy the cell walls of certain bacteria), acid and mucus. NO plays an important role in maintaining the integrity of this barrier.

An adequate supply of blood is vital to the integrity of the GI mucosa and NO (produced by eNOS) dilates mucosal blood vessels via activation of GC. NO is also an important regulator of the mucus secretion, especially by the epithelial cells in the stomach, again via the stimulation of GC.[151] Investigations in which segments of the intestine were exposed to inhibitors of NOS indicate that NO also participates in maintaining the barrier and regulating the permeability of the gut by helping to maintain a tight and intact mucosal barrier.[29,77] However, the mechanism by which NO influences epithelial permeability remains unknown. It is well known that NO relaxes smooth muscles (see above) and it is possible that similar effects on contractile proteins within the epithelial cells that regulate intercellular tight junctions can account for the effects of NO on intestinal permeability.

Mucosal defenses

NO exerts a profound influence on the functions of mesenchymal cells, enhancing collagen deposit by fibroblasts and stimulating endothelial cells to perform angiogenesis and thereby affecting the ability of the GI mucosa to resist injury and undergo repair.[151] Moreover, NO has potent effects on immune cells within the lamina propria of the GI tract, which also has an impact on the resistance of the mucosa to injury. One such example involves mast cells, which coordinate the inflammatory response by releasing numerous chemical signals (primarily cytokines) in response to antigens, bacterial products and various other factors. Mast cells have been shown to release NO and the rate of this production can be enhanced by cytokines. Interestingly, the NO released by mast cells appears to attenuate the release of numerous other

inflammatory mediators from these cells.[55,125] In addition, NO reduces mast cell degranulation and increases leukocyte adherence processes, which further protect the mucosa.[67,78,151]

Moreover, NO can inhibit the production of various immunomodulatory cytokines by macrophages, as well as modulate the action of macrophage-derived cytokines on their target cells.[40,59,104,139] One of the most important functions of GI macrophages is to kill and remove bacteria that have penetrated the epithelium and this function is dependent on NO as well. *In vitro* studies indicate that NO itself is not very toxic, but that formation of $ONOO^-$ is responsible for this bactericidal activity.[17]

INFLAMMATORY BOWEL DISEASE AND IRRITABLE BOWEL SYNDROME

Considering the numerous functions of NO in the GI tract and the cytotoxic potential of NO-derived species, it is not surprising that NO has been implicated in the pathogenesis of numerous GI diseases, some of which will be highlighted here.

IBD is the collective name for a group of disorders of the GI tract of unknown etiology, characterized by intestinal inflammation and chronic relapses associated with local and systemic complications.[19,112] Traditionally, IBD is divided into two main categories: ulcerative colitis (UC) and Crohn's disease (CD). The symptoms commonly associated with UC and CD are diarrhea, usually involving discharge of blood and mucus, and abdominal pain. In more severe cases, signs of systemic inflammation such as fever, malaise and weight loss may also be present.

At the same time, these two different categories of disease differ in several respects. In the case of CD, the entire GI tract, from the oral cavity to the anus, can be affected (Fig. 3), but, most commonly, only the terminal ileum, ceacum and colon are invloved.[15] The inflammation is distributed unevenly ('skip lesions') and occurs in all layers of the intestinal wall (transmural).[15] In Sweden the annual incidence of CD varies between 4.5-6.7/100 000, with an overall prevalence of 117/100 000.[35,79,83]

In contrast, the inflammation associated with UC is, by definition, restricted to the mucosa of the colon. Typically, this inflammation is distributed continuously throughout the rectum and, to a variable extent, the colon in the posterior direction (Fig. 3). The annual incidence of UC in Sweden is approximately twice that of CD, i.e., 12-13/100 000 with an overall prevalence of 235/100 000 inhabitants.[35,149] When inflammation is present only in the rectum and colon of a patient, it can be impossible to discriminate between UC and CD, which has led to the diagnosis of indeterminate colitis. Approximately 10% of the patients suffering from colonic IBD receive this diagnosis.[135]

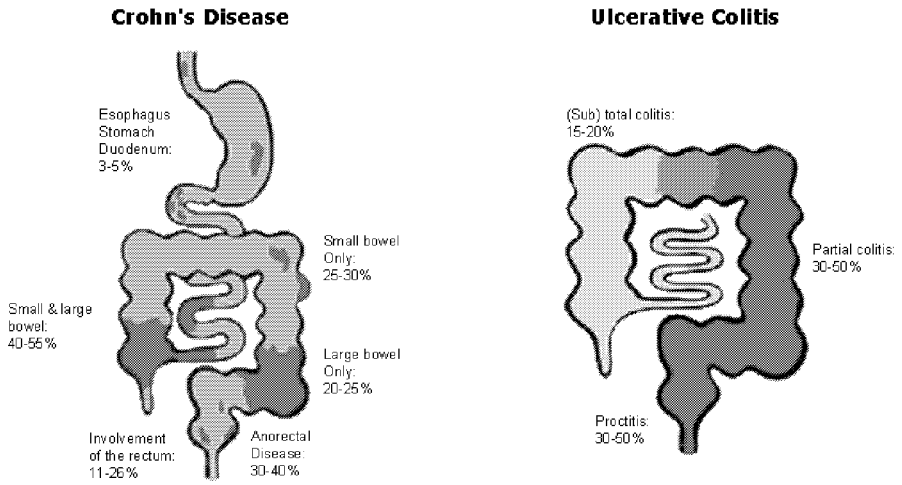


Figure 3: Schematic drawings showing which parts of the gastrointestinal tract that are affected in Crohn's disease and ulcerative colitis.

Microscopic colitis, a term that includes lymphocytic colitis and collagenous colitis (CC), is characterized clinically by chronic, non-bloody and watery diarrhea and by a macroscopically normal or almost normal colonic mucosa in which characteristic histopathological abnormalities can be found.[41,81] These disorders are sometimes grouped together with inflammatory bowel disease, since histopathological evidence of infiltration of inflammatory cells into the colonic mucosa is present. However, in the present discussion, microscopic colitis will be considered as a distinct entity.

Irritable bowel syndrome (IBS), a chronic disorder associated with a variety of symptoms, is characterized by abdominal pain and alterations in bowel habits without apparent organic disease.[24] This is one of the most frequent causes of chronic diarrhea in adult patients and the most common diagnosis assigned to out-patients by gastroenterologists, despite the fact that this condition remains seriously under-diagnosed.[86] The Rome II criteria[143] are commonly used to diagnose IBS, but clinical differentiation between IBS and IBD remains problematic, since these diseases have many symptoms in common.

A small, relatively homogenous subgroup of patients describe an acute onset of symptoms following an episode of gastroenteritis, to which their bowel habits have been entirely regular. These patients are classified as suffering from post-infectious IBS (PI-IBS). Accordingly, PI-IBS is now defined as an acute onset of persistent symptoms conforming to the Rome II criteria following an episode of gastroenteritis associated with 2 or more of the following symptoms: fever, vomiting, diarrhea or the presence of pathogenic bacteria in a stool culture.[143]

Diagnosis and assessment of disease activity

Because IBD and IBS share similar symptoms, including abdominal pain and alterations in bowel habits, distinguishing between these two entities clinically can be problematic. Many IBS patients are examined extensively with invasive, unpleasant

expensive radiographic and endoscopic imaging procedures with biopsy sampling.[147] Thus, in the clinical setting it would be of great value to develop a reliable and feasible procedure distinguishing between a disorder of inflammatory nature (e.g., IBD) and disorders presumed to be of non-inflammatory origin, such as IBS.

The different pathophysiologies underlying IBS and IBD require different treatments. Treatment of IBS, a functional disorder, aims at relief of the symptoms. In contrast, medical management of IBD aims at ameliorating or eliminating underlying inflammation, although assessment of the degree of inflammatory activity remains a difficult challenge. Endoscopy with biopsy sampling is currently the ″gold standard″ for such assessment. Various serological and haematological parameters are also employed in clinical practice, but the correlation between these values and mucosal inflammation is poor.[20]

Moreover, clinical indices of disease activity are widely used to assess the response to treatment, an approach which originated from the search for indicators of improvement in connection with clinical trials. The first to describe such a disease index for UC were Truelove and his colleague Witts who, in 1955, developed a three-grade scale that is still in use today.[148] Later, several other numerical indices for UC have been developed, including the Powell-Tuck index in 1979[115] and the Disease Activity Index formulated by Sutherland in 1987[140].

In 1976, the CD activity index (CDAI) was developed on the basis of the American National Cooperative CD study.[10,11] Harvey and Bradshaw[52] later demonstrated that a simplified version of the CDAI, which they called the Harvey Bradshaw Index (HBI), accurately reflects the clinical activity of CD. In 1994, Lichtiger[82] developed a disease activity scale for UC similar to the HBI (index of Lichtiger), which made it easier to perform statistical comparisons of the disease activity in UC and CD patients in connection with clinical studies.

In general, these indices of clinical disease activity reflect and correlate reasonably well with the clinical assessment and the patient's feeling of well-being. All these indices are however, based largely on subjective factors and may therefore be easily biased by non-inflammatory processes.

Markers of inflammation

In order to overcome the subjectivity of clinical indices and to make the assessment of disease activity more reliable, fecal markers were developed. These markers consist of a heterogeneous group of substances that are generated by and/or leak from the inflamed intestinal mucosa. In addition to their usefulness in diagnosing and assessing disease activity, such markers may also be helpful in monitoring the effect of treatment and in predicting relapses.

The ideal marker should be accessible by a non-invasive procedure, give reproducible results, and be sensitive and specific. Thus, the levels of the marker in normal individuals and subjects with active and inactive inflammatory bowel disease should be clearly different and sample acquisition should be straightforward.[113] Excretion of leukocytes labeled with ^{111}indium correlates well with histopathological indices of inflammation and is currently the ″gold standard″ among fecal markers of inflammation.[128,154] Unfortunately, this approach is cumbersome, costly and time-consuming.

Several serological parameters have been evaluated including the level of C-reactive protein, rate of erythrocyte sedimentation, levels of anti-*Sacchromyces cerevisiae* antibodies and peri-nuclear antineutrophil cytoplasmic antibody. Disappointingly, none of these tests have lived up to expectations.[126,155] Another potential stool marker of inflammation described recently is calprotectin. First reported on by Fagerhol et al.[39], calprotectin is a calcium- and zinc-binding protein present in the cytoplasm of neutrophils and in the membrane of monocytes. By competing for zinc, calprotectin inhibits zinc-dependent enzymes and matrix metalloproteinases, which play important roles in angiogenesis, wound healing, inflammation, and induction of apoptosis in both normal and malignant cells. In this manner calprotectin is capable of regulating many important processes in the body.[137]

Upon activation of neutrophils or adhesion of monocytes to endothelial cells, calprotectin is released and can be detected in the serum and body fluids [137] Fecal levels of calprotectin are currently being assessed as a potentially useful clinical screening test for intestinal inflammation.[38] This test can be performed on a very small amount of stool sample that can be sent to the laboratory by regular mail, due to the high stability of calprotectin in feces.[114,123] Furthermore, this diagnostic test exhibits a high degree of sensitivity and specificity for IBD.

In the case of lactoferrin, an iron-binding neutrophil protein and another interesting potential marker of inflammations present in stool, less information is available regarding its stability. Most determinations of stool levels of lactoferrin have been performed in relationship to infectious GI disorders, but some investigations with regards to IBD and IBS have also been carried out. Stool levels of lactoferrin appear to allow differentiation between inflammatory and non-inflammatory colonic disorders, but cannot be employed to distinguish active from inactive disease in a reliable manner. Further studies are necessary to evaluate the usefulness of this marker in a clinical setting.

NO AND INFLAMMATION OF THE GI TRACT

As early as 1986 Roediger et al. reported high levels of NO_2^- in rectal dialysates from patients with IBD, but at that time the importance of endogenous NO was not yet known. In the early 1990's, various studies involving both animal models and humans indicated that NO may be involved in GI inflammation.[99]

Production of NO by the GI tract can be monitored by direct measurement of luminal NO, measurement of mucosal NO using electrodes, and/or quantitation of metabolites of NO. Our research group, as well as others, has demonstrated that patients with GI inflammation exhibit high luminal levels of gaseous NO. Direct measurements of mucosal NO production by Japanese investigators, employing NO sensitive electrodes introduced through the biopsy channel of a colonoscope, revealed elevated production in patients with UC compared to healthy controls.[62,116] Furthermore, monitoring of the formation of L-citrulline, the other product of the reaction catalyzed by NO synthases, also indicated the involvement of NO in GI inflammation.[14,99]

Thus, it appears that the level of intestinal NO is elevated during intestinal inflammation. However, what is the normal level of NO in healthy humans, and how much does this level vary over time?

iNOS and gastrointestinal inflammation

The most likely source of the elevated levels of NO associated with GI inflammation is thought to be iNOS. Indeed, expression of the iNOS protein has been shown to be increased in circulating monocytes, on the surface of epithelial cells and within the infiltrate in the lamina propria of UC and CD patients.[30,45,80] Apparently, the expression of cNOS is also altered in connection with UC; nNOS expression in the muscularis mucosae was shown to be decreased, while expression of eNOS within the lamina propria was increased in these patients.[74] In healthy subjects, iNOS is expressed within the colonic epithelium of the normal mucosa.[132]

The pathways regulating iNOS expression appears to vary in different cells and species. In general, activation of the transcription factors NF-κB and STAT-1α, and subsequent activation of the iNOS promoter seems to be essential steps in regulation of iNOS expression in most cells.[72] These transcription factors can be activated by various stimuli, including microbial products, pro-inflammatory cytokines, and physical or chemical stress.[64]

It is reasonable to assume that the up-regulation of NO production associated with IBD has some functional significance. Initial efforts to unravel the role played by NO in intestinal inflammation involved non-specific inhibitors of NOS. In animals in which acute intestinal inflammation is induced chemically, both non-selective inhibition of NOS and selective inhibition of iNOS ameliorate the signs of inflammation.[49,56,100,101,117,118]

In all of these studies the NOS inhibitor was administered prior to induction of the acute inflammation. Subsequently, Kiss et al.[70] demonstrated that the time-point of NOS inhibition influences the outcome of the colitis induced. When administrated prior to the inflammatory agent, L-NAME aggravated the colitis, whereas administration 6 hours after the inducer of inflammation attenuated the colitis.[70] In addition, recent studies have also revealed beneficial effects of 1400W and L-NIL, more selective inhibitors of iNOS, on rat models of colitis.[5,66,98,159]

Despite the seemingly compelling evidence that induction of iNOS has a deleterious effect on acute colitis, at least in animal models, contradictory findings have also been reported. There are numerous reports of beneficial effects of NO on experimental IBD, as indicated by aggravation of the symptoms by NOS inhibitors or amelioration by NO donors.[31,58,70,157] The findings with iNOS$^{-/-}$ knock-out mice are also contradictory and appear to depend on the chemical used to induce acute colitis.[57,76] Moreover, in IL-10-deficient mice, which spontaneously develop intestinal inflammation, deletion of the iNOS gene had no effect on the inflammation.[95]

In attempting to explain these discrepancies, it is important to consider the potential differences between species, strains and animal models, as well as the differences in experimental procedures. Thus, the time-point of administration of a substance may have a crucial influence on the results obtained, as shown by Kiss et al.[70] For instance, inhibition of NOS prior to induction of iNOS by the colitis-inducing

agent could result in selective inhibition of cNOS and thus decrease the protective effects of this enzyme. It is also important to understand that the role of NO within a given tissue may differ, depending on the cell type present and local concentration.[28] These findings illustrate clearly the complex biology and diverse roles played by NO.

Thus, NO appears to influence virtually every aspect of the mucosal defenses, it can be protective, as well as cytotoxic, possessing both anti- as well as pro-inflammatory properties.[108] In light of these dual roles of NO and the abundant presence of this messenger throughout the human body in both sickness and health, it could be questioned whether this little radical could ever be a useful marker for intestinal inflammation.

AIMS OF THE STUDY

The overall aim of this work has been to further evaluate the potential usefulness of measurements of rectal NO in diagnosing and therapeutic monitoring of inflammatory conditions in the bowel, as well as to examine the source of NO in the GI tract. More specifically, our goals have been:

- to determine the level of rectal NO in healthy individuals and the variations over time
- to evaluate the usefulness of rectal levels of NO in differentiating between IBD and IBS
- to assess NO as a marker for treatment efficacy in patients with active IBD
- to examine the effects of inhibition of NOS on NO production in connection with collagenous colitis
- to determine whether GI bacteria represent a potential source of rectal NO
- to compare the levels of rectal NO and fecal calprotectin in patients with IBD

METHODOLOGICAL CONSIDERATIONS

In this section I will present an overview of the most important methods used throughout this project. For more detailed information, please see the relevant articles. Permission to perform all of these studies was obtained from the regional ethics committee and all subjects gave their informed consent prior to entering the studies.

STUDY SUBJECTS

Diagnosis of all the patients with IBD (89 with UC and 49 with CD) or with CC (n=28) included in these studies (**Papers I, II and IV**) was based on previous endoscopic and microscopic examinations. The patients with IBS (n=39, **Paper I**) were diagnosed according to the Rome II criteria.[143] A total of 59 healthy volunteers, 36 from Sweden and 23 from the Netherlands, also participated. We examined the effect of an NOS inhibitor (L-NMMA) on CC patients, since these individuals displayed the highest levels of rectal NO during active disease and the precise source of their intestinal NO has not yet been identified. In addition, despite the inconvenience and social restrictions caused by frequent eliminations of loose stools (sometimes more than 20 times a day) during active periods the disease is considered benign and almost never life-threatening.

ASSESSMENT OF DISEASE ACTIVITY

For assessment of disease activity in patients with CD, we employed either the HBI (**Paper I**) or a modification of this index (**Paper IV**). For patients with UC, the Truelove-Witts index was used in **Paper I** and the van Lichtiger index in **Paper IV**. The disease activity of patients with CC was graded on the basis of the number of diarrheic bowel movements per day (**Papers I and IV**).

The factors on which the HBI[52] is based are presented in Table 1. In **Paper IV** the number of points for loose stools each day (normally one point per stool) was limited to 5, thereby affecting the maximum score. A score of > 4 was considered to reflect active disease in **Paper I**, while a score of > 3 on the modified index was considered to be active disease in **Paper IV**.

The Truelove-Witts criteria include the following parameters: number of bowel movements each day, presence of blood in the stool, body temperature, heart rate and hemoglobin concentration.[148] Patients with active disease are classified as experiencing a mild, moderate or severe flare-up.

The components of the Lichtiger score are shown in Table 2,[82] with a score of > 3 being considered as active disease. The Lichtiger score was used in **Paper IV** to comparisons between patients with UC and CD.

Patients with CC were considered to be in remission when they did not experience any loose stools. Once active, the disease was classified as exhibiting a mild (< 4 stools/day), moderate (4-5 stools/day) or severe (≥ 6 stools/day) flare-up.

Table 1: Components of the Modified Harvey Bradshaw Score[82]

Category	Variable	Points
General well-being	Very good	0
	Good	1
	Moderated	2
	Bad	3
	Very bad	4
Abdominal pain	None	0
	Mild	1
	Moderate	2
	Severe	3
Number of loose stools a day	0-1	0
	2-3	1
	4-5	2
	6-7	3
	8-9	4
	10 +	5
Complications		
	Arthralgias	1
	Uveitis/iritis	1
	E. nodosum	1
	Aphthous ulcer	1
	Pyroderma gangrenosum	1
	Anal fissur	1
	Draining fistula	1
	Abscess	1
Abdominal mass		
	None	0
	Dubious	1
	Definite	2
	Definite and tender	3
Max. Total		23

Table 2: Components of the Lichtiger Score[82]

Category	Variable	Points
General well-being	Excellent	0
	Very good	1
	Good	2
	Moderate	3
	Bad	4
	Very bad	5
Abdominal pain	None	0
	Mild	1
	Moderate	2
	Severe	3
Number of loose stools a day	0-2	0
	3-4	1
	5-6	2
	7-9	3
	10+	4
Visible blood in stool	None	0
	<50%	1
	≥50%	2
	100%	3
Nocturnal diarrhea	No	0
	Yes	1
Fecal Incontinence	No	0
	Yes	1
Use of anti-diarrhea agents	No	0
	Yes	1
Abdominal tenderness	None	0
	Mild and localized	1
	Moderate/diffuse	2
	Severe or rebound	3
Max. Total		21

CHEMILUMINESCENCE ASSAY FOR NO

In this work we have employed three different chemiluminescence analyzers for measuring gaseous NO, two from Aerocrine AB (Stockholm, Sweden) and one from Eco Physics (Dürnten, Switzerland). In **Papers I, II** and **III** the exhaled breath analyzer model 1 (EBA 1®) from Aerocrine AB was used, whiles in **Paper IV** a NIOX® from Aerocrine AB and a CLD 700® from Eco Physics were used.

Measurement of gaseous NO by chemiluminescence is based on the reaction of this molecule with an excess of ozone (O_3) to produce NO_2, some of which is in the excited state (NO_2^*). When NO_2^* returns to its ground state, the excess energy is released as a photon and light (luminescence) with wavelengths of 640-3000 nm is emitted. This light is quantitated by a photosensitive surface and the signal subsequently amplified through a cascade of electron releases in a photomultiplier. The amount of light produced is directly proportional to the concentration of NO and the intensity of the luminescence can be converted into an electrical signal and displayed as NO levels, with a response time of less than 0.7 seconds. This chemiluminescence assay is extremely sensitive, with a detection limit of 1 ppb, and exhibits a linear response to NO concentrations between 1-100 000 ppb. Furthermore, the assay is highly specific for NO, without interference from other nitrogen oxides.[4]

All of the analyzers used were calibrated with the same concentration of NO gas for measurements of rectal and nasal NO (10 000 ppb) or of exhaled NO (100 ppb). The differences in these chemiluminescence analyzers lies in the sensitivity of the output signal, the volume of gas required to perform the measurement and manner in which the concentration of NO is displayed. The EBA 1® NO analyzer is most sensitive and requiring the smallest sample of gas (10 ml of air) to provide a correct

measurement, but the concentration of NO must be recorded manually. The NIOX® NO analyzer required 30 ml of gas, so in this case the original rectal sample was diluted 3-fold with NO-free air to obtain a total volume of 30 ml. With this analyzer the NO concentration is displayed as the result of a regression analysis of the last 80-90% of the gas sample, i.e., when the chemiluminescence reaction is most constant. The CLD 700® NO analyzer requires the largest volume of gas (50 ml) and displays the NO concentration in 0.00 ppm. Thus, the lowest concentration of NO measurable with this chemiluminescence is 50 ppb

SAMPLING OF RECTAL NO

Diffusion of NO provides the basis for rectal sampling with a balloon catheter (Fig. 4_A). The catheter is composed entirely of silicone in order to minimize allergic reactions and the method has been described in detail elsewhere.[54] Briefly, the catheter (Argyle, Sherwood Medical, Ireland) was inserted into the rectum using a lubrication gel free from anesthetics to a level 15 cm above the anal sphincter. This balloon was then inflated with 10 ml NO-free air and left to equilibrate with gases in the rectum for 10 minutes. The gas sample was subsequently withdrawn from the balloon and injected into a rapid-response chemiluminescence system (Aerocrine AB, Stockholm Sweden) to determine the NO concentration. Depending on the chemiluminescence detection system employed, this sample was diluted 3- or 5-fold (**Paper IV**), or not at all (**Papers I and II**) before injection.

A B

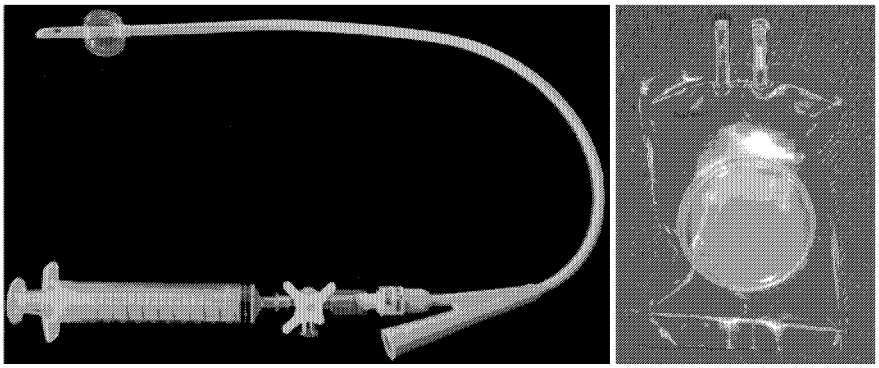


Figure 4: **A:** The balloon-tipped catheter, connected to a syringe, employed for sampling of rectal nitric oxide.
B: The procedure for sampling of bacterial nitric oxide generation. The agar plate was inserted into a gas-tight infusion bag, which was sealed prior to inflation with 300 ml of air.

SAMPLING OF BACTERIAL NO

For the investigation documented in **Paper III** we developed a new procedure in order to be able to measure gaseous NO produced either by individual strains of bacteria or by the mixed fecal flora (Fig. 4_B). This procedure involved 500-ml, gas-tight infusion bags, made of multilayer double-wound film (M312), together with two flexible co-extruded tubes (M916) of Cryovac (Infubags®, S.E. Nündel Kunststoff-Technik GmbH, Germany), into which the agar plates of interest were inserted, together with an anaerobic pouch system and anaerobic indicator (AnaeroGen™ compact AN0020C,

and BR0055, Oxoid, Basingstoke, England) whenever anaerobic conditions were desired. Some of these agar plates were supplemented with 0.1 mM sodium nitrite ($NaNO_2$) or sodium nitrate ($NaNO_3$).

Following inoculation of the agar plates with a defined amount of bacterial suspension and insertion of all the necessary items, the bags were immediately closed with an impulse sealer and filled with 300 ml NO-free air. After 1, 6, 18 and 24 hours of incubation at 37° C, 10 ml of gas was removed from each bag at each time-point. The NO concentrations of these samples were immediately measured with a rapid-response chemiluminescence system (Aerocrine AB, Stockholm, Sweden), as described above.

SAMPLING OF EXHALED AND NASAL NO

In the study described in **Paper II** we measured the levels of NO in the respiratory tract according to the recommendations of the ATS and the European Respiratory Society Task Force Report.[1,68] Thus, NO exhaled orally was quantified employing an on-line, single-breath procedure. After inhalation, the subjects were asked to exhale against a resistance into the EBA at a target flow rate at 50 ml/s for a period of 10 seconds.

For determination of nasal NO, air was aspirated from one nostril at a constant flow rate of 2.0 L/min using a pump connected to a nasal olive. The sampling tube of the NO analyzer (aspiration flow of 0.1 L/min) was connected to a side-arm of this pump. The subjects were asked to inhale via the nose and then to hold their breath with their mouth closed. The nasal olive containing a central lumen was that then placed securely in one nostril and nasal sampling continued for 15 seconds, when a plateau was reached. The nasal NO levels obtained were corrected for ambient levels of this gas.

ANALYSIS OF NITRITE, NITRATE AND CALPROTECTIN

In the studies reported on in **Paper III** and **IV,** the concentrations of NO_3^- and NO_2^- were determined by reductive cleavage and subsequent determination of the NO released into the gas phase by chemiluminescence. The samples were introduced directly via a gas-tight syringe into a reducing solution (consisting of 45 mmol potassium iodide and 10 mmol iodine per liter in glacial acetic acid at 60°C in the case of NO_2^- and vanadium (III) chloride in 1 N hydrochloric acid (saturated solution) at 95° C for nitrate) in a micro-reaction purge vessel coupled to condenser and heating units both equipped with jackets for temperature control (Sievers, Boulder, CO, USA). The temperature of the condenser unit was controlled by a continuous flow of cold water while the temperature of the heating unit was controlled by a flow of warm water from a constant-temperature circulating bath (MGW Lauda M3, Germany). The reducing solution was bubbled continuously with nitrogen, used as a carrier gas for NO, at a flow rate of 192 ml/min. The outlet of the gas stream was passed through a scrubbing bottle containing sodium hydroxide (1 M, 0° C) in order to trap traces of acid, before transferring the gas into the rapid-response chemiluminescence system (Aerocrine AB, Stockholm Sweden) to determine the level of NO.

The data obtained were processed further with Origin for Windows, version 7.0 (Microcal, Northamton, MA, USA). The quantity of NO_2^- was obtained by calculation

of the area under the curve and comparison to calibration curves for a freshly prepared standard solution of NO_2^- and NO_3^- in ultra-pure water. Since vanadium (III) chloride also converts NO_2^- to NO, NO_3^- was quantitated by subtraction of the NO_2^- concentration from the total NO generated in this case. The levels of NO_3^- and NO_2^- are expressed as nanomoles per gram wet-weight of feces.

Calprotectin was measured using a commercial enzyme-linked immunoassay (ELISA, Calpro A.S. Norway), according to the manufacturer's instructions. The faecal levels of calprotectin are expressed as µg per gram wet-weight faeces, with normal values being <50 µg/g.[27]

ANIMAL MODELS

Animal models were used to determine whether the NO produced by the bacterial strains and the total fecal flora *in vitro* could also be detected *in vivo* (**Paper III**). For this purpose, adult, germ-free Agus rats (weight 315 ± 15 g, n = 12) were compared to conventional rats of the same strain (weight 315 ±10 g, n = 10). In addition, 10 germ-free Agus rats were mono-associated with L. rhamnosus (ATCC 53103) for 7 days, while 4 others were colonized conventionalized with the fresh caecal content obtained from 2 conventional Agus rats. Samples of the luminal content from different parts of the GI tract were cultured in order to verify the establishment of lactobacilli *in vivo*. The germ-free animals have been inbred for more than 90 generations at the Karolinska Institutet and were maintained under standard conditions in lightweight, stainless-steel isolators. These animals received sterile rodent food and their germ-free status was checked weekly.

On the day when NO was to be measured, the animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital and laparotomy then performed. The abdomen was opened, caecum identified and luminal NO gas measurements carried out described in detail elsewhere.[133] Briefly, employing a syringe with a thin needle, NO-free air was used to inflate the caecum (Fig. 5). After allowing 15 seconds for mixing this intestinal gas was aspirated and immediately injected into the chemiluminescence analyzer in order to obtain the peak concentration of NO. Thereafter, the rats were sacrificed with an overdose of sodium pentobarbital injected intra-peritoneally.

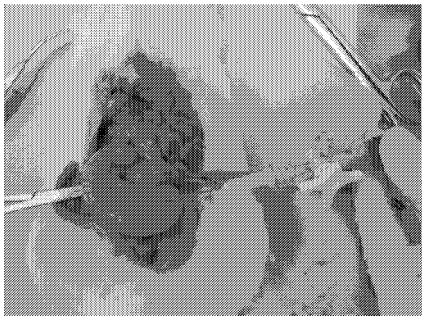


Figure 5: Measurement of nitric oxide in the caecum

STATISTICAL ANALYSIS

When the distribution of all of our data was examined with the Kolmogorov-Smirnov test, only the data resulting from the *in vitro* tonometric balloon testing in **Paper I**, turned out to be normally distributed. Therefore, while these later data are presented as means ± standard errors of the mean, all of other data are expressed as medians and the 25^{th} and 75^{th} percentiles. In **Paper I**, the independent sets of data were compared using either the Kruskal-Wallis (for more than 2 sets) or the Mann- Whiney *U* test (2 sets). The Wilcoxon signed-rank test was employed to analyze the data from the related groups in **Paper II**. In **Papers III** and **IV** correlations were evaluated with the Spearman rank test. For all statistical analyses conducted, a p-value of less than 0.05 was considered to be significant.

RESULTS AND COMMENTS

RECTAL LEVELS OF NO

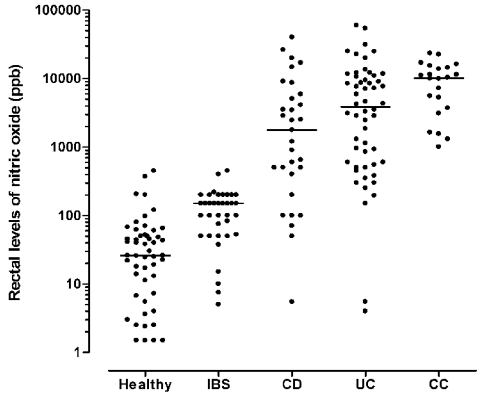


Figure 6: Rectal levels of nitric oxide in healthy volunteers and patients with irritable bowel syndrome (IBS), Crohn's disease (CD), ulcerative colitis (UC) and collagenous colitis (CC). Data from Papers I, II and IV

Healthy volunteers

Healthy adult volunteers displayed rectal levels of NO ranging from 7, 3-25 ppb (median, 25th - 75th percentiles (**Paper IV**)) to 45, 34-64 ppb (**Paper I**; Fig.6). This relative wide range of values probably reflects our use of different NO analyzers and methods of interpreting NO recordings as discussed above in the section on methodological considerations. The same type of catheter and same incubation time were employed for all of our NO measurements. The intra-individual variation from the median of rectal NO detected in 7 healthy volunteers over a period as long as two weeks was low (i.e., 27%; see Fig. 7).

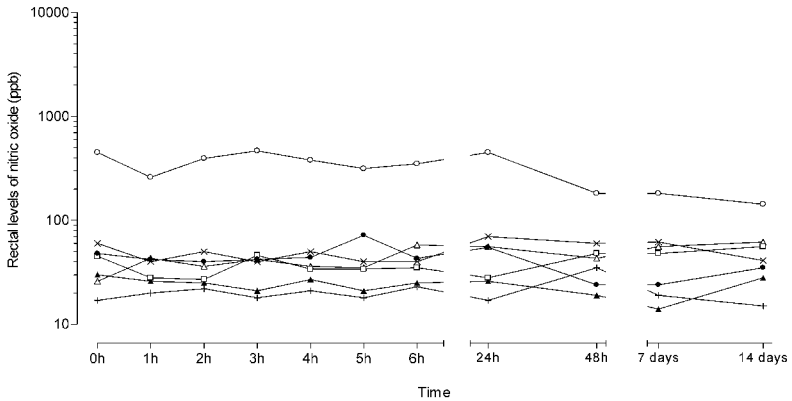


Figure 7: Individual levels of rectal NO detected in healthy volunteers by repeated measurements during a 2-week period.

Patients with IBD or collagenous colitis

Patients with active UC or CD exhibited median rectal NO levels ranging from 600 (200-5475) ppb (**Paper I**) to 927 (71-8978) ppb (**Paper IV**). Thus, these groups of patients demonstrated levels that were significantly higher than those of either healthy volunteers (see above) or patients with disease in remission 200 (100-400) ppb in the case of healthy volunteers (**Paper I**) and 96 (11-595) ppb for patients with disease in remission (**Paper IV**)). Nonetheless, the rectal levels of NO in patients in remission was significantly elevated in comparison to that of the healthy volunteers in both of these studies ($p<0.001$ in **Paper I** and $p=0.045$ in **Paper IV**). There was no significant difference between the levels of NO for UC and CD patients.

Patients with active CC displayed median rectal NO levels ranging form 1625 (1000-11050) ppb in **Paper IV** and 9950 (4475-19750) ppb in **Paper I** to 10850 (1300-16200) ppb in **Paper II**. All of these values were significantly higher than those of healthy volunteers and of subjects with CC in remission, with the later two groups exhibiting the same range of rectal NO levels (rectal levels of NO in patients with CC in remission: 50 (43-125) ppb (**Papers I and IV**)).

In **Paper IV** we also determined the content of NO_3^-, one of the end products of NO metabolism in the human body, in the fecal samples from healthy volunteers and all groups of patients. We expected to observe higher fecal NO_3^- levels in patients with high rectal levels of NO and, indeed, these fecal levels were higher in IBD patients than in healthy volunteers ($p=0.015$). Even patients with IBD in remission demonstrated elevated fecal levels of NO_3^- ($p=0.009$). There was no significant difference between the fecal levels of either NO_3^- or NO_2^- for patients with UC and CD. Patients with active CC had elevated levels of NO_3^- in comparison to CC patients in remission.

Patients with IBS

In **Paper I** we report that patients with IBS demonstrated a median rectal NO level of 150 (53-200) ppb, which was significantly higher than the corresponding level in the healthy volunteers ($p<0.001$), but lower than in all other groups of patients ($p<0.0001$; Fig. 6).

RELATIONSHIPS AND CORRELATIONS

The discriminatory power of rectal levels of NO

The ability of NO to discriminate between active bowel inflammation and IBS on the basis of rectal levels of NO is depicted in Figure 8. Employing a cut-off level of 250 ppb, the highest degree of sensitivity (95%) and specificity (91%) in discriminating between patients with active bowel inflammation (i.e., patients with active UC, CD and CC) and IBS on the basis of rectal NO was obtained. The positive predictive value (i.e., the probability that the disease is present when this NO level is >250 ppb) and negative predictive value (the probability that the disease is not present when the NO level is <250 ppb) were 97% and 86 %, respectively.

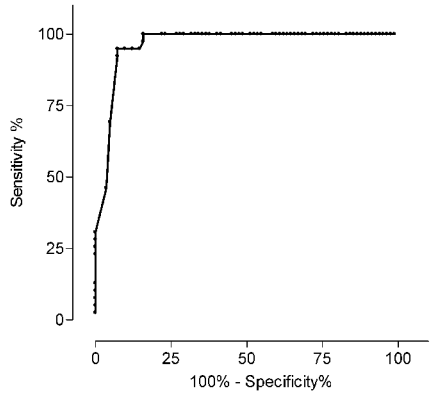


Figure 8:
Receiver-operating characteristics analysis of the ability of rectal NO levels to discriminate between patients with active bowel inflammation (Crohn's disease, ulcerative colitis and collagenous colitis) and IBS. A cut-off value of 250 ppb gives a sensitivity and specificity of 95% and 91%, respectively.

Effects of NOS inhibition

Systemically administration of L-NMMA to patients with collagenous colitis significantly inhibited their NO synthesis as reflected in the significant and uniform decreases in the levels of exhaled and nasal NO and the rise in mean arterial pressure. In contrast, the median rectal level of NO did not decrease significantly after administration of this inhibitor of NOS. However, in 5 out of 10 patients examined this rectal level did decrease significantly following L-NMMA infusion, supporting the suggestion that mucosal NOS is the source of this NO.[105,110] In the other 5 patients, rectal NO was unchanged or even increased by this treatment.

NO and disease activity

In **Paper I**, the rectal levels of NO in patients with UC experiencing an acute flare-up of the disease were determined, before and after a period of aggressive medical treatment. In parallel, i.e., on the day of admission to the hospital and one month later, the HBI was employed to evaluate the severity of their disease activity. The median rectal NO level for the entire group (n=19) the day of admission was 13 700 (4 600-22 600) ppb and this value decreased to 1 100 (350-10 000) ppb following 28 days of treatment ($p<0.01$).

Moreover, the day of admission, the mean Truelove-Witts score for disease activity for all 19 patients was 2.3. On day 28, twelve patients were in complete remission according to this index (with a score of 0), whereas remission in the remaining 7 patients was incomplete (mean score = 1.6). Retrospective analysis revealed that the disease activity of these two sub-groups did not differ significantly on day 1. In the case of the sub-group in complete remission, rectal NO levels had decreased by almost 95% during the study period ($p=0.002$), whereas the values of this parameter did not change significantly for patients with incomplete remission. A correlation between rectal NO levels and disease activity in IBD patients could also be demonstrated (Fig. 9_A).

NO and the daily number of diarrhea

Assessment of disease activity in patients with CC is based upon the daily number of defecations involving loose stools. In **Paper I**, we documented quite a strong correlation between rectal NO levels and this measure of disease activity ($r=0.78$, $p<0.0001$). Moreover, in **Paper IV,** we found a significant, but weaker correlation between this number and rectal NO levels in patients experiencing active IBD ($r=0.44$, $p<0.05$). Figure 9_B shows the correlation between NO and the daily number diarrhea in all patients with IBD and CC included in this thesis

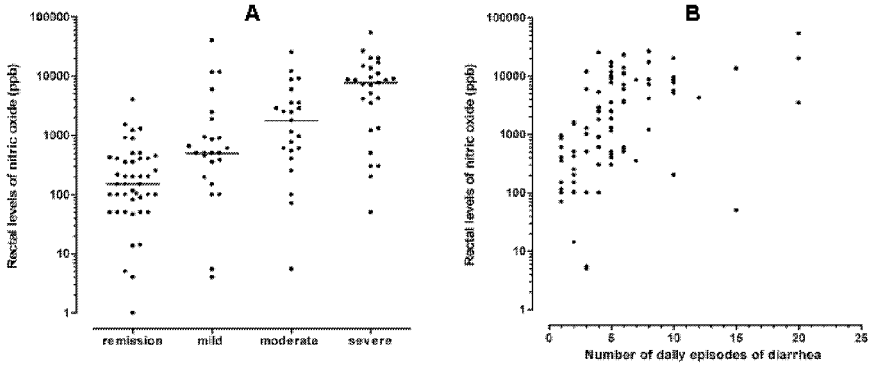


Figure 9: Plots documenting the correlations between the rectal levels of nitric oxide and disease activity (**A:** r= 0.63) or the daily number of episodes with diarrhea (**B:** r=0.58)

CALPROTECTIN

In the study described in **Paper IV** we determined fecal levels of calprotectin, a well-established marker for bowel inflammation. As was the case for rectal NO levels, patients with IBD displayed greatly enhanced levels of fecal calprotectin compared to healthy volunteers ($p<0.001$). The Individual fecal levels of calprotectin for healthy volunteers and patients with active IBD or CC are depicted in Figure 10.

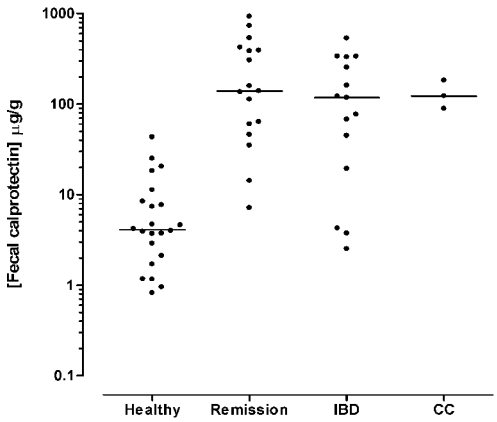


Figure 10: Individual and median levels of fecal calprotectin for healthy volunteers, patients with inflammatory bowel disease (IBD) in clinical remission, active IBD and active collagenous colitis (CC).

The fecal levels of calprotectin in healthy volunteers and patients with active IBD or CC were 4 (2-9) µg/g, 117 (19-330) µg/g and 123 (89-183) µg/g, respectively. Surprisingly, patients with IBD in clinical remission had a median value of 140 (53-408) µg/g, which is actually higher than the value associated with active disease and significantly elevated in comparison to healthy volunteers ($p<0.001$). There was no difference between the fecal levels of calprotectin for patients with CD and UC. Correlations between fecal calprotectin and the number of defecations involving loose stools (Fig. 11_A) or age (Fig. 11_B) were found ($r=-0.46$ and 0.51, respectively; $p<0.01$).

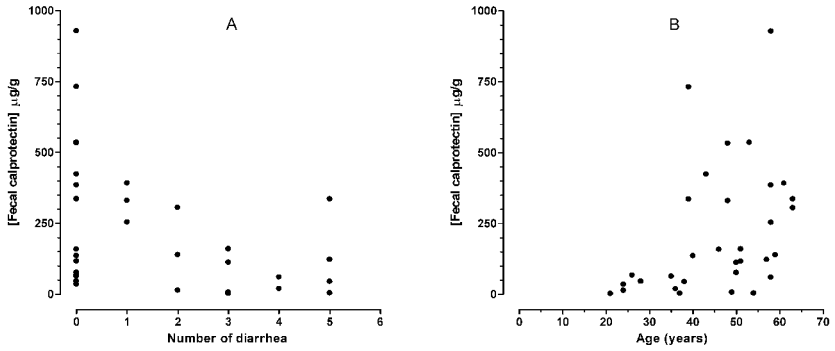


Figure 11: Scatter plots documenting the correlations between the fecal levels of calprotectin and the number daily of episodes of diarrhea (**A**: r = -0.46) or age (**B**: r = 0.51) in patients with IBD.

PRODUCTION OF NO BY BACTERIA (PAPER III)

In feces

During 24 hours of anaerobic incubation on ISO agar plates, the fecal flora of healthy volunteers generated small amounts of NO (~75 ppb), a value which was potently increased upon addition of NO_2^- or NO_3^- to the medium (to ~2000 and ~900 ppb, respectively). There was pronounced individual variation in the level of NO production by these fecal samples: three of the 8 samples examined did not produce any NO at all; whereas the remaining 5 samples produced NO in the range of 32-420 ppb on regular plates, 1 680-6 500 ppb when NO_2^- was added and 29-3 600 ppb when NO_3^- was added to the medium.

As described earlier, NO can also be generated by the spontaneous reduction of NO_2^- in an acidic environment, and we wanted to test how much of the NO generated in the presence of added NO_2^- arose from this phenomenon in these experiments. We observed a clear correlation between the pH of the agar and the level of NO detected, with no significant generation (<100 ppb) at pH values above 5.5. At a pH of 5, approximately 425 ppb NO was generated solely from the reduction of NO_2^- under the condition employed.

However, as stated above, 5 of 8 fecal samples produced 1 680-6 500 ppb NO when NO_2^- was added to the medium. Since the pH on the plates was 5-5.5 after 24 hours of incubation, fecal generation of NO under these circumstances does not seem to be due

solely to the acidic reduction of NO_2^-. Moreover, the generation of NO by feces was highly correlated to the amount of feces present on the plate, as well as to the amount of NO_2^- or NO_3^- added to the medium.

By individual types of bacteria

None of the bacteria tested produced NO on regular agar plates; without addition of NO_2^- or NO_3^- to the medium. In the presence of NO_3^- only a few of these bacterial strains (*L. reuteri*, *L. acidophilus*, *bifidobacteria* and *S. thermophilus*) produced some NO (33-45 ppb), whereas, when NO_2^- was added, NO generation by lactobacilli and bifidobacteria was potentially enhanced (up to >5000 ppb). None of the *E. coli* strains tested generated levels of NO above 5 ppb. Incubation with almost all of these bacterial strains reduced the initial pH of the agar plates to a level that made NO production by reduction of NO_2^- possible. However, in this case as well, the NO levels detected when NO_2^- is added to the medium were higher than could be accounted for solely by NO_2^- reduction.

***In vivo* experiments**

Germ-free rats demonstrated caecal NO level of approximately 9, 7-12 ppb.[133] When these animals were colonized with the intestinal flora from normal rats, this level rose to 93, 83-160 ppb, which is somewhat closer to the corresponding level detected in conventional rats of the same breed (i.e., 317, 64-455 ppb). In germ-free rats colonized by *Lactobacillus rhamnosus* alone, we found caecal NO levels of 14 (3-18) ppb, a value similar to that for untreated germ-free rats.

GENERAL DISCUSSION

The GI tract is truly a unique environment, constituting a site of connection with the outside world. This organ system protects the body from harmful microorganisms and, at the same time, it establishes a symbiotic relationship with the resident microflora. However, given the opportunity, certain components of this microflora will try to expand their habitats and trespass across the intestinal borders, thereby turning the normally peaceful situation at the mucosal surface into open war. Accordingly, a fine line seems to exist between the homeostatic balance maintained in the presence of commensal gut flora and the responses designed to destroy bacteria that invade the GI mucosa.

In light of these observations, it is not surprising that disturbance of this delicate balance can result in chronic inflammation detrimental to the host. Although the exact etiology of IBD remains unclear, the pathological mechanisms involved are slowly being unraveled and are doubtless multifactorial.[127] One current hypothesis is that IBD is the result of an over-exaggerated mucosal immune response against constituents of the commensal gut flora.[48,153]

One of the phenomenon most consistently associated with both experimental and human IBD, as reported by our own research group and others, is up-regulation of iNOS and consequent overproduction of NO, indicating that this messenger molecule is involved in the GI inflammatory response.[14,54,62,69,88,89,99,119,121] The stability of NO in the gaseous phase has allowed direct quantification of its level in the intestine and, as mentioned above, this level is significantly elevated in patients suffering from IBD and CC. The primary aim of the present thesis was to evaluate the possible clinical value of utilizing rectal levels of NO to diagnose IBD, as well as to monitor the response of this disease to treatment. Our findings to date are encouraging and support the use of NO as a surrogate marker for bowel inflammation. During this work, we also discovered an alternative source of NO in the large intestine, namely, the commensal bacteria. Although intraluminal generation of NO by bacteria represents a potential problem in connection with the determination of mucosal NOS activity during inflammation, this phenomenon could be highly interesting from a physiological point of view.

The most significant findings of this project are discussed in detail below. I have divided this discussion into three major areas; first, the potential sources of NO in the GI tract during health and disease, thereafter, a more speculative section on the possible role of NO in connection with IBD, IBS and CC; and finally, the possible clinical application of our findings.

SOURCES OF RECTAL NO

An important aspect of evaluating NO as a possible marker for inflammation is to pinpoint its origin. Where is the NO we are measuring coming from and by what mechanisms is it produced? One likely source is a NOS present in the gut mucosa and, indeed, all three isoforms of NOS are expressed throughout the GI tract. Furthermore, the expression of mucosal iNOS is clearly up-regulated in IBD[14,97] and CC[105]. Surprisingly, however, when we attempted to block NO production in patients with CC

by i.v. administration of L-NMMA, only half of these subjects exhibited a reduction in rectal NO, despite clear evidence of effective systemic inhibition of NO production. The several possible explanations for these findings include methodological issues and poor penetration of the drug into the cells of the colonic mucosa that produce NO.

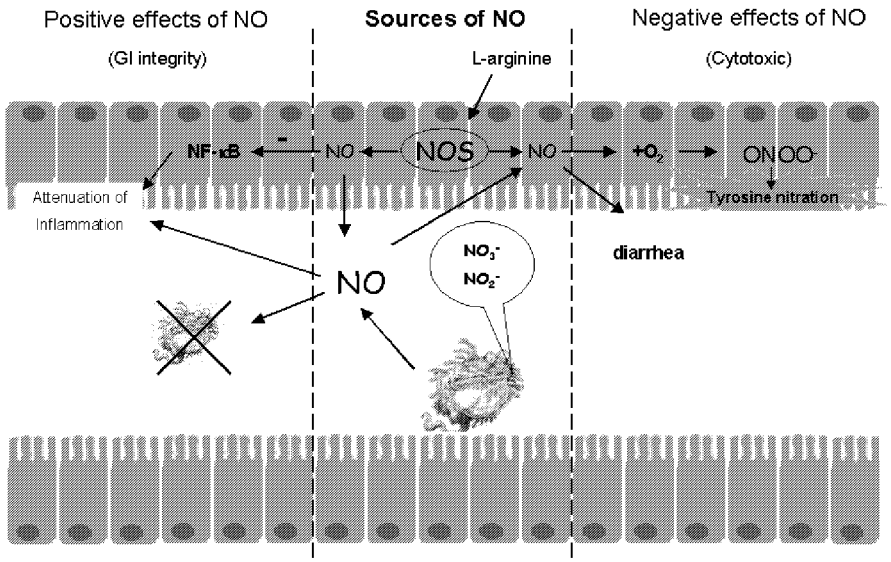
Interesting alternative sources of intestinal NO are the gut bacteria. Anaerobic denitrifying bacteria present in, e.g., soil and sediment can produce NO as an intermediate in the nitrogen cycle, through which NO_3^- is reduced step-wise to N_2.[90] Therefore, we decided to determine whether commensal gut bacteria are capable of generating NO via a similar pathway. In Paper III we show that both human feces and certain of the individual anaerobic bacterial strains present in the gut flora do indeed produce considerable amounts of NO in the presence of NO_3^- or NO_2^- *in vitro*. NO generation was also observed in the gut lumen of conventional rats, but not in germ-free rats, further demonstrating the involvement of bacteria in the generation of intestinal NO.

Another issue of interest is the anatomical localization of the source responsible for producing the NO that we detect in the rectum. Is this NO being produced locally or does it originate from a more proximal location? For instance, bacteria in the oral cavity convert dietary NO_3^- into NO_2^-, which is converted to NO in the acidic gastric environment. Indeed, the high levels of NO normally present in the gastric lumen could suggest that this is the source of NO found in the colon in patients with IBD.[9,91,96] However, this seems unlikely since the levels of NO in the small intestine of healthy control subjects are low.[36] Within a more limited region of the GI tract, such as the colon itself, NO can probably spread rather uniformly as a result of diffusion in the gas phase. The environment in the colon is ideal for NO stability since the O_2 tension there is very low, and therefore, NO produced more proximately should be found in the rectum as well. Indeed, the rectal levels of NO in adults with CD affecting small bowel and ileo-caecal region as well as in children with CD localized to the ileo-caecal region are elevated.[85]

Although we now know that bacteria can be an important source of NO in the GI tract, we still cannot say exactly how much these microorganisms contribute to the elevated NO levels associated with inflammatory bowel disease.

NO AND IBD

NO undoubtedly plays an important role in modulating several key physiological functions of the digestive system. However, it has also been suggested that NO is a mediator of the tissue injury associated with several intestinal disorders (Fig. 12). Here, I will discuss how NO might be involved in the pathogenesis of IBD.



Figuur 12: Schematic drawing illustrating NO generation in the GI tract and its possible effects. In the mucosa NO is generated from L-arginine by NO synthases while bacteria in the gut lumen can generate NO from nitrate (NO_3^-) and nitrite (NO_2^-). Positive effects of NO may include attenuation of inflammatory responses and killing of gut pathogens. Negative effects can also be foreseen for example generation of tissue-damaging radicals or induction of diarrhea.

Bacterial NO production

The finding that commensal bacteria in the gut produce significant amounts of NO is of interest for several reasons. First, such production is a possible source of error when trying to determine mucosal NO production in connection with IBD, as discussed above. A more intriguing possibility is that bacterial generation of NO and other nitroso species plays a role in physiological processes in the gut.

For example, one could speculate that the NO produced by certain residents in the commensal flora can inhibit the immune responses mediated by NF-κB and thereby help maintain a 'peaceful atmosphere' at the mucosal surface. Thus, an imbalance in the GI flora could either result in failure to suppress these immune responses due to insufficient generation of NO, or cause tissue damage by elevated levels of NO. Furthermore, the NO produced by certain gut commensal could influence the growth of other bacteria, including pathogenic species, and thereby play a role in controlling bacterial overgrowth. Recently, we observed greater numbers of bacteria in the feces of patients with active IBD than in the feces of healthy volunteers. However, the feces from healthy subjects appeared to be able to produce more NO than that of IBD patients (unpublished findings), suggesting that bacterial NO plays a protective role in microbial homeostasis of the gut.

Clearly, the biological significance of the NO produced by the gut microflora remains to be established.

Regulation of mucosal immunity and of apoptosis

In the acute inflammatory response of the GI mucosa, NO appears to play a protective role, reducing the adherence of leukocytes to the endothelium and inhibiting the production of cytokines by the pro-inflammatory Th1 cells and IL-12 (the major cytokine activating Th1 cells) by macrophages. NO also decreases the release of mediators from mast cells, inactivates oxidants and eliminates monocytes, such as macrophages, by inducing apoptosis.[6]

However, what happens when the inflammatory process becomes uncontrolled? Why is the rectal level of NO in patients with IBD so high and how might NO influence the pathogenesis of this diseases? It is well known that cytokines released by Th1 cells and macrophages up-regulate the expression of iNOS via NF-κB, in addition to stimulating the inflammatory response. Active IBD is associated with local overproduction of pro-inflammatory cytokines and with activation of NF-κB in macrophages of the lamina propria and epithelial cells, which might explain the enhanced mucosal production of NO [122,130]

Recently, it has also been suggested that aberrations in the control of programmed cell death in mucosal T-cells are involved in the pathogenesis of IBD. Induction of apoptosis in these cells is severely impaired in connection with IBD and significant differences in the regulation of apoptosis in the mucosa affected by UC or CD have been observed.[109]

The overall role of NO connected with apoptosis remains controversial. A certain level of NO is required for cell survival, whereas exposure to donors of NO under certain conditions enhances the incidence of apoptosis.[22,131,150,158]

The cytotoxic action of NO

In addition to acting as signaling molecule, NO (and/or its reaction products) also exert direct cytotoxic effects. At high concentrations, NO may interact with various target molecules or groups, including O_2, thiol groups, and metal atoms in the prosthetic groups of various proteins, resulting in their activation or inactivation. High levels of NO can also inhibit essential components of cellular respiration causing depletion of cellular energy stores and consequent cytotoxicity.

However, findings from investigations on knockout mice lacking iNOS,[75,95] as well as the observation that the high rectal levels of NO in patients with CC are not associated with tissue injury[89,105,111], suggest that NO alone does not cause intestinal inflammation directly. However, this radical may well potentiate the toxicity of other radicals. For example, when NO reacts with O_2^-, $ONOO^-$ is formed and can trigger lipid oxidation, protein nitration and DNA damage, thereby leading, among other things, to increased permeability in epithelial cells.[94] Indeed, enhanced levels of tyrosine nitration (a marker of $ONOO^-$ formation) have been detected in biopsy samples from patients with IBD.

All together, the available evidence suggests that NO cannot be viewed as being all ″good″ or ″bad″. The biological effects of NO will depend on several parameters including the amount generated, the time-course of this generation and, of course, its localization and interactions of NO with other radicals. It seems unlikely that the enhanced rectal levels of NO associated with IBD are solely detrimental. A gradient of

action, ranging from physiological effects at low levels to toxic effects by highly elevated levels of NO may exist.

NO, IBS AND COLLAGENOUS COLITIS

Irritable bowel syndrome (IBS) and its etiopathogenesis remain a controversial topic, with certain investigators favoring an underlying a pathophysiological mechanism, while others propose a psychological etiology.[51] Very little is known at present concerning the relationship between IBS and NO. One report has documented plasma levels of NO_3^- in patients with IBS within the normal range.[34]

As discussed above, there is a subgroup of patients with IBS referred to as PI-IBS patients. Recent evidence suggests that low-grade intestinal inflammation could be the cause of the symptoms in a small proportion of patients with IBS, especially IBS characterized by extensive diarrhea.[25,26,53] Indeed, Parry and co-workers[107] demonstrated that PI-IBS patients exhibit elevated levels of fecal calprotectin. In agreement with this finding we also found slightly elevated levels of rectal NO in such patients.

PI-IBS is associated with a modest increase in the number of mucosal T-lymphocytes and serotonin-containing enteroendocrine cells (EC).[134] Mucosal T-lymphocytes can up-regulate the expression of iNOS, thereby giving rise to excess production of NO. EC are the source of 90% of the mucosal serotonin. A study by Stoner and collegues[136] in rats suggests that NO is the predominant secretomotor neurotransmitter mediating the effects of serotonin on chloride secretion. NO is a terminal neurotransmitter in the neural-mucosal reflex and acts therefore directly on the enterocyte to induce secretion, a possible mechanism for diarrhea. If this is also the case in humans, this phenomenon could explain why patients with PI-IBS have higher levels of luminal NO than healthy control subjects.

It is possible that NO contributes to the pathophysiological events underlying CC. The level of intestinal NO is very high in patients with this disease, in fact, even higher than in the case of UC. Moreover, since several animal studies implicate that NO plays a role in causing diarrhea,[63,93] an intriguing possibility is that NO produced in the lumen or by the superficial epithelial cells contributes to the pathophysiology of this disorder. NO appears to stimulate collagen production by fibroblasts[29] and immunohistochemical studies have revealed the presence of iNOS in the superficial epithelial cells of patients with CC.[105,110] As suggested by Closs et al,[23] one possibility is that NO causes diarrhea via activation of a soluble GC present in intestinal epithelial cells, this process would thus be similar to cGMP-dependent diarrhea caused by the heat-stable *E.coli* exotoxins, which are known to activate GC.[152]

CLINICAL APPLICATIONS

The findings discussed above indicate that high levels of NO appear to be involved in the disturbances in the inflammatory and apoptotic processes that are important factors in the pathophysiology of IBD. Theoretically, the level of NO should provide information concerning the state of the GI mucosa.

NO as a diagnostic tool

Sampling of rectal NO employing the balloon technique described here is convenient for the examiner and relatively non-invasive for the patient. In the more than 600 such measurements performed in our laboratory to date, not one subject has had any complications and both adults and children experience the procedure as being tolerable. In order for rectal levels of NO to serve as a useful surrogate marker for bowel inflammation, the normal level of NO present in the gut had to be established. In Paper I we report that healthy volunteers have uniformly low levels of rectal NO and that the hourly, daily and weekly intra-individual variations are small. These low rectal levels of NO in healthy volunteers are in striking contrast to the increased levels observed in patients with GI inflammation.

Abdominal discomfort, with symptoms such as diarrhea and pain, is one of the most common reasons for a primary visit to a physicians and subsequent consultation with a gastroenterologist. An important initial task for the consulting physician is to establish whether the symptoms are caused by an inflammatory condition, since such conditions require special treatment. Diagnosis of inflammation is not, however, always straightforward. Standard diagnostic procedures include taking the patient's history, physical examination, endoscopy, tests of blood chemistry and radiological examination, which are time-consuming and relatively expensive.

The use of rectal levels of NO as a surrogate marker for bowel inflammation has several advantages. This procedure is rapid and minimally invasive and, moreover, reflects processes occurring locally in the affected organ itself. This later consideration probably explains why NO and other local markers, such as the level of calprotectin in faeces,[144,147] appears to be far superior to unspecific serologic markers of inflammation in terms of sensitivity and specificity. In Paper I we clearly demonstrated that the rectal level of NO is a sensitive and specific biomarker for inflammation in the gut. The overlap in this value for patients with active bowel inflammation and those with a functional bowel disease (i.e., IBS) is minimal and, employing a cut-off level of 250 ppb, this marker exhibits high degree of sensitivity and specificity in discriminating between active bowel inflammation and IBS.

NO and monitoring treatment

Once the diagnosis has been established, the physician is dependent on a reliable marker of the on-going inflammatory activity to be able to optimize treatment. Both our research group and several others have attempted to detect a relationship between the severity of IBD and the rectal level of NO. Such a relationship appears to exist in the case of UC. In patients with this disease, Rachmilewitz and co-workers[116] found a correlation between rectal levels of NO (measured using NO-sensitive electrodes) and in clinical and endoscopic indices of disease activity.[116] Furthermore, serum levels of NO metabolites in these same patients correlate to the level of C-reactive protein; and serum levels of NO_3^- in patients with UC have been reported to be directly proportional to the erythrocyte sedimentation rate, leukocyte, and platelet counts.[46,120]

In the case of CD, it is less clear that there is a correlation between rectal levels of NO and disease activity. In Papers I and IV, we did observe a correlation between rectal NO levels and clinical indices of disease activity in patients with UC and CD as did Ljung et al.[85] in children with CD. In addition, these levels decreased markedly in

patients with UC who responded well to anti-inflammatory treatment (Paper I). In contrast, an investigation from France, did not find any correlation between these parameters and disease activity in patients with CD, despite their increased levels of iNOS.[50]

In the case of CC the daily number of diarrheic bowel movements is employed as an indicator of disease activity (Paper I) and the rectal levels of NO in patients with this disease were shown here to be correlated with disease activity as well. The daily number of bowel movements is also an important parameter in the clinical indices of disease activity applied to UC and CD. One possible explanation for this correlation is that NO plays a role in intestinal water transport and high levels of NO could thus cause diarrhea via activation of soluble GC.

NO and calprotectin

NO and calprotectin have been investigated independently as promising non-invasive surrogate markers for bowel inflammation. The rectal/fecal levels of both these markers are potentially elevated in IBD, but no investigators have attempted previously to measure both of these parameters in the same groups of patients (Paper IV). Here, we were unable to detect a correlation between fecal levels of calprotectin and disease activity.

It could be argued that this is not surprising, since indices of clinical disease activity are not directly related to inflammatory activity.[44] Indeed, it is known that indices of clinical disease activity correlate poorly with endoscopic indications of inflammation, whereas the fecal level of calprotectin correlates well to both endoscopic and histological grading of disease activity.[123,146] The observation that NO plays a role in intestinal water transport could explain why we were able to detect a correlation (albeit weak) between the rectal level of NO and indices of clinical disease activity.[116]

The fecal level of calprotectin was negatively correlated to the daily number of diarrheic bowel movements, a finding that is difficult to explain. One possibility is that the presence of looser stools results in dilution of the calprotectin.[114] We also found a correlation between fecal calprotectin levels and age, which was not observed in the case of NO. This raises the question as whether a fixed cut-off value can be employed to discriminate between patients with IBD and patients with a functional bowel disorder regardless of age.

The fact that the rectal levels of NO and fecal levels of calprotectin are not correlated to one another, or to the same additional parameters, indicates that these parameters may be markers for two different aspects of the inflammatory process. Whereas NO could be a marker for ongoing inflammation, calprotectin might serve as predictor of relapse. However, at present, this is only speculation based on our single study. Several examples of a good correlation between faecal levels of calprotectin and ongoing inflammation have been reported.[2,37,44,145]

In summary, inflammatory activity in the intestinal mucosa is associated with pronounced increases in luminal levels of NO. The most promising clinical potential for measurements of rectal NO thus lies in screening for bowel inflammation. This parameter might also be a useful complement for monitoring disease activity, although this remains less clear. An extensive prospective study in which the rectal level of NO is compared to traditional procedures for diagnosis of intestinal inflammation in undiagnosed individuals is strongly warranted.

SUMMARY AND CONCLUSIONS

NO is a pluripotent biological messenger involved in numerous physiological and pathological processes in the GI tract. In the intestinal mucosa NO is synthesized from the amino acid L-arginine via a reaction catalyzed by NOS. The levels of gaseous NO in the GI tract can be measured directly using a balloon-tipped catheter. Employing this procedure, the present thesis was designed to further evaluate the potential usefulness of measurements of rectal levels of NO in connection with the diagnosis of IBD and monitoring the response of this disease to treatment.

We have demonstrated that intra-individual variation in the rectal NO level of healthy subjects over time is small. Increased rectal levels of NO can be utilized to discriminate between IBS and active IBD with a sensitivity of 95% and specificity of 91%. In addition, the rectal level of NO was correlated to disease activity in patients with IBD and CC in several of the studies conducted here. Furthermore, the NO levels decreased markedly in IBD patients responding well to treatment

The lack of effect of intravenous administration of L-NMMA, an inhibitor of NOS, on rectal levels of NO in patients with active CC indicated that this messenger may be produced by other sources as well. Since denitrifying bacteria present in, e.g., soil have the ability to generate NO from nitrite as a part of the nitrogen cycle, one such source could be the microflora of the GI tract. We found that human feces and individual strains of representative gut bacteria are, indeed, able to generate NO in the presence of nitrate and/or nitrite *in vitro*.

Our comparison of NO and calprotectin as markers for inflammation revealed that the levels of these substances may reflect different aspects of the inflammatory process, since these levels were not correlated to one another or the same additional parameters. Whereas NO appears to be a marker for ongoing inflammation, calprotectin might serve as a predictor of relapse. However, at present this is a speculation based on the findings from our single study.

Measurement of rectal levels of NO can be used as a rapid and minimally invasive procedure for discriminating between active bowel disease and IBS, as well as, possibly, to obtain useful supplementary information in the monitoring of the response of patients with IBD to treatment. The anaerobic gut flora can generate some of the rectal NO detected, especially when nitrate and/or nitrite is present. The role played by the large amounts of NO produced in connection with intestinal inflammation in this disease itself and in host defense remains unclear. On the one hand, NO is involved in the killing of pathogenic microorganisms, but on the other hand, NO and/or its metabolites can also be cytotoxic to host cells, making it a double-edged sword. Future studies will reveal the possible biological significance of NO generated by GI bacteria with respect to regulation of GI integrity, as well as the potential diagnostic usefulness of calprotectin and NO and the exact role of these substances in the pathogenesis of bowel inflammation.

SUMMARY IN SWEDISH

Magtarmbesvär är en av de allra vanligaste orsakerna till besök hos läkare men att ställa rätt diagnos bara utifrån symptom kan ibland vara väldigt svårt. Icke desto mindre är detta viktigt eftersom olika sjukdomar kräver helt olika behandling. Oftast hittar man ingen självklar orsak till besvären men ibland döljer sig en mer allvarlig underliggande sjukdom. Ett sånt exempel är inflammatorisk tarmsjukdom; en grupp sjukdomar som drabbar yngre människor och som kräver mycket speciell behandling ofta under lång tid.

Vi har utvecklat en metod för att enkelt kunna avgöra om en person har en pågående inflammation i sin tarm. Metoden bygger på att mäta halterna av en gas, kvävemonoxid (NO) i ändtarmen. NO är en signalsubstans med en mängd funktioner i magtarmkanalen, bland annat i immunförsvaret. En inflammerad tarmslemhinna producerar enormt mycket mer NO än en frisk och denna gas kan fångas upp av en liten ballong som blåses upp inne i tarmen. Själva mätningen är helt smärtfri och kan bäst liknas med att ta tempen i ändtarmen.

I denna avhandling har jag försökt utvärdera NO mätningar för att få en bättre uppfattning om detta test kan bli användbart i kliniken som ett diagnoshjälpmedel. Vi fann att normala NO halter är låga och varierar ganska lite med tiden. Halterna hos patienter med tarminflammation var däremot kraftigt förhöjda och högre ju sjukare patienterna var. När medicinsk behandling startade sjönk NO värdena betydligt i takt med att patienterna förbättrades. NO halten korrelerade också till en viss del till andra kliniska parametrar såsom antalet diarréer per dag.

Vi försökte också få en bättre uppfattning om var det NO vi mäter egentligen bildas. Den självklara huvudkällan torde vara ett enzym (så kallat NO syntas) i den inflammerade slemhinnan men till vår förvåning upptäckte vi att bakterier i tarmen också är kapabla att bilda NO. Exakt hur mycket bakterier bidrar till de nivåer vi uppmäter vid sjukdom är fortfarande oklart men detta bifynd är mycket intressant också ur andra aspekter. NO är en högaktiv biologisk budbärare och om den bildas i stora mängder från bakterier kan detta tänkas påverka fysiologiska processer i tarmen. En intressant möjlighet är att den inflammatoriska processen i slemhinnan påverkas av NO-bildning från bakterier, något som framtida studier får utvisa.

Sammanfattningsvis har vi visat att mätningar av NO kan ge värdefull information om huruvida en patient har inflammation i tarmen eller inte. Detta kan annars vara svårt för en läkare att avgöra bara utifrån patientens symptom. Vi tror att detta enkla test kan bli till nytta inom sjukvården i diagnos och uppföljning av misstänkt tarminflammation.

SUMMARY IN DUTCH

Problemen met de maag of darmen is één van de meest voorkomende redenen dat een patiënt een bezoek brengt aan zijn huisarts. Het stellen van de juiste diagnose gebaseerd op de symptomen kan heel moeilijk zijn, maar is erg belangrijk omdat de verschillende aandoeningen van het maag/darmstelsel verschillend behandeld moeten worden. Vaak wordt er geen vanzelfsprekende oorzaak gevonden voor de klachten, maar soms houdt er zich toch een ernstige ziekte schuil achter de symptomen. Een voorbeeld van zo'n ziekte is chronische darmontsteking, een aandoening die vaak optreedt bij jonge mensen en die een speciale, vaak langdurige, behandeling vraagt.

Wij hebben een methode ontwikkeld om gemakkelijk te kunnen beoordelen of een persoon lijdt aan actieve darm ontsteking. De methode is gebaseerd op het meten van hoeveelheden gas, stikstofmonoxide (NO), in het rectum. NO is een zogenaamde "boodschapsubstantie" met vele functies in het maag/darm kanaal, ondermeer het reguleren van ons immuunsysteem. Een ontstoken darmslijmvlies produceert veel meer NO dan gezond darmslijmvlies. Deze verhoogde hoeveelheid NO kan worden gemeten doormiddel van een klein opgeblazen ballonnetje in het laatste stukje van het rectum.

In dit proefschrift hebben we geprobeerd te evalueren of de rectale NO metingen door een arts gebruikt zouden kunnen worden als een potentieel diagnostisch hulpmiddel voor het opsporen van ontstekingen in de darm. We hebben gezien dat de normale NO waarden in de darm van gezonde personen laag zijn en dat ze weinig variëren in de tijd. De NO niveaus in de darm van mensen met darmontsteking daarentegen, zijn erg verhoogd, en hoe hoger deze waarden zijn, hoe zieker de patiënten. Tijdens medische behandeling van de ontsteking daalt het NO niveau naar mate de patiënt verder herstelt. NO niveaus correleren ook tot op zekere hoogte met andere klinische parameters zoals het aantal maal per dag dat een patient met diarree te kampen heeft.

We hebben ook geprobeerd een beter beeld te krijgen van de herkomst van het NO dat we meten. Een enzym (NOS) wordt gedacht de grootste bron te zijn van NO in het ontstoken darmslijmvlies. Tot onze verwondering ontdekten we dat bacteriën die leven in onze darmen ook de mogelijkheid bezitten om NO te produceren. Precies hoeveel bacteriën kunnen bijdragen aan de NO niveaus die we meten tijdens darmontstekingen is nog onzeker. De ontdekking dat bacteriën NO kunnen produceren is ook interessant vanuit een andere invalshoek. NO is een zeer actief biologische boodschapper, en daar waar dit gas in grote hoeveelheden geproduceerd wordt kan het fysiologische processen beïnvloeden. Een interessante hypothese is dat de ontstekingsprocessen in het darmslijmvlies beïnvloed kan worden door NO geproduceerd door bacteriën. Toekomstige studies zullen dit moeten uitwijzen.

Samenvattend kunnen we zeggen dat we hebben laten zien dat de rectale NO meting waardevolle informatie geeft omtrent de vraag of een patiënt al dan niet een ontsteking heeft in de darm. Dit is normaal gezien moeilijk te diagnosticeren enkel gebaseerd op de symptomen. Wij denken dat deze eenvoudige test nuttig is voor artsen bij het stellen van de diagnose, en tijdens de opvolging van de behandeling, bij patiënten met darm ontsteking.

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