Nitric Oxide in

Inflammatory Bowel Disease

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Abstract

The aim of this study was to study nitric oxide (NO) in inflammatory bowel disease (IBD) with a special reference to disease activity and disease location, in both an adult and a pediatric patient population, using rectal gas sampling. Furthermore, we investigated the cellular source of the measured NO as well as the relationship between the expression of inducible NO synthase (iNOS) and the proinflammatory cytokines TNF-α, IL-1β and INF-γ. Increased NO production was found in active IBD but not inactive disease. Rectal NO levels correlated with disease activity expressed both as clinical activity score and endoscopic inflammatory score in IBD suggesting that rectal NO measurements could be used as an objective quantitative marker of disease activity. We also showed a subgroup of severely ill IBD patients, not responding to glucocorticosteroid treatment, to have low (< 1000 ppb) initial levels of rectal NO upon onset of flare, suggesting that rectal NO measurements could be used to identify corticosteroid refractory IBD patients, later subjected to colectomy. Increased rectal NO levels were seen in active ulcerative colitis (UC), colo-rectal Crohn’s disease (CD) as well as ileo-cecal CD showing that rectal gas sampling for NO analysis could be used to detect both active ileo-cecal and colo-rectal disease. By the correlation between rectal NO levels and mucosal iNOS expression in polymorphonuclear cells in the lamina propria of the colonic mucosa we conclude that the induction of iNOS in these cells plays a major role in the production of the increased luminal NO seen in active IBD. The degree of mucosal iNOS expression displayed significant correlation to the degree of TNF-α and IL-1β but not INF-γ expression. To conclude, our present data in IBD specify NO to be a reliable marker of inflammatory activity as indicated by its elaboration by iNOS released from polymorphonuclear leukocytes in the colonic mucosa.

Key words: Inflammatory bowel disease, Crohn’s disease, Ulcerative colitis, Inflammation, Nitric oxide, Nitric oxide synthase, Cytokine, Tumor necrosis factor-α, Interleukin-1β, interferon-γ, Human

“Don’t be too humble, you’re not that great”

Golda Meier
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<td>Crohn’s disease activity index</td>
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<tr>
<td>cGMP</td>
<td>cyclic monophosphate</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>cNOS</td>
<td>constitutive nitric oxide synthase</td>
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<td>DAI</td>
<td>disease activity index</td>
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<td>EDRF</td>
<td>endothelium-derived relaxing factor</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
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<tr>
<td>FAD</td>
<td>flavine adenine dinucleotide</td>
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<td>FMN</td>
<td>flavine mononucleotide</td>
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<td>Hb</td>
<td>hemoglobin</td>
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<td>HBI</td>
<td>Harvey-Bradshaw index</td>
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<td>IBD</td>
<td>inflammatory bowel disease</td>
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<td>interleukin-1β</td>
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<td>INF-γ</td>
<td>interferon-γ</td>
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<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<tr>
<td>nNOS</td>
<td>neural nitric oxide synthase</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood count</td>
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<tr>
<td>5-ASA</td>
<td>5-aminosalicylic acid</td>
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Original papers


V. Ljung T, Axelsson LG, Herulf M, Lundberg JON, Hellström PM. Early changes in rectal nitric oxide levels and mucosal inflammatory mediators in Crohn’s colitis in response to TNF-α antibody (infliximab) treatment. (Manuscript).

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Introduction

Inflammatory bowel disease

Ulcerative colitis (UC) and Crohn’s disease (CD), together comprising the entity inflammatory bowel disease (IBD) are chronic inflammatory diseases of unknown etiology. The majority of patients with IBD have an intermittent course with relapses and clinical remission.\(^1\)\(^,\)\(^2\) Common symptoms found in UC and CD are diarrhea (typically with blood and mucus) and abdominal pain. In more severe cases signs of systemic inflammatory involvement such as fever, malaise, anorexia and weight loss supervene. Both UC and CD have a bimodal age distribution with the highest incidence in young adults.\(^3\)\(^,\)\(^4\) In Sweden an incidence of 12 to 13/100 000 per year and a point prevalence of 235/100 000 inhabitants is found for UC.\(^6\)\(^,\)\(^7\) The incidence and prevalence figures in population-based studies for CD are often approximately half compared with UC; corresponding to reported incidence rates varying between 4.5 and 6.7/100 000 per year in Swedish studies.\(^7\)\(^,\)\(^8\) In gastroenterological hospital units conditions are reversed, due to the fact that CD allocates two to four times the health care resources compared with UC.\(^10\)

Definition of ulcerative colitis and Crohn’s disease

The diagnoses of UC and CD are based on clinical symptoms, endoscopical, histological, or radiological findings.\(^11\)\(^-\)\(^13\) UC is per definition restricted to the colon and the distribution is typically continuous, involving the rectum and to variable extent the colon in oral direction. The inflammation is restricted to the mucosa, and the histological changes are continuous in concordance with macroscopic findings. However, no certain features of the inflammatory reaction are specific for UC.

CD can involve the whole gastrointestinal tract, from the oral cavity to the anus. The endoscopical findings with uneven distribution (“skip lesions”) correspond to the histological patchy inflammatory appearance. The inflammation is transmural. In case of
established IBD, the finding of granuloma is pathognomonic for CD, but granulomas are
found only in 50-70% of CD. If the disease is confined to the rectum and colon it can be
impossible to discriminate between UC and CD, which has led to the diagnosis
indeterminate colitis, applicable to 10% of patients having colonic IBD.

*Inflammatory cells in intestinal tissue in IBD*

UC is a nonspecific inflammation involving leukocytes, plasma cells and lymphocytes
infiltrating the lamina propria. The neutrophils and eosinophils are predominately a
feature of the acute phase of the disease and correlate to the severity of the
inflammation. The cells participating in the inflammatory response in CD are basically the same
nonspecific constellation, however with predominance for lymphocyte infiltration.

*Assessment of disease activity in IBD*

The need for activity indices originate from the search for objective markers of
improvement in controlled clinical trials. Sidney Truelove therefore constructed the first
system for expressing degree of illness; the three-category scale for UC. Truelove’s
category scale, still in use, has been followed by numerical indices like the Powell-Tuck
index and the Disease Activity Index (DAI) by Sutherland. In 1976 the Crohn’s
disease activity index (CDAI) was developed by the group conducting the American
National Cooperative Crohn’s Disease Study. Harvey and Bradshaw later
demonstrated that a simplified version of CDAI accurately expressed the clinical activity
(HBI), and amending growth a pediatric CDAI was established. These clinical indices
all weigh subjective factors highly; hence they quantify the patient’s degree of illness
rather than the severity of inflammation.

However, as treatment in IBD is primarily aimed at reducing inflammation an objective
marker of intestinal inflammation is warranted. Various blood test are used in clinical
practice, but the correlation to mucosal inflammation is poor. Stool tests have the
obvious advantage of measuring the inflammation on site, and many promising surrogate
markers have been presented such as calprotectin and excretion of $^{111}$In-tagged autologous granulocytes.\textsuperscript{27,28}

\textit{Treatment of ulcerative colitis}

Sulfasalazine was the first drug used to induce remission in active UC\textsuperscript{29} and is still in use. 5-ASA compounds have been developed in order to omit the side effects associated with the sulphonic component of sulfasalazine. 5-ASA has been proven effective for induction, as well as for maintenance of remission in UC.\textsuperscript{30,31} In moderate to severe active UC the mainstay treatment is glucocorticosteroids\textsuperscript{19,32} If maintenance therapy with 5-ASA fails, immunomodulators such as 6-mercaptopurine/azathioprine are often applied, as well as in corticosteroid-refractory or corticosteroid-dependent cases.\textsuperscript{33} Glucocorticosteroids have not proven to be useful for preventing relapse in UC.\textsuperscript{34,35}

\textit{Treatment of Crohn’s disease}

The pharmacological treatment used in CD basically consists of the same drugs applied in UC with addition to metronidazole and recently the chimeric monoclonal tumor necrosis (TNF)-\(\alpha\) antibody (infliximab). Results from randomized controlled trials, as well as the clinical use of the different compounds differ slightly between UC and CD: 5-ASA has a proven efficacy for induction of remission also in CD, but only minor benefit has been shown for 5-ASA in maintenance therapy of CD.\textsuperscript{36} As in UC, glucocorticosteroids are effective in inducing remission, but can not be used to prevent relapse in CD.\textsuperscript{37,38} Even if glucocorticosteroids can preserve corticosteroid-achieved remission, their clinical usefulness is limited by the severe long-term side effects.\textsuperscript{39} Azathioprine is effective for the maintenance of remission in CD as well as in UC, but lacks clinical potential for induction of remission, basically due to slow onset of action.\textsuperscript{30,41} Metronidazole has been shown to be equipotent to sulfasalazine for treatment of active CD and is also used for perianal CD.\textsuperscript{32,41} Infliximab has rapidly been incorporated in the standard therapy of active, corticosteroid-dependent or -refractory CD, and might be useful in preventing relapse as well as in fistulous CD.\textsuperscript{34-47}
Nitric oxide

Previously recognized as a component of smog, automobile exhaust and cigarette smoke, the biological effects of NO were recognized, initially as endothelium-derived relaxing factor (EDRF) by Furchgott and Zawadzki, and later identified as NO by two independent groups.49, 50

Chemistry of NO

NO is one of the simplest molecules in nature (molecular weight 30 D). Despite having an unpaired electron, hence being a free radical, its reactivity is quite limited. Under physiological circumstances NO reacts with (Fig. 1): (1) Other radicals, for e.g. with the superoxide ion (O$_2^-$) thereby acting as an antioxidant.52 (2) Oxygen; this reaction results in the formation of nitrite (NO$_2$), which rapidly consumes more NO to form dinitrogen trioxide (N$_2$O$_3$). NO is relatively stable; at an NO concentration of 5 parts per billion (ppb) in ambient air (21% O$_2$) less than 10% NO is converted to NO$_2$ within 2 hours.53 (3) Transition metals, such as iron, which is the way NO activates soluble guanylyl cyclase, interacting with the heme group resulting in generation of guanosine 3',5'-cyclic monophosphate (cGMP), by which many of the biological actions of NO are mediated. As pointed out above, NO is fairly stable at low concentrations in ambient air, but in aqueous solution the half-life is in the range of seconds, and in vivo in the presence of scavengers even shorter.55

[Diagram showing the reaction pathways of NO]

**Figure 1.** Schematic drawing demonstrating three different principal NO pathways under physiological circumstances: (1) NO reacts with other radicals, thereby acting as an antioxidant, (2) NO reacts with oxygen forming reactive oxygen species, yielding cytotoxic properties, and (3) NO reacts with transition metals, activating soluble guanylyl cyclase, by which many of NO's homeostatic functions are mediated.
Enzymatic production in mammals

NO is synthesized by oxidation of the guanidine terminal nitrogen in L-arginine by NO synthase (NOS), yielding equimolar amounts of citrulline, a reaction dependent on nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrate and flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin as co-factors. NOS exist in three different isoforms; two calcium-dependent, constitutive forms (cNOS), and one calcium-independent inducible form (iNOS). The two different isoforms of cNOS are named after the cell types in which they were first characterized; endothelial NOS (eNOS, NOS III) and neural NOS (nNOS, NOS I).

The activity of cNOS is regulated by agonists (e.g. acetylcholine and glutamate) or physical events (e.g. shear stress) resulting in increased intracellular calcium concentration generating bursts of NO in the picomolar range. Despite the nomenclature, eNOS is found in smooth muscle cells, mucosal cells and epithelial cells in addition to endothelial cells, and nNOS is expressed in platelets, skeletal muscle cells besides neurons.

iNOS is absent in resting cells, hence NO production by iNOS requires de novo protein synthesis. The expression of iNOS is controlled by the transcription factor nuclear factor (NF)-κB, which is activated by lipopolysaccharide and proinflammatory cytokines such as TNF-α, interleukin (IL)-1β and interferon (INF)-γ (Figure 2).
Figure 2. The expression of iNOS and proinflammatory cytokines such as TNF-α, IL-1β and INF-γ is controlled by NF-κB. Under basal conditions NF-κB forms an inactive complex with inhibitor IκB proteins (IκB) in the cytoplasm. Inflammatory activation signals such as LPS, TNF-α, IL-1β and INF-γ activates IκB kinases which results in the release of NF-κB. Once released, NF-κB tranlocate to the nucleus and binds to specific DNA sequences leading to activation of transcription of iNOS, and proinflammatory cytokines.

Once in function iNOS will produce large amounts of NO for extended period of time. The primary cell types expressing iNOS are macrophages and neutrophils, but virtually all mammalian nucleus-containing cells can be stimulated to express iNOS.  

**Extracellular NO production**

Dietary nitrate (NO₃⁻) is concentrated in the saliva, and rapidly reduced to nitrite (NO₂⁻) in the oral cavity by bacterial reductases, and subsequently the acidification of NO₂ in the gastric lumen results in the formation of NO. NO may also be formed as a result of a direct reaction between HO⁻ and L- or D-arginine. Furthermore, in the colon, NO generation is a consequence of anaerobic microorganism respiration using NO₂ and NO₃ as substrates.
**Biological activity of NO**

NO is recognized as an important modulator of a vast number of physiological events, ranging from cardiovascular homeostatic regulations to neural transmission, immunomodulation and antimicrobial activity. This diversity of actions can be understood regarding NO being a small, highly lipophilic molecule, which readily diffuses between cells. NO does not exert its effects via a specific receptor binding, rather the actions attributed to NO are dependent on which chemical reaction is evoked (Fig. 1).

NO’s homeostatic properties have generally been associated with picomolar concentrations produced by eNOS, maintenance of adequate circulation, regulation of microvascular permeability, modulation of platelet aggregation and adhesion and leukocyte-endothelial interaction.\textsuperscript{76-79}

On the other hand, induction of iNOS has been blamed for the deleterious effects seen at high concentrations of NO, especially in inflammation. This concept has several exceptions, like in pregnancy where iNOS is constitutively expressed and seems to regulate vascular tone in the fetus.\textsuperscript{80-83}

Even in inflammation the role of NO seems to be dichotomous, which has labeled this tiny molecule with epithets such as “the good, the bad and the ugly” and “Jekyll and Hyde”.\textsuperscript{84, 85}

**NO in IBD**

The role of NO in IBD has been demonstrated using a variety of techniques. High concentrations of nitrite in rectal dialysates from patients with IBD has been reported\textsuperscript{86} as well as elevated levels of citrulline in rectal biopsies from patients with UC,\textsuperscript{87} and increased NOS activity in UC.\textsuperscript{88} It is likely that the generation of the high levels of NO seen in IBD are elaborated by induction of iNOS,\textsuperscript{67, 89, 90} even if increased eNOS activity also has been shown.\textsuperscript{91, 92} Induction of iNOS occurs in infiltrating inflammatory cells in the intestinal wall as well as in epithelial cells.\textsuperscript{54, 67, 89, 90, 93, 94}
Aims of the study

To develop a feasible method for assessing luminal concentrations of NO.

To evaluate NO production in UC and CD in relation to inflammatory disease activity.

To investigate if rectal NO measurements are able to detect inflammatory activity in areas from the ileum to the rectum in IBD.

To determine whether rectal NO sampling is applicable to monitor inflammatory activity in children with UC and CD.

To identify the cellular source of the measured luminal NO.

To examine the association between the expression of iNOS and the proinflammatory cytokines TNF-α, IL-1β and INF-γ in the mucosa from patients with UC and CD.
Materials and Methods

Patients and healthy controls (papers I-V)

We studied adult and pediatric patients with UC and CD in remission as well as in active phase. The diagnoses were defined according to established clinical practice, and based on clinical symptoms, histological, endoscopical or radiological findings as described by Leonard-Jones and later Shivanada.\textsuperscript{11-13}

The study population comprised of 151 cases; 54 with UC and 97 with CD. Among the UC population 14 patients were in remission and 40 had active disease; the corresponding figures for CD was 10 cases in remission and 87 with active disease. With the exception of six pediatric patients with ileo-cecal CD (paper II), all patients had colo-rectal CD. The distribution of UC and CD was based on ileo-colonoscopy in all cases, with additional scintigraphy in 14 children with CD (paper II).

Discrimination between UC in remission and active phase was based on the category scale according to Truelove-Witts\textsuperscript{19} in paper I, III, and on the numerical DAI by Sutherland\textsuperscript{21} in paper IV and V. DAI was also used to analyze correlations between disease activity and other parameters in the case of UC. According to the Truelove-Witts’ criteria, remission was defined as absence of any activity, and according to DAI remission was defined as a score ≤ 2.

The numerical HBI\textsuperscript{24} was used to separate CD in remission from active disease, as well as to analyze correlations between disease activity and other parameters. An HBI score ≤ 4 defined remission.

The degree of endoscopical inflammation was categorized according to a four-step scale, 0 (normal mucosa), 1 (erythema and edema), 2 (in addition petechial bleeding and friable mucosa), 3 (in addition spontaneous bleeding and ulcers), previously used by Baron and Ginsberg among others.\textsuperscript{95, 96} This scale is also a part of the DAI.\textsuperscript{21}
Standard blood test (white blood cell count (WBC), hemoglobin (Hb), platelet count, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)), were used as surrogate markers of inflammation for correlation analyses.

The control group used for rectal NO measurements consisted of 54 healthy individuals. In case of immunohistochemical staining the control biopsies were sampled from individuals (n=12) having colonoscopy performed due to suspected polyps or colorectal cancer. Mucosal biopsies were only taken from patients were colonoscopy was either normal or showed minor benign polyps, hence no case of malignancy was included in the control group.

_Sampling of rectal NO (papers I – V)_

The basic concept of applying rectal gas sampling with a balloon catheter was: (1) NO is stable at low concentrations in ambient air,\(^5\) (2) NO diffuses easily and (3) the gas in the colon is spread within the extension of the lumen.

Pre-experimental assessment of different balloon catheters preceded the studies. Two different catheter types were used. In paper I we used a tonometry catheter developed for CO\(_2\) sampling from the sigmoid colon of patients in intensive care units; the TRIP\(^\text{TM}\) Sigmoid Catheter (Tonometrics Division Instrumentarium Corp., Helsinki, Finland). The Argyle\(^\text{TM}\) catheter (Sherwood Medical, Tullamore, Ireland), originally developed for catheterization of the urinary bladder, was used in papers II – V. Both catheters are made of 100% silicone, the Argyle\(^\text{TM}\) catheter having thinner silicone dimension.

The catheter was inserted into the rectum, using lubrication gel free of local anesthetics, to a level 20 cm (paper I) or 10 cm (papers II-V) above the anal sphincter. The balloon was inflated with 5 ml (paper I) or 10 ml (papers II–V) of ambient air (< 5 ppb NO), and left for 15 min (paper I) or 10 min (papers II–V) to equilibrate with gases in the rectum. Thereafter, the gas was withdrawn from the catheter balloon and diluted to a final volume of 50 ml before chemiluminescence analysis with correction for dilution. Analyses were performed within 15 min of sampling.
**Assessment of balloon catheter recovery rate (papers I and II)**

The catheters were inserted in a canister with known concentrations of NO gas (AGA AB, Lidingö, Sweden), ranging from 100 to 100,000 ppb. The NO concentrations were kept stable, continuously monitored with a chemiluminescence analyzer (CLD 700, Eco Physics, Dürnten, Switzerland). The catheter balloons were filled with different volumes of ambient air (< 5 ppb NO) and incubated inside the canister for 1, 5, 10, 15, 20, 30, 45, 60 and 90 min. Gas samples were withdrawn from the balloons and subsequently analyzed for NC. The recovery was calculated as percentage of the actual NO concentrations (papers I and II).

**Estimation of the contribution of fecal NO production**

Two indirect methods were used. In paper I, patients and controls prepared for colonoscopy with water enema and ingestion of isotonic polyethylene glycol solution were compared with subjects with no preparation.

In paper III, fresh fecal samples from four patients with active IBD were incubated in a closed canister (volume 400 ml) for 30 min and NO content was thereafter sampled with Argyle™ catheters incubated in the canister for 10 min.

**Immunohistochemistry (papers IV and V)**

Rectal mucosal biopsies were sampled through flexible sigmoidoscopy at day 1, 3, 7 and 28. Biopsy specimens were always taken in the vicinity of lesions. In case of colectomy (n = 8) (paper IV), the surgical specimens were additionally used for analyses. All endoscopical, as well as surgical biopsies were kept in Histolab (Histolab, Göteborg, Sweden) on ice and snap-frozen in liquid nitrogen within 30 min pending immunohistochemical evaluation.

**NOS, TNF-α, IL-1β and INF-γ immunohistochemistry staining (papers IV and V)**

Six μm thick cryostat sections were mounted on gelatin-coated glass slides, air-dried and stored at -80 °C. Before staining, the slides were thawed at room temperature and subsequently fixed in cold 2% formaldehyde in phosphate buffered saline (PBS). The
following incubation and washing steps were performed in PBS supplemented with 0.1% saponin (Sigma Chemicals, St Louis, MO, USA) to permeabilize cellular membranes. Endogenous peroxidase activity was blocked with a combination of 0.3% hydrogen peroxides and 0.1% sodium azide. The sections were treated with normal human serum and normal goat serum (Vector laboratories Inc, Burlingame, CA, USA) to reduce unspecific binding of the antibodies and with Avidin/Biotin blocking kit (Vector laboratories) in order to reduce unspecific binding to the ABC-elite reagents. The sections were incubated over night at +4 °C with mouse monoclonal antibodies to iNOS (NOS-1N; 20 μg/mL, Sigma), nNOS (NOS-BI; 73 μg/mL, Sigma), eNOS (NOS-EL; 17 μg/mL, Sigma), IL-1β (2D8; 1.4 μg/mL, ImmunoKontact, Abingdon, Oxon, United Kingdom), TNF-α (Mab I; 10 μg/mL + Mab II; 14 μg/mL, Pharmigen, San Diego, CA, USA), and to INF-γ (7-B6; 10 μg/mL+ 1-DIK; 10μg/mL Mabtech AB, Nacka, Sweden). Mouse IgG1 (28 μg/mL, Dako, Glostrup, Denmark) served as an isotype-matched negative control. Biothionylated goat-anti mouse IgG1 (4 μg/mL, Caltag laboratories, Burlingame, CA, USA) was used as secondary antibody. For the eNOS stainings an IgA antibody was used which requires another common biothionylated goat-anti mouse immunoglobulin (11 μg/mL, Dako). The biothionylated secondary antibodies were followed by horse-radish peroxidase-conjugated avidin/biotin-complex (Vectastain, ABC-elite, Vector laboratories). Positive peroxidase staining was developed with 3, 3'-diaminobenzidine (DAB peroxidase substrate kit, Vector laboratories) and counterstained with haematoxylin (Histolab).

Quantification of cells stained by immunohistochemistry (papers IV and V)
All microscopic evaluations were performed by one investigator (T.L.), who was blinded to the clinical data as well as analyzed parameter (eNOS, nNOS, iNOS, TNF-α, IL-1β, INF-γ and IgG1, respectively). Sections were analyzed using light microscope (Nikon Ltd, Tokyo, Japan). For each section three different grid-areas rich in positive cells, in the lamina propria and of sufficient technical quality were chosen for quantitative analysis of NOS and cytokine expression. High power magnification (X400) was used. For each area the number of positive cells was registered, thereafter the total number of positive cells
was divided with the total grid-area. For each section the result is expressed as the mean number of positive cells per grid-area.

*Study design (papers I – V)*

In papers I - III single measurements were performed in each individual and the results are based on comparisons between study groups; i.e. healthy controls, UC and CD in remission and in active phase, respectively, and ileo-cecal versus ileo-colonic CD (paper II). In paper III repeated measurements were additionally performed in a small subgroup (n = 4) of patients in both active phase and clinical remission.

In papers IV and V the time-course of rectal NO and mucosal expression of NOS and cytokines were assessed using repeated measurements; baseline values (day 1), day 2, day 7 and day 28 during glucocorticosteroid treatment (paper IV) and infliximab treatment (paper V). These studies were performed in an open-labeled, prospective manner, however the evaluation of the immunohistochemistry staining was assessed using a single-blinded procedure.

*Statistical analysis (papers I – V)*

For statistical analysis and graph plotting GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used. One-way analysis of variance (ANOVA), Wilcoxon paired t-test, Mann-Whitney test and Kruskal-Wallis test were applied as described in each paper. Correlation was analyzed with the Spearman rank-test. Data are expressed as mean ± SEM and range, where appropriate. P < 0.05 was considered significant.
Results and Discussion

Recovery rate of the balloon catheters (papers I and II)
The TRIP\textsuperscript{TM} catheters had a recovery rate of 33\%\pm2.4\% at 15 min \textit{in vitro}. The values obtained \textit{in vivo} were therefore corrected with a factor of 3 (paper I). The Argyle\textsuperscript{TM} catheters used in the subsequent papers had a recovery rate ranging from 62\%\pm0.8 to 66\%\pm1.7 \% depending on NO concentration. No correction factor was used for the values obtained \textit{in vivo} (papers II-V).

Rectal NO levels in healthy controls (papers I – V)
Using the Argyle\textsuperscript{TM} catheters adult healthy controls displayed stable mean rectal NO values of 89\%\pm13 ppb (paper V) and 100\%\pm17 ppb (paper IV). Somewhat higher values (681\%\pm91 ppb) were obtained using the TRIP\textsuperscript{TM} catheters. This difference is partly explained by the fact that no correction factor was used with the Argyle\textsuperscript{TM} catheters, which should have been approximately 1.5 in accordance with the \textit{in vitro} experiments. But even if such a correction factor was added, there is still a difference between the levels recorded using the Argyle\textsuperscript{TM} and the TRIP\textsuperscript{TM} catheters. This might be due to differences in time to achieve steady-state. Thus, we considered the Argyle\textsuperscript{TM} catheters be more feasible for rectal sampling of NO.
In the pediatric control group the Argyle\textsuperscript{TM} catheters were used. Low rectal NO levels of 60\%\pm16 ppb (paper II) and 75\%\pm17 ppb (paper III) were found.

Rectal NO levels in IBD (papers I – V)
In adults, greatly increased mean rectal NO levels were found in active UC ranging from 6983\%\pm2215 ppb (paper I) to 13270\%\pm3600 ppb (paper II). Children with active UC displayed NO levels of the same magnitude, 5500 (range 950-34000) ppb (paper II) and 8842\%\pm5120 ppb (paper III). The rectal NO levels found in patients with UC in remission were significantly lower (593\%\pm60 ppb in adults; paper I, and 338\%\pm116 ppb in children; paper III).
In active CD, rectal NO levels were comparable with those in UC, in adults ranging from 3578±1199 ppb (paper V) to 15895±4297 ppb (paper I), and in children 4550 (range 300-49350) ppb (paper II) to 12820±4497 ppb (paper III). As in UC, the rectal NO levels found in CD patients in remission were significantly lower (184±53 ppb in children, paper III).

Rectal NO in relation to disease localization (paper II)
Rectal NO measurements in children with active ileo-cecal CD showed significantly increased levels 2625 (range 300-15000) ppb (p<0.01) as well as children with active colorectal CD 5675 (range 300-49350) ppb (p<0.001) compared to controls. Hence, rectal NO measurement was able to reveal ileo-cecal inflammation, but with less power compared colo-rectal disease.

Rectal NO measurements as a marker of disease activity in IBD (papers III - V)
Repeat rectal NO measurements in adults with UC showed that the NO levels decreased when patients achieved remission (from 20017±5886 ppb to 917±495 ppb, p<0.01) (paper IV). In CD the corresponding values were 10178±2706 ppb when in active phase and 4203±2858 ppb in remission, p<0.01 (paper IV) and 3926±1687 ppb in active phase versus 1050±428 ppb in remission, p<0.05 (paper V). All of the four children with CD evaluated in both active phase and remission showed decreased values in remission; mean NO levels of 17830±7110 ppb and 300±143 ppb, respectively (paper III).

By applying numerical clinical activity scores we tested the hypothesis that rectal NO measurements could serve as an objective quantitative marker of disease activity in UC and CD with colo-rectal distribution. Rectal NO levels correlated with DAI in UC (r=0.34, p<0.01), and with HBI in CD (r=0.48, p<0.01). This association was strengthened in the groups achieving remission (r=0.72, P<0.001 for UC and r=0.64, p<0.001 for CDI (paper IV). In paper V correlation between rectal NO levels and HBI could not be demonstrated in spite of a significant decrease of rectal NO levels in patients with active disease at baseline (day 1) and in remission at follow-up (day 28). This finding is in line with the results in paper IV showing that rectal NO levels did not
correlate with HBI in patients not responding to glucocorticosteroid treatment, as the studied population in paper V consisted of corticosteroid-refractory patients. Further, the HBI score is more an assessment of symptom burden, i.e. illness, than one of inflammatory activity. We found that rectal NO levels correlated to the endoscopic score, i.e. the ongoing inflammation in this subgroup of patients (r=0.42, CL 0.22 – 0.59, p<0.001) (paper V). No relationship between rectal NO levels and blood chemistry values such as WBC, Hb, platelet count or CRP were found. This disparity in results may be due to the fact that NO measures inflammatory activity on site, whereas the other variables reflect the systemic response to a severe inflammatory disease.

Of special interest, among corticosteroid-refractory patients no association was found between rectal NO levels and disease activity (papers IV and V). This is explained by a sub-group of patients where colectomy had to be carried out due to treatment-refractory severe disease. This group of patients displayed a different pattern of rectal NO levels in relation to disease activity, having significantly lower rectal NO levels (1110±510 ppb) at baseline compared to patients responding to the treatment (p<0.05) (paper IV). Using a cut-off level of rectal NO ≤ 1000 ppb for patients with severe CD (HBI ≥ 10) at baseline, the rectal NO-test detected 6 out of 7 patients later operated with colectomy due to treatment failure. Only one case of CD with NO ≤ 1000 ppb and HBI ≥ 10 at entry of the study managed without surgery within the forthcoming 28 days (paper IV).

Cellular source of luminal NO (papers I, III, IV and V)
The fecal contribution to luminal NO production is minor. In paper II, we compared rectal NO levels in subjects where the fecal load had been removed due to colonoscopy preparation with water enema in combination with ingestion of polyethylene glycol to those without preparation. Neither for patients with IBD, nor for the control group, were any differences seen depending on preparation or not. Furthermore, in patients with active IBD in vitro studies with incubated fresh feces resulted in low concentrations of NO (136±92 ppb) in the sampling canister, indicating that feces does not significantly contribute to the increased luminal NO levels seen in active IBD (paper III).
To which extent eNOS-produced NO contributes to the basal low levels of luminal NO in healthy subjects and patients with IBD in remission was not assessed in depth in this thesis. However, as eNOS was abundantly expressed, mainly in epithelial cells in all subjects including healthy controls, and that iNOS at the same time was rarely expressed in healthy controls it seems reasonable that the low levels of luminal NO found under physiological conditions are derived from eNOS (paper IV). As we did not find any up-regulation of neither eNOS nor iNOS in IBD, it is not likely that eNOS plays a major role in the production of the greatly increased luminal NO levels seen in active IBD. This conclusion is however hampered by the methods used in paper IV, i.e. quantification of eNOS expression might not reflect the total production capacity as the production is governed by intracellular calcium levels and not by transcription.

The results in papers IV and V indicate that iNOS is the major contributor to the elevated NO production seen in active IBD, as: (1) iNOS expression was significantly increased at baseline in both UC and CD compared to controls (p<0.001 for UC, paper IV and p<0.001 and p<0.01 for CD, paper IV and V, respectively), (2) iNOS expression decreased profoundly in association with therapy. This down-regulation reached statistical significance when UC and CD patients were pooled, comparing baseline values with follow-up at day 28 (p<0.05) (paper IV) and (3). iNOS expression correlated with rectal NO levels for patients with UC (r=0.53, p<0.01). This finding was even more pronounced by sub-analyzing patients achieving remission (r=0.85, p<0.01) (paper IV).

Due to technical limitations we were not able to evaluate the possible induction of iNOS in the epithelial lining, as our isotype-matched negative controls showed unspecific staining of the epithelium. Therefore we had to restrict the immunohistochemical assessments to the lamina propria in our studies. Even so, our results indicate that infiltrating immune cells are of importance for the NO generation seen in IBD. Morphologic characterization of iNOS positive cells in the lamina propria revealed that the majority of these cells were polymorphonuclear leukocytes (paper IV, V).
These findings together indicate that the increased luminal NO levels seen in active IBD are enzymatically produced primarily by iNOS, and that iNOS induction in polymorphonuclear leucocytes is of major importance for the increased NO production in active IBD.

_Cytokine expression (papers IV and V)_

Both TNF-α and IL-1β expression had a similar pattern as iNOS expression, being down-regulated in response to glucocorticosteroid treatment (paper IV) and infliximab treatment (paper V). In case of glucocorticosteroid treatment the decline of cytokine expression was most pronounced at follow-up on day 28, whereas in response to infliximab an early profound down-regulation was seen, reaching maximum on day 3, followed by ε re-accumulation during the following assessments on day 7 and 28. In paper IV only IL-1β demonstrated statistically increased expression at baseline compared to the control group (p<0.05), but in paper V both TNF-α and IL-1β expression at baseline were significantly increased (p<0.01 and p<0.05, respectively).

Expression α:iNOS correlated with that of TNF-α both in UC and CD (r=0.46, p<0.05 and r=0.44, p<0.05, respectively, paper IV). In the case of IL-1β UC and CD patients were pooled to demonstrate a correlation with iNOS (paper IV). INF-γ expression was found to be of less importance compared to TNF-α and IL-1β expression as none (paper IV) or minor (paper V) down-regulation was seen in response to therapy. Neither did INF-γ correlate with iNOS expression nor with IL-β or TNF-α (paper IV).
General discussion

Inflammation, the hallmark of IBD, is a dynamic complex process crucial for survival. The inflammatory response is considered beneficial, aiming at repair of tissue injury and defending the host against invading microorganisms. Under physiological conditions the intestinal mucosa is in an intricate state of controlled inflammation, with cross-talk between mucosal cells and bacteria adherent to the epithelium of the host.\(^9\)

One might regard NO as a pivotal marker of intestinal inflammation. Thus, NO measurements might serve as a surrogate marker of inflammatory activity. It is by now established that UC and CD are associated with substantial overproduction of NO, and that this overproduction is a feature of the active phase of these diseases.

Whether NO act primarily aggressive or protective in IBD is unclear. NO has pro-inflammatory properties by stimulating chemotaxis of neutrophils and monocytes,\(^{100,101}\) enhance cytokine production\(^{102,103}\) and generate superoxide ions.\(^{104}\) Peroxynitrite (OONO\(^-\)) is produced when superoxide (O\(_2^*\)) reacts with NO. Peroxynitrite exerts cytotoxic actions via lipid peroxidation and sulphydryl oxidation\(^{105}\) and enhances the local inflammatory response.\(^{106}\) In addition, NO might contribute to the edema and perturb the intestinal motility associated with IBD.\(^{107,108}\)

In contrast to these deleterious effects, NO also has the capacity to exert anti-inflammatory actions by down-regulating leukocyte-endothelial cell adhesion,\(^{109}\) decrease aggregation of platelets,\(^6\) decrease microvascular permeability\(^{110,111}\) and down-regulate NF-\(\kappa\)B.\(^{112,113}\) This controversy has not been bypassed by applying different experimental animal models of IBD. While early studies showed that inhibition of NOS could ameliorate the inflammation,\(^{114-116}\) the advent of iNOS-deficient mice demonstrates that NO has anti-inflammatory properties in gut inflammation.\(^{117}\) In fact, the field is even more obscure as apparently identical models have generated contradictory results: in the case of hapten-induced colitis, NOS inhibition has resulted in amelioration,\(^{116}\) as well as
exacerbation of the inflammation, and in iNOS deficient mice, using the similar hapten-mode to induce colitis, NO has additionally been ascribed to exert harmful effects.

Several papers have investigated intestinal NO production in active phase and in remission, but little was known about NO production in relation to remission-inducing therapy and degree of severity of inflammation. Our data confirm that active UC and CD result in increased NO production, measured as increased rectal NO levels (papers I – V). Furthermore, we were able to show that increased NO production is not merely a feature separating patients with active IBD from those in remission, rather luminal NO production was shown to correlate with the degree of disease activity measured either with a clinical activity score or with an endoscopic score but not with blood chemistry markers of inflammation. Hence it is tempting to speculate that rectal NO measurements might serve as an objective quantitative marker of disease activity; specifically intestinal inflammation.

As rectal NO levels represent a locally produced gas, it is of interest to evaluate how far from the site of production it is possible to detect increased NO production with rectal measurements. Previous work has demonstrated high intragastric NO concentrations, in the range of 2600 ppb in healthy subjects, which should be compared to rectal levels in the range of 100 ppb in healthy subjects. In celiac disease a 20-fold increase of NO concentrations were seen in the jejunum, but the rectal NO levels in this patient group did not differ from values in healthy controls. Using gas sampling at colonoscopy it has shown that higher NO values were obtained from the lumen in close vicinity to areas of intense macroscopic inflammation than farther from lesions. Nevertheless we demonstrate that ileo-cecal inflammation is readily detected by rectal NO measurements, even if the power was inferior to that found for colo-rectal inflammation. Taken together these data imply that the method to assess intestinal inflammation by measuring rectal NO has sufficient sensitivity to detect intestinal inflammation from the terminal ileum and distally.
It is also important to highlight that increased NO production is an unspecific marker of intestinal inflammation seen in celiac disease as well as in infective gastroenteritis. Hence, the clinical usefulness of rectal NO measurements lies within its ability to monitor inflammatory disease activity, whereas the diagnoses of UC and CD still rest on clinical, histological, endoscopic or radiological grounds.

We single out that iNOS induction in polymorphonuclear leukocytes in the lamina propria are an important source of the increased luminal NO levels seen in active UC and CD, as the iNOS expression in these cells showed significant correlation with rectal NO levels. This seems to be a reasonable hypothesis as the invasion of polymorphonuclear leukocytes in the mucosa is an early feature of relapse in IBD. Furthermore, the most prominent histological feature found in response to infliximab treatment in CD was the reduction of neutrophils in the epithelium and lamina propria. To which extent iNOS in epithelial cells contribute to the NO response seen in IBD could not be addressed in our experiments as these cells showed unspecific background staining. From literature it is likely to assume that epithelial cells may contribute to NO production as well.

Due to NF-κB’s central role as a key regulator of the inducible expression of vast number of genes involved in the inflammatory response such as iNOS, TNF-α and IL-1β, the global down-regulation of iNOS, TNF-α and IL-1β expression in response to glucocorticosteroid treatment seen in paper IV could be explained by the finding that glucocorticosteroids keep NF-κB in its inactive cytoplasmatic form. The corresponding down-regulation of iNOS, TNF-α and IL-1β expression shown in response to infliximab treatment (paper V) is due to the fact that TNF-α is known to induce NF-κB activation, hence anti-TNF-α therapy leads to a global down-regulation of genes controlled by NF-κB.

An intriguing finding in our study was the unexpected low rectal NO values (1100±510 ppb) found in a sub-group of patients referred to colectomy within 28 days from onset of glucocorticosteroid treatment due to failure to control disease activity.
This finding implicates that low rectal NO levels before onset of treatment in combination with high disease activity might predict unfavorable outcome of glucocorticosteroid treatment, i.e. being suggestive for an alternative first-line therapy.

Our finding also fits in the context where NO per se is attributed with protective properties in the inflammatory response. Indeed, several actions exerted by NO act defensively; by regulating mucus secretion, decreasing microvascular permeability, and regulating leukocyte-endothelial cell interactions.

How could then the Janus face of NO in intestinal inflammation be explained in a comprehensive way? One previously favored explanation was that low concentrations of NO, preferably synthesized via eNOS should have protective properties of crucial importance in gut homeostasis, whereas high concentrations produced by iNOS may have deleterious effects. As the amount of information has grown this hypothesis seems too simplistic; high NO production is abundant under physiological conditions, i.e. in the paranasal sinuses, in the stomach and in the maternal uterus, placenta and fetal organs during pregnancy. Having said that, discriminating between the beneficial role of low NO concentrations from eNOS and the harmful effect of the high NO concentrations yielded from iNOS probably holds some truth as the protective properties assigned to NO in experimental models of inflammation are almost uniformly carried out in models mirroring the acute inflammatory response. As NO production via iNOS requires de novo protein synthesis, this means that in acute experiments there is invariably insufficient time for iNOS to be expressed. Thus, the result of NOS inhibition is highly dependent on the timing of the inhibition; early inhibition should lead to exaggerated cell injury and delayed inhibition should result in diminished cell damage.

The cell injury seen in connection with high NO output is not mediated by the molecule itself, but rather by its cytotoxic byproducts generated when NO reacts with oxygen. Reactive nitrogen species are under control by antioxidative enzymes (superoxide dismutase (SOD), catalase, glutathione peroxidase) and free radical scavengers (α-
tocopherol, ascorbate) during physiologic conditions. Inflammation will occur when these antioxidants and scavengers are overloaded or depleted. Hence, it is logical to assume that conditions generating a high output of reactive nitrogen species will lead to inflammation and consequently cellular injury and dysfunction.

The reactive nitrogen species dinitrogen trioxide ($\text{N}_2\text{O}_3$) is known to exert deleterious actions by nitrosation. The production of $\text{N}_2\text{O}_3$ is facilitated at high concentrations of NO and O$_2$. The predominant location for this reaction would be in a lipophilic milieu, like cell membranes where a 310-fold acceleration has been found compared to the cytosol (Fig. 3).$^{133}$

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\begin{align*}
\text{NO} + \text{O}_2 \rightarrow 2\text{NO} + \text{O}_2 \rightarrow \text{N}_2\text{O}_3
\end{align*}
\]

**Figure 3.** The formation dinitrogen trioxide ($\text{N}_2\text{O}_3$) in which the initial oxygenation of NO is rate-limiting. High concentrations of NO and O$_2$ facilitate this reaction, and are found in a lipophilic milieu, such as cell membranes.

Together with a 50-fold increase in NO synthesis during inflammation due to iNOS expression, the potential rate of NO oxygenation could be accelerated 15,000-fold in membranes during inflammation, compared to the cytosol under basal conditions.$^{134}$ Another hypothesis is that NOS exerts a dual function depending on the access of L-arginine (Fig. 4). Under basal conditions, with physiological intracellular concentrations of L-arginine, NOS will generate NO, but as NOS activity is enhanced during inflammation a state of intracellular L-arginine depletion leading to production of $\text{O}_2^{-\bullet}$ in
parallel to NO occurs. $O_2^*$ will then immediately react to form OONO which is known to exert cytotoxic properties.\(^{135,136}\)

**Figure 4.** Nitric oxide synthase (NOS) exerts dual functions dependent on access to L-arginine. (A) Under physiological intracellular concentrations of L-arginine, NO will be generated, (B) In case of intracellular L-arginine depletions NOS will generate superoxide ($O_2^*$) in parallel with NO.

To conclude, one might speculate that the induction of iNOS in inflammation contributes to the cellular injury and dysfunction due to production and effects of reactive nitrogen species such as dinitrogen trioxide and peroxynitrite under specific conditions, whereas NO hardly exhibits deleterious properties by itself. Hence, the favorable outcome of glucocorticosteroid treatment in patients with high rectal NO levels compared with those with low levels $\leq 1000$ ppb could be understood as a marker of a deleterious state with excessive dinitrogen trioxide and peroxynitrite formation and subsequently uncontrolled inflammation in IBD.
Conclusions

Rectal gas sampling via all-silicone balloon catheters is a feasible method to assess luminal NO levels in the colon.

Greatly increased NO-production is found in active UC, and ileo-cecal and colo-rectal CD. Rectal NO levels correlate with disease activity assessed with clinical activity scores and endoscopic inflammatory scores, but not with blood chemistry markers of inflammation in UC and CD. Rectal NO measurements could serve as an objective quantitative marker of disease activity, assessing response to medical treatment. Low (<1000 ppb) initial levels of rectal NO in severely ill IBD patients might signal a corticosteroid-refractory course.

Rectal NO measurements were able to detect ongoing inflammation not only in colo-rectal IBD, but also in ileo-cecal disease, but with less power.

Rectal gas sampling was a feasible method for detecting active UC and CD in children as well as in adults.

Induction of iNOS in polymorphonuclear cells in the lamina propria plays a major role in the production of the increased luminal NO levels in active UC and CD. Bacterial NO production does not contribute to the increased NO production in IBD. The degree of mucosal iNOS expression correlated to the degree of TNF-α and IL-1β, but not INF-γ expression.

Rectal NO measurements thus seem to be a promising aid in monitoring the clinical course of IBD.
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References


105. Beckman J, Beckman T, Chen J, Marshall P, Freeman B. Apparent hydroxyl radical production by peroxynitrite; implication for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620-1624.


