ON THE ROLE OF THE INDUCIBLE ENZYMES iNOS AND COX-2 IN COLITIS

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (I-IV):

I Kankuri E, Asmawi MZ, Korpela R, Vapaatalo H and Moilanen E (1999) Induction of iNOS in a rat model of acute colitis. *Inflammation* **23**:141-152.

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III Kankuri E, Vaali K, Knowles RG, Lähde M, Korpela R, Vapaatalo H and Moilanen E (2001) Suppression of acute experimental colitis by a highly selective iNOS inhibitor N-[3-(aminomethyl)benzyl]acetamidine. *J Pharmacol Exp Ther* **298**:1128-1132.

IV Kankuri E, Hämäläinen M, Hukkanen M, Salmenperä P, Kivilaakso E, Vapaatalo H and Moilanen E (2002) Suppression of pro-inflammatory cytokine release by selective inhibition of iNOS in mucosa of patients with ulcerative colitis. *Submitted manuscript.*

ABSTRACT

Conventional therapy of inflammatory bowel diseases, IBD (Crohn's disease and ulcerative colitis), is currently based on aminosalicylates, steroids, and immunosuppressants like azathioprine. Despite intensive therapy the disease may relapse leading to surgical bowel resections or proctocolectomy. Even though surgery is highly effective in terms of controlling inflammation, novel therapies based on accumulating knowledge of the features of IBD and gut inflammation are needed.

The inflamed mucosa in IBD produces high amounts of prostaglandins and nitric oxide through the inducible enzymes: cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), respectively. The expression and activity of these enzymes are associated with disease severity implicating them as potential anti-inflammatory drug targets. COX-2 or iNOS -related treatments in different models of IBD have yielded ambiguous results ranging from exacerbation of disease to abolition of inflammation. The purpose of the present studies was to provide additional information about the roles of COX-2 and iNOS in gut inflammation.

Induction of COX-2 and iNOS protein and enzyme activity in acute inflammation of the colon was shown using a chemically-induced animal model of colitis. A preferential inhibitor of COX-2, nimesulide, inhibited inflammatory edema formation, infiltration of pro-inflammatory leukocytes and production of prostaglandin E_2 by the inflamed mucosa. However, no beneficial effect on macroscopic disease was found either by treatment with preferential or unselective inhibition of the prostaglandin producing enzymes.

Treatment of acute experimental colitis with a highly selective inhibitor of iNOS not only inhibited pro-inflammatory leukocyte infiltration and formation of inflammatory edema, but also suppressed macroscopic inflammation. This effect of selective iNOS inhibition was studied further *in vitro* in colon mucosal incubations. Expression of iNOS was increased in inflamed as compared with macroscopically uninflamed mucosa of patients with ulcerative colitis. The selective inhibitor of iNOS reduced the release of pro-inflammatory cytokines associated with disease activity from the inflamed samples.

These results suggest that iNOS expression and activity in active gut inflammation are associated with inflammatory damage, and that selective inhibition of iNOS may be beneficial in treatment of colitis. Some anti-inflammatory effect may also be achieved by treatment with selective inhibition of COX-2, however treatment of acute colitis with drugs that inhibit cyclooxygenase enzymes in this study had no effect on macroscopic inflammation. Altogether, the role of iNOS in gut inflammation and related therapeutic strategies warrant further investigation.

ABBREVIATIONS

1400W	N-[3-(aminomethyl)benzyl]acetamidine
AA	arachidonic acid
ASA	acetylsalicylic acid
5-ASA	5-aminosalicylic acid
cAMP	cyclic adenosine 3',5'-monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine 3',5'-monophosphate
COX	cyclooxygenase
COX-1	constitutive cyclooxygenase
COX-2	inducible cyclooxygenase
CD	Crohn's disease
DHA	docosahexaenoic acid
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EET	epoxyeicosatrienoic acid
ELISA	enzyme linked immunosorbent assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GI	gastrointestinal
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
IBD	inflammatory bowel disease
IFN-γ	interferon-gamma
I-kappaB	inhibitor of nuclear factor kappa B
IL	interleukin
IL-1, IL-6	interleukin-1, interleukin-6
IL-1Ra	interleukin-1 receptor antagonist
L-NAME	N^G-nitro-L-arginine methyl esther
LOX	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene; e.g. LTB_4, leukotriene B_4
MPO	myeloperoxidase

mRNA	messenger ribonucleic acid
NF-kappaB	nuclear factor kappa B
NO	nitric oxide; NO^+ nitrosonium cation; NO^-, nitroxyl anion
NOS	nitric oxide synthase
cNOS	constitutive nitric oxide synthases, includes eNOS and nNOS
eNOS, NOS-III	endothelial nitric oxide synthase
iNOS, NOS-II	inducible nitric oxide synthase
nNOS, NOS-I	neuronal nitric oxide synthase
NSAID	non-steroidal anti-inflammatory drug
$ONOO^-$	peroxynitrite anion
PAF	platelet activating factor
PARS	poly(adenosinediphosphate-ribose) synthetase
PG	prostaglandin; e.g. PGE_2, prostaglandin E_2
PGEM	PGE_2 metabolite, 13,14-dihydro-15-keto-prostaglandin E_2
PPAR-γ	peroxisome proliferator activated receptor-gamma
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SASP	salazosulfapyridine, sulfasalazine
sGC	soluble guanylate cyclase
SNAP	S-nitroso-N-acetyl-penicillamine
T_H1	T-helper cell subtype 1
TX	thromboxane; e.g.TXB_2, thromboxane B_2
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF-α	tumor necrosis factor alpha
UC	ulcerative colitis

1 INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic relapsing inflammatory bowel diseases (IBD). In UC, mucosal inflammatory lesions or ulcerations are usually inconsistently found in the large intestine, while in CD they may exist throughout the gastrointestinal tract. The lesions in UC are relatively shallow mucosal ulcerations while in CD they are transmural and deep. The clinical characteristics of both diseases include rectal bleeding, bloody stools, diarrhea, and abdominal pain. Relief is provided by treatment with corticosteroids, aminosalicylates or immunosuppressive agents. However, long-term and high-dose medical treatment for relapsing and active disease frequently has adverse effects. Patients may require surgical removal of the inflamed bowel or the whole large intestine; an operation which can provide considerable relief, but can also cause disability.

In the inflamed mucosa in chronic IBD, the expression and activity of the inducible enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are increased. The former produces large amounts of nitric oxide (NO) aimed at defensive purposes, but resulting in aggravation of inflammation through its reaction with superoxide in an inflammatory focus. The latter enzyme, COX-2, is a key enzyme in prostaglandin synthesis. Production of prostaglandin E_2 (PGE_2) is associated with increased COX-2 expression and activity. Like NO, also PGE_2, has anti-inflammatory and pro-inflammatory properties. It inhibits infiltration of inflammatory cells and production of inflammatory cytokines. On the other hand, in inflammation PGE_2 retains vasodilation and enhances vascular permeability thus contributing to the formation of inflammatory edema.

Pharmacological inhibitors of iNOS are not yet in clinical use while selective inhibitors of COX-2 have recently been launched on the market. In most normal tissues, the expression of these inflammation-associated enzymes is hardly detectable, but is induced in response to proinflammatory stimuli e.g. various bacterial products and endogenous lymphocyte-derived activators of immune responses e.g. interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α).

Many studies so far have addressed the effects of different drugs in respect to nitric oxide synthase (NOS) or cyclooxygenase (COX) inhibition in experimental colitis with conflicting results. Regulation of excess NO or prostaglandin production has however been suggested to provide therapeutic benefit. In order to elaborate on the roles of the inducible enzymes, iNOS and COX-2 in colon inflammation the effects of selective inhibitors of iNOS and COX-2 were studied in an experimental model of rat acute colitis as well as in incubations of colon mucosal tissue samples from patients with UC.

2 REVIEW OF THE LITERATURE

2.1 The inflammatory cascade

The body's inflammatory response is its effort to gain restitution, to reconstitute tissue integrity and to heal the tissue after a detrimental and destructing insult. It is aimed at disposing of foreign and potentially harmful material from the damaged tissue. According to the persistence of the stimulus, this reaction can be divided into acute and chronic stages, which show distinct differences, but also similarities in cellular activation as well as in the release of inflammatory mediators. Regardless of the tissue involved, the inflammatory response utilizes many analogous processes and mechanisms.

2.1.1 Acute inflammation

The acute inflammatory reaction is triggered by a vast array of injurious stimuli such as mechanical or chemical trauma, extensive heat or cold, hypoxia, nutrient deficiency, and microbes. This initial, innate response to an environmental challenge is non-specific and its course is usually similar in all vascularized tissue. Phagocytizing cells are attracted to the site of inflammation through activation of the plasma complement and kallikrein-kinin cascades, clotting system, and the release of cytokines (e.g. IL-8) and leukotriene B_4 (LTB_4) from activated, injured, or dying inflammatory or tissue cells (Table 1). In response to these various chemotactic stimuli, neutrophil granulocytes, capable of ingesting and destroying foreign material and bacteria, are the first to be recruited and penetrate the vascular wall from the systemic circulation. Local inflammatory mediators facilitate this neutrophil emigration, and contribute to the formation of inflammatory edema through the dilation and increased permeability of postcapillary venules resulting in formation of heat and hyperemia. Bradykinin and prostaglandins activate and sensitize tissue peripheral sensory nerves contributing to the generation of inflammatory pain.

Upon activation the phagocytes produce high amounts of reactive oxygen and nitrogen species, and utilize proteolytic enzymes (e.g. elastase) and bactericidal agents (e.g. hypochlorous acid from myeloperoxidase) for defensive purposes, but nevertheless also cause host tissue damage and destruction. They aggravate the acute inflammatory response through the release of chemotactic substances, cytokines, and lipid mediators. The classic hallmarks of inflammation: reddening, edema, heat, pain, and loss of function are all present in acute inflammation.

In order to endogenously limit the inflammatory response, tissue cells produce anti-inflammatory agents, which can inhibit the actions of pro-inflammatory cytokines, proteases, and oxygen radicals. Such mediators include cytokines (IL-4, IL-10), cytokine antagonists (IL-1 receptor antagonist, soluble TNF-α receptor), and enzymes (e.g. antiproteases and superoxide dismutases).

The acute, innate inflammatory reaction ends favorably with its resolution, clearance of foreign material, and healing through regeneration or fibrosis. It may also progress to a more long lasting response, chronic inflammation or abscess formation.

2.1.2 Chronic inflammation

After the acute phase inflammation persists if the initial stimulus is not removed, and the chronic phase of inflammation ensues. Using the stage set by the acute response, increased infiltration and activation of lymphocytes and monocyte/ macrophages overcome the neutrophil granulocyte dominance of acute inflammation. The chronic inflammatory reaction is usually more specifically oriented against components of the primary insult than the acute response, and is therefore characterized as adaptive immunity or the “second line” of defense. This type of specificity relies on the clonal expansion of a specific subset of lymphocytes capable of recognizing the foreign antigen and eliciting a cytotoxic, an immunomodulatory, or an antibody secretory response.

In addition to the subsiding neutrophils, immune effector functions in the chronic phase are elicited through the phagocytic and killing actions of macrophages, cytotoxic T-cells, and natural killer cells. They regulate the functions of, and their functions are regulated by T-helper-cells and B-cells, which secrete cytokines and antibodies, respectively. Activating cytokines e.g. IL-1, IL-6, or TNF-α augment many cell mediated immune functions while inhibitory cytokines e.g. IL-4, IL-10, or transforming growth factor beta suppress them. As in acute inflammation, the process is driven by the interaction of many mediators, but still by using the same effector pathways. The most favorable outcome of chronic inflammation is healing either through scarring or regeneration.

Granulomatous inflammation is a hallmark of some chronic inflammatory processes, and is characterized by the presence of an aggregate of activated macrophages (some joint together as epithelioid giant cells) encompassed by lymphocytes. Granulomas are found in tuberculosis and sarcoidosis, but are a distinct feature of CD as well. Interestingly, unregulated cellular activation and unbalanced cytokine profile are implicated in e.g. UC and CD and are regarded to ultimately contribute to the perpetuation and chronic relapsing nature of these diseases.

Table 1. Some chemical mediators of inflammation and their inflammatory properties. Adapted from Cotran *et al.* (1994).

Source		Agent		Actions
CELLS	Preformed	Histamine		Vascular dilatation and permeability ↑
		Serotonin		Vascular permeability ↑
		Lysosomal enzymes		Vascular permeability ↑, chemotaxis, tissue destruction, anti-microbial
	Newly synthesized	Prostaglandins	PGE_2	Vasodilation, pain
			TXA_2	Vasoconstriction
		Leukotrienes	LTB_4	Chemotaxis, leukocyte activation
			LTC_4, LTD_4, LTE_4	Vasoconstriction, vascular permeability ↑
		Lipoxins		Neutrophil chemotaxis ↓, macrophage activation
		Platelet-activating factor		Vascular dilatation and permeability ↑, chemotaxis, neutrophil activation and degranulation
		Cytokines		Divergent effects e.g. IL-8: chemotaxis ↑, IL-1, IL-6, TNF-α: Inflammatory response ↑
		Nitric oxide		Leukocyte migration ↓, anti-microbial, tissue destruction (metabolism) ↑
		Oxygen metabolites		Anti-microbial, tissue destruction ↑
PLASMA	Complement activation	Anaphylatoxins		Leukocyte chemotaxis↑ and activation, degranulation of mast cells and neutrophils
		Membrane attack complex		Phagocytosis
	Hageman factor activation	Kallikrein-kinin pathway		
			Kallikrein	Positive feedback activation of kallikrein-kinin pathway
			Bradykinin	Vascular permeability and dilatation ↑, pain
		Coagulation/fibrinolysis system		
			Plasmin	Complement and fibrin cleavage and activation
			Fibrinopeptides	Chemotaxis ↑, vascular permeability ↑
			Thrombin	Chemotaxis ↑, fibrosis ↑

↑, increase or activation; ↓, decrease or inactivation; IL-1, interleukin-1;PGE_2, prostaglandin E_2; LTB_4, leukotriene B_4; TNF-α, tumor necrosis factor alpha; TXA_2, thromboxane A_2

2.2 Nitric oxide

Nitric oxide (NO) is a radical, highly reactive and diffusible gas (dissolved nonelectrolyte in biological fluids), which is formed in the body in a number of different cell types through a reaction catalyzed by homodimeric, nitric oxide synthases (NOS) (Moncada *et al.*, 1991; Moncada, 1992; Änggård, 1994; Schmidt and Walter, 1994). The reaction catalyzed by NOS (Figure 1) requires a number of different co-factors (Table 2) (Knowles and Moncada, 1994; Marletta *et al.*, 1998). Three types of NOS have been identified, two isoforms of which are constitutive and calcium-dependent, and one is inducible and calcium-independent (Table 2) (Alderton *et al.*, 2001). The expression of the inducible isoenzyme (inducible NOS, iNOS, NOS-II) is induced by bacteria-derived lipopolysaccharide, mitogenic stimuli, and proinflammatory cytokines (Förstermann and Kleinert, 1995; Farrell and Blake, 1996). iNOS was originally found in activated macrophages (Table 2) (McCall *et al.*, 1989; Yui *et al.*, 1991). It produces high amounts of NO, at approximately 1000 times the concentrations achieved by the constitutive enzymes, for immunodefensive purposes (Nathan, 1997; Coleman, 2001). The expression of iNOS has been found to be increased in sites of active inflammation in many inflammatory diseases e.g. inflamed synovia in rheumatoid arthritis (Stichtenoth and Frolich, 1998), and inflamed colon mucosa in colitis (Singer *et al.*, 1996; Guslandi, 1998). A low level of physiological iNOS expression is also present in the normal (Roberts *et al.*, 2001b) and uninflamed colon (Colon *et al.*, 2000).

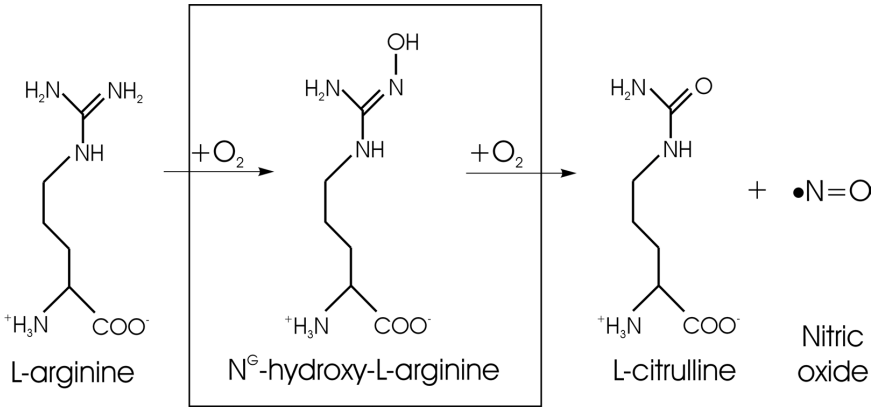

Figure 1. The reaction catalyzed by nitric oxide synthases.

NO for the regulation of physiological responses is produced by consitutively expressed NOS enzymes found in many cell types (Table 2) (Förstermann and Kleinert, 1995). One of the NOS enzymes was initially found in nerve cells, thus it was named neuronal NOS (nNOS or NOS-I) (Garthwaite *et al.*, 1988; Bredt *et al.*, 1991; Mayer and Andrew, 1998). nNOS derived NO functions as a neuronal messenger and serves diverse functions related to memory and neuronal signaling (Brenman and Bredt, 1996; Prast and Philippu, 2001). In the periphery, NO is one of

the transmitters released from the non-adrenergic, non-cholinergic (NANC) nerves (Lefebvre, 1995) and it regulates gastrointestinal (Bult *et al.*, 1990; Sanders and Ward, 1992), airway (Belvisi *et al.*, 1992) and cerebral artery (Toda and Okamura, 1996) smooth muscle tone, and penile erection (Burnett *et al.*, 1992). Endothelial NOS (eNOS or NOS-III) is a NO-producing enzyme found initially in vascular endothelial cells (Marsden *et al.*, 1992; Knowles and Moncada, 1994). NO produced by eNOS acts as a vasodilating and antiaggregatory substance (Moncada *et al.*, 1991; Schmidt and Walter, 1994). A portion of NO in the body is formed by a NOS-independent mechanism. NO is formed e.g. through the reduction of nitrite by bacterial nitrite reductase in the gut (Duncan *et al.*, 1995), or in acidic conditions of the stomach and skin (Weitzberg and Lundberg, 1998). *In vivo*, preformed nitric oxide can be stored in and released from nitrosothiols (Hogg, 2002).

Table 2. Some features of nitric oxide synthases.

	nNOS, NOS-I	**eNOS, NOS-III**	**iNOS, NOS-II**
Molecular weight	160 kDa	130 kDa	130 kDa
Expression	Constitutive	Constitutive	Inducible
Expressional control			+: LPS, IL-1, TNF-α -: glucocorticoids
Expressing cells	Neurons	Endothelial cells	Macrophages, lymphocytes, epithelial cells, smooth muscle cells etc.
NO production	Picomoles	Picomoles	Nano- to micromoles
Cellular localization	Cytosolic	Membrane associated	Cytosolic
Cofactors	Calmodulin, tetrahydrobiopterin, NADPH, FAD, FMN, heme		
$[Ca^{2+}]_i$-activated	+	+	-
Post-translational modifications	Homodimerisation	Homodimerisation, Myristoylation	Homodimerisation

FAD, flavine-adenine dinucleotide; FMN, flavine mononucleotide; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor alpha; NOS, nitric oxide synthase; NADPH, reduced form of nicotine adenine dinucleotide phosphate; $[Ca^{2+}]_i$, intracellular calcium

In relation to its oxidation state, nitric oxide may exist as the nitroxyl anion (NO^-), NO, or the nitrosonium cation (NO^+) (Gow and Ischiropoulos, 2001). Due to this chemical variability, it has been suggested that the NOS enzymes should therefore be called nitrogen oxide synthases (Alderton *et al.*, 2001). The nitrosonium character of NO is thought to be responsible for nitrosothiol formation and subsequent modifications in the activity of ion channels, enzymes, G-proteins, or neuronal N-methyl-D-aspartate receptors (Broillet, 1999). The reactions of NO^- have not yet been clearly dissected and defined. However, the cytotoxic effects of the NO^- -donor, Angeli's salt may help

clarify some aspects of this putatively detrimental side of NO-biochemistry (Colton *et al.*, 2001; Miranda *et al.*, 2001). For the sake of clarity, if not otherwise stated, the term NO is used throughout the text for all these molecular species.

The effects of NO can be divided into either direct or indirect forms (Table 3), and are associated with low or high NO-concentrations, respectively (Broillet, 1999; Davis *et al.*, 2001). The principal actions of NO are mediated by its binding to the heme-moiety of the soluble guanylate cyclase (sGC) leading to enzyme activation and the subsequent increase in intracellular cyclic guanosine 3',5'-monophosphate (cGMP) concentrations (Katsuki *et al.*, 1977; Hobbs, 1997; Lucas *et al.*, 2000). cGMP is a second messenger of NO functions and regulates the activity of cGMP-dependent protein kinases, cGMP-gated ion channels, and phosphodiesterases (Biel *et al.*, 1998; Smolenski *et al.*, 1998; Lucas *et al.*, 2000). In addition to activating sGC, NO may affect the functions of other heme containing enzymes such as cyclooxygenases (Salvemini *et al.*, 1993) or mitochondrial aconitases (Gardner *et al.*, 1997). NO has also been shown to have direct effects on ion-channels (Volk *et al.*, 1997; Janssen *et al.*, 2000). Indirect effects of NO are mediated through the formation of reactive nitrogen species (RNS) (Davis *et al.*, 2001). Oxidation of NO results in formation of RNS either through auto-oxidation (reaction with molecular oxygen) or through the reaction with superoxide (Hughes, 1999).

Table 3. Effects of nitric oxide at the cellular level.

Direct effects mediated by NO in low NO concentrations
- Reactions with Fe- proteins e.g. hemeproteins (guanylate cyclase)
- Reactions with Zn- proteins e.g. Zn-finger proteins (p53)
- Formation of nitrotyrosine with tyrosyl radicals
- Anti-oxidative effects through reactions with e.g. lipid peroxides
- Nitrosothiol formation, S-nitrosylation
Indirect effects, mediated by NO metabolites at high NO concentrations
- N_2O_3 (derived from NO auto-oxidation) mediated DNA deamination and nitrosylation (nitrosothiol, nitrosamine formation) e.g. nitrosylation of cysteine residues

- Peroxynitrite (derived from the reaction with superoxide) mediated:
  - protein nitration (formation of nitrotyrosine)
  - lipid oxidation and nitration
  - DNA oxidation and nitration, DNA strand breaks
  - irreversible inhibition of mitochondrial respiration

NO, nitric oxide

In inflammation, excessively high concentrations of NO are produced by iNOS for mainly defensive purposes (Nathan, 1997). For example, in an experimental model of colitis iNOS deficient mice showed an increased inflammatory response and delayed

healing when subjected to colitis induction suggesting that iNOS has anti-inflammatory activity and is important in resolving inflammation (McCafferty *et al.*, 1999). However, a dualistic role of iNOS-derived NO in inflammation presents itself through NO-metabolism. Under oxidative stress, NO rapidly reacts with superoxide (O_2^-) to produce the toxic, oxidizing and nitrosating peroxynitrite anion ($ONOO^-$) (Koppenol *et al.*, 1992; Beckman and Koppenol, 1996). The reaction between NO and O_2^- occurs more rapidly than the neutralization of superoxide through superoxide dismutases (Pryor and Squadrito, 1995). It has been shown that iNOS may produce O_2^- instead of NO when depleted of substrates or co-factors e.g. L-arginine or tetrahydrobiopterin (Xia *et al.*, 1998; Vasquez-Vivar and Kalyanaraman, 2000), and thereby directly contribute to oxidative state. Peroxynitrite is highly toxic and causes mutations in DNA (Epe *et al.*, 1996; Szabo *et al.*, 1996; Burney *et al.*, 1999), disruptions in mitochondrial function and respiration (Castro *et al.*, 1994; Sharpe and Cooper, 1998; Brown, 1999; Bringold *et al.*, 2000), and modulations in protein thiol, tyrosine and prosthetic groups (Beckman and Koppenol, 1996; Bouton, 1999). The role of peroxynitrite is further complicated by the fact that it also has other functions including being an antimicrobial agent (Xia and Zweier, 1997; Akaike and Maeda, 2000).

NO has a clearly defined physiological mechanism of action. It binds to its intracellular receptor, sGC leading to stimulation of cGMP production. Beyond this, NO has complex diverse functions related to its local concentrations and metabolism (Table 3). Even at high concentrations NO has anti-oxidative, anti-microbial, and anti-viral effects that benefit the host. However, the production of NO through iNOS and of O_2^- in an inflammatory focus result in increased formation of peroxynitrite and RNS, which are considered detrimental. Moreover, nitrosylation of amines by RNS may yield potent mutagens and carcinogens (Jaiswal *et al.*, 2001).

2.2.1 Nitric oxide synthase inhibitors

The increased production and the detrimental metabolism of NO in inflammation, and the fact that iNOS is almost exclusively expressed in inflamed tissue provide the basis for development of iNOS inhibitors as anti-inflammatory drugs. Inhibition of NOS is achieved through either a mechanism-based, or a cofactor inhibiting or antagonizing manner (Bryk and Wolff, 1999; Hobbs *et al.*, 1999; Alderton *et al.*, 2001). Some of the most used mechanism-based NOS inhibitors are shown in Table 4. Due to similarities in reaction mechanisms between constitutive and inducible NOS, selective inhibition of iNOS has been difficult to achieve. The majority of NOS-inhibitors are substrate analogs i.e. their inhibitory action on NOS can be antagonized with high concentrations of L-arginine (Bryk and Wolff, 1999). One of the oldest NOS-inhibitors is N^G-nitro-L-arginine (L-NNA), which shows selectivity for the constitutive enzymes, particularly for nNOS over eNOS and iNOS in many experimental models (Alderton *et al.*, 2001). L-NNA produces a competitive

irreversible inhibition of both eNOS and nNOS, and is a competitive reversible inhibitor of iNOS (Dwyer *et al.*, 1991; Moore and Handy, 1997). The L-arginine analog, N^G-nitro-L-arginine methyl esther (L-NAME) is a more soluble derivative of L-NNA. L-NAME is hydrolyzed by cellular esterases to form L-NNA (Pfeiffer *et al.*, 1996). In addition to L-NNA and L-NAME many non-selective inhibitors of NOS have been identified. These include the arginine analog N^G-methyl-L-arginine (L-NMA, L-NMMA), and the heme-binding thiocitrulline derivatives L-thiocitrulline (L-TC) and S-alkyl-L-thiocitrulline (Mayer and Andrew, 1998). Treatment with non- or cNOS-selective NOS-inhibitors results in increased vascular smooth muscle tone and blood pressure (Aisaka *et al.*, 1989; Rees *et al.*, 1989), an effect not seen with selective iNOS inhibitors (Garvey *et al.*, 1997).

Recently, a novel highly selective inhibitor of iNOS, N-[3-(aminomethyl)benzyl]-acetamidine (1400W), has become available (Garvey *et al.*, 1997). The non-amino acid structured 1400W has been shown to inhibit iNOS at 50-5000 times lower concentrations than cNOS (Garvey *et al.*, 1997; Alderton *et al.*, 2001). 1400W is an irreversible, or slowly reversible inhibitor of iNOS whereas its inhibitory effect on the constitutive isoforms is more readily reversible (Garvey *et al.*, 1997). Since selective iNOS inhibition is considered a promising field of drug research novel compounds (e.g. isoquinolinamine and thienopyridine -derivatives) are constantly developed (Beaton *et al.*, 2001a; Beaton *et al.*, 2001b).

Other NOS-inhibitors with varying selectivity for iNOS over the constitutive enzymes include aminoguanidine, L-N^G-(1-iminoethyl)lysine (L-NIL), and L-N^G-(1-iminoethyl)-ornithine (L-NIO) (Mayer and Andrew, 1998; Bryk and Wolff, 1999). Aminoguanidine for example is also an inhibitor of diamine oxidase (Kusche *et al.*, 1987) and an antioxidant (Yildiz *et al.*, 1998). These NOS-unrelated effects may complicate interpretation of experimental results when using NOS-inhibitors in experiments (Zhou *et al.*, 2002).

Table 4. Some of the experimentally most used nitric oxide synthase inhibitors and their isoform selectivities. Adapted from Alderton *et al.* (2001).

	Selectivity (fold)		
	iNOS vs. nNOS	iNOS vs. eNOS	nNOS vs. eNOS
L-NNA	0.09	0.11	1.2
L-NMMA	0.7	0.5	0.7
L-NIL, L-NIO	20	30-50	1.3
1400W	32	>4000	>130
Aminoguanidine	5.5	11	1.9

1400W, N-[3-(aminomethyl)benzyl]acetamidine; L-NNA, N^G-nitro-L-arginine; L-NMMA, N^G-methyl-L-arginine; L-NIL, L-N^G-(1-iminoethyl)-lysine; L-NIO, L-N^G-(1-iminoethyl)-ornithine

2.3 Eicosanoids

The 20 carbon backboned fatty acid, arachidonid acid (AA; 5,8,11,14-eicosatetraenoic acid), is a crucial component of the eucaryotic cell membrane. It resides estherized in membrane phospholipids, preferably in phosphatidylcholine or phosphatidylethanolamine. Unesterified, free AA is cleaved from these structures by the actions of phospholipases, mainly by phospholipase A_2 (PLA_2). When released intracellularly, AA is a substrate in the synthesis of a wide spectrum of cellular lipid mediators (Brash, 2001).

2.3.1 Prostanoids

It was found in the 1970s that large amounts of primary prostanoids, prostaglandin D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 (TXA_2) are present and produced in inflammation (Moncada *et al.*, 1973; Velo *et al.*, 1973), and that their production is inhibited by treatment with acetylsalicylic acid –like anti-inflammatory drugs (Collier and Flower, 1971; Vane, 1971; Smith and Willis, 1971).

Prostanoids have many modulatory functions in the inflammation process (Tilley *et al.*, 2001). The main E-series prostaglandin, PGE_2, for example has hyperalgesic effects (Ferreira *et al.*, 1978; Stock *et al.*, 2001) and it is involved in the generation of fever (Aronoff and Neilson, 2001). However, PGE_2 also has anti-inflammatory effects: it inhibits leukocyte and lymphocyte functions in inflammation (Wheeldon and Vardey, 1993; Kaur *et al.*, 1999; Hori *et al.*, 2000; Nataraj *et al.*, 2001).

Prostaglandins are formed from their primary substrate, arachidonic acid (Figure 2). Under certain conditions, other fatty acids such as eicosapentaenoic acid or dihomo-gamma-linolenate can also be utilizied as substrates by the prostaglandin synthesizing cyclooxygenase (COX) enzymes as well. Endogenous arachidonic acid is stored in plasma membranes in phospholipids, and is enzymatically released by the actions of different phopholipases (PL), mainly by PLA_2. Free AA is then converted to PGG_2 and PGH_2 by the COX (also known as prostaglandin endoperoxide H synthase) -enzymes (Figure 2).

To date, two isoforms of COX have been identified (Table 5) (Vane *et al.*, 1998). These enzymes use identical processes in the catalysis of PGH_2, which is then further metabolized either by enzymatic or non-enzymatic pathways into different prostanoids (Smith *et al.*, 1996; Smith *et al.*, 2000). The constitutive isoform of COX, COX-1, is constantly expressed in most tissues under physiological conditions. COX-1 –derived prostaglandins are most important in the regulation of arterial tone, platelet aggregation, mucosal integrity of the gastrointestinal tract, and perfusion maintenance in the kidneys (Eberhart and DuBois, 1995; DuBois *et al.*, 1998; Vane *et al.*, 1998). The expression of the inducible isoform, COX-2, is increased in

response to pro-inflammatory stimuli e.g. IL-1, TNF-α, IL-6, and bacterial lipopolysaccharide (LPS) (Jones *et al.*, 1993; Wu, 1996; Mitchell and Warner, 1999).

Some degree of constitutive COX-2 expression is, however, normally present in different tissues (Vane *et al.*, 1998). Of utmost importance in this respect are the kidneys, the central nervous system, and the female reproductive organs. At these sites, COX-2 activity and prostaglandin production is required for homeostatic and physiological processes (DuBois *et al.*, 1998; Funk, 2001; Hinz and Brune, 2002).

Table 5. Some features of cyclooxygenases.

	COX-1	**COX-2**
Molecular weight	72 kDa	72 or 74 kDa
Expression	Constitutive	Inducible
Expressional control	-	+:LPS, IL-1, TNF-α -: Glucocorticoids
Expressing cells	Ubiquitous	Ubiquitous (macrophages, smooth muscle cells, fibroblasts, epithelial cells, etc.)
Cellular localization	Endoplasmic reticulum	Endoplasmic reticulum, nuclear envelope
Cofactors	Heme, glycosylation required for optimal activity	
$[Ca^{2+}]_i$ –activated	Yes	Yes
Post-translational modifications	Homodimerisation, N-glycosylation	Homodimerisation, N-glycosylation (72kDa species 3 groups, 74 kDa species 4 groups)
Substrate specificity	Narrow (AA, dihomo-γ-linoleate)	Wide due to larger catalytic cleft (AA, dihomo-γ-linoleate, α-linolenate, eicosapentaenoic acid)

AA, arachidonic acid; IL-1, interleukin-1; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha;$[Ca^{2+}]_i$, intracellular calcium

During the first stages of inflammation prostanoids are produced via the constitutive COX-1 activity in response to increased substrate availability due to the activation of phopholipases (Smith *et al.*, 2000). Induction of COX-2 expression requires new protein synthesis and thus time, and is a more delayed process (Wu, 1996). Later on, the increased COX-2 activity is the principal contributor to the production of inflammatory prostanoids (DuBois *et al.*, 1998; Herschman, 1999). It has been suggested, that increased COX-activity and PGH_2 production saturates the isomerase-derived primary prostanoid formation and leads to non-enzymatic production of PGI_2 and PGE_2, the major inflammatory prostaglandins (Brock *et al.*, 1999; Mitchell and Warner, 1999). Also the expression of the PGE_2 synthetizing enzyme (PGE-synthase) has been found to be inducible (Jakobsson *et al.*, 1999).

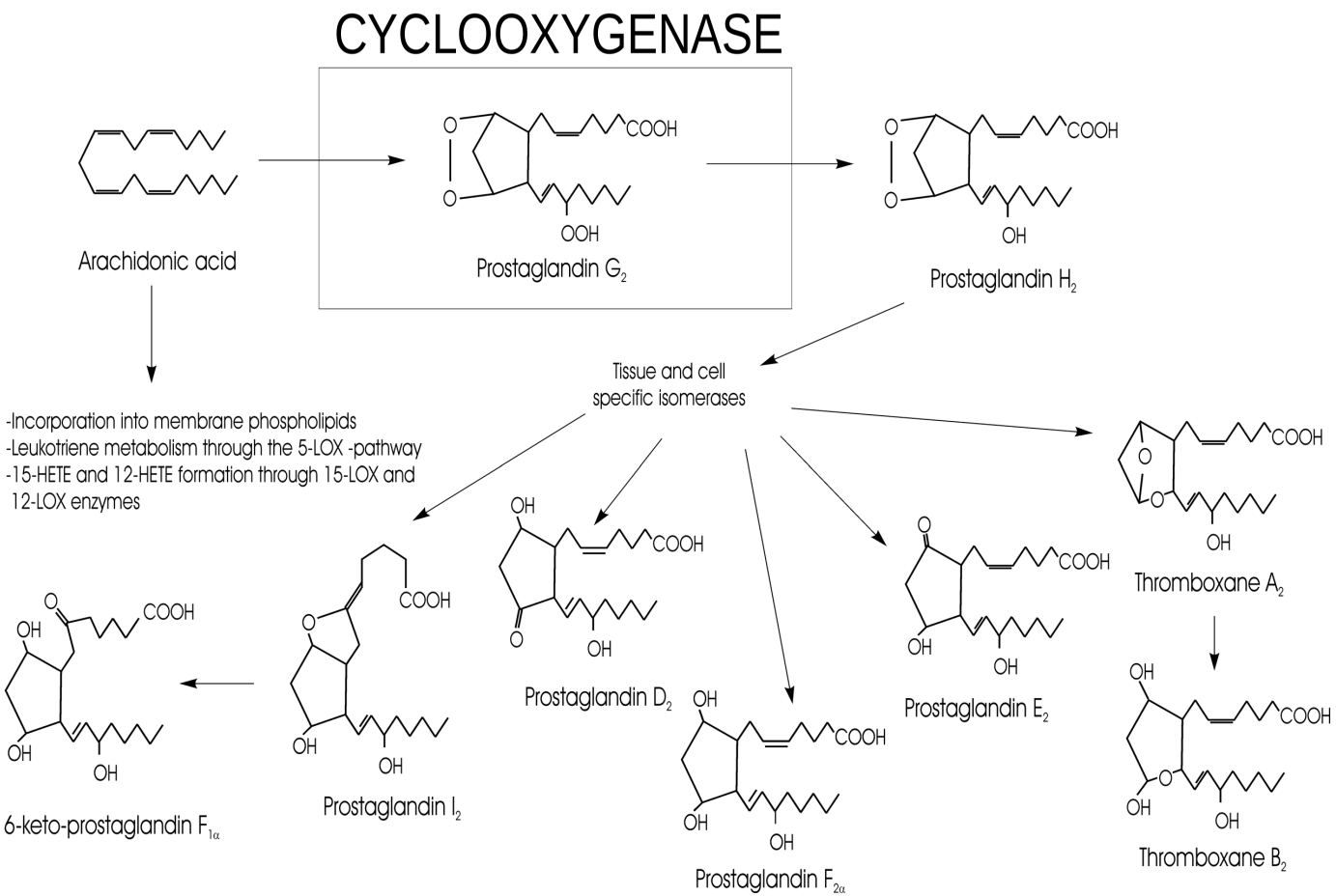

Figure 2. The reaction catalyzed by cyclooxygenase (prostaglandin H endoperoxidase synthase) enzymes and the biosynthesis of primary prostanoids and some of their main metabolites.
HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase

The effects of prostaglandins are mediated through stimulation of their respective receptors (Table 6). For example, stimulation of PGE_2-receptors EP_2 or EP_4 results in increase of intracellular cyclic adenosine monophosphate (cAMP) and subsequent relaxation of smooth muscle. EP_2 activation is also associated with anti-inflammatory effects of PGE_2 such as the inhibition of leukocyte activation. EP_3 stimulation is mediated by a decrease in intracellular cAMP concentrations, and is associated with inhibition of acid secretion from gastric parietal cells. (Narumiya *et al.*, 1999)

Through stimulation of their receptors the prostanoids mediate and regulate many physiological functions in the gastrointestinal tract (Whittle and Vane, 1987; Eberhart and DuBois, 1995). They inhibit gastric acid secretion, increase the secretion of protective mucus, blood flow, and modulate intestinal motility (Eberhart and DuBois, 1995).

Table 6. Prostaglandin receptors, signal transduction and tissue distribution. Adapted from Narumiya *et al.* (1999).

Receptor type	Subtype	Second messenger	Tissue distribution
DP		cAMP ↑	Brain, meninges, intestine
EP	EP_1	Ca^{2+}	Kidney, lung, intestine
	EP_2	cAMP ↑	Uterus
	EP_3	cAMP ↑/↓/PI *)	Kidney tubuli, brain, smooth muscle, intestine, enteric ganglia
	EP_4	cAMP ↑	Kidney glomeruli, intestine
FP		PI	Corpus luteum, kidney, heart, lung, intestine
IP		cAMP ↑/ PI	Platelets, smooth muscle
TP	TPα	PI/cAMP ↓	Lung, kidney, heart, thymus, spleen
	TPβ	PI/cAMP ↑	Lung, kidney, heart, thymus, spleen

↑/↓, increase/decrease; cAMP, cyclic adenosine-3',5'-monophosphate; DP, prostaglandin D_2 receptor; EP, prostaglandin E_2 receptor; FP, prostaglandin F_2 receptor; IP, prostaglandin I_2 receptor; TP, thromboxane receptor; PI, phosphoinositol cascade. *) isoform (e.g. $EP3_{A-D}$) specific action

2.3.2 Other arachidonic acid derivatives

In addition to COX-enzymes, arachidonic acid is metabolized by different lipoxygenases (LOX) to form leukotrienes, hydroxyeicosatetraenoic acids (HETEs), hydroperoxyeicosatetraenoic acids (HPETEs), lipoxins, hepoxilins, and oxylipins, and by cytochrome P450 epoxygenases to form epoxyeicosatrienoic acids (EETs), HETEs and diHETEs (Eberhart and DuBois, 1995; Brash, 1999; Zeldin, 2001). Except for the 5-lipoxygenase (5-LOX) -derived leukotrienes (Funk, 2001), the roles and functions of these other lipid mediators have been less intensively studied, but

there is emerging interest in their mechanisms of action in different physiological and pathophysiological states (Levy *et al.*, 2001).

The 5-LOX enzymes catalyze oxygenation of position 5 in AA and the formation of leukotriene A_4 (LTA_4) (Brash, 1999). LTA_4 may undergo subsequent enzymatic conversion to LTB_4 by LTA_4 hydrolase or to the cysteinyl leukotriene (LTC_4, LTD_4, and LTE_4) precursor LTC_4 by LTC_4 synthase (Funk, 2001).

The granulocytes, mast cells and macrophages express 5-LOX and possess leukotriene synthesis capacity far greater than parenchymal or matrix cells (Peters-Golden, 1998; Funk, 2001). However, LTA_4 secreted by macrophages or neutrophils can be converted by parenchymal cells to these downstream metabolites (Feinmark and Cannon, 1986; Peters-Golden, 1998). LTB_4 is a potent chemotactic activator of neutrophil granulocytes and macrophages in inflammation (Yokomizo *et al.*, 2001), while the cysteinyl leukotrienes increase vascular permeability and induce smooth muscle contraction (Busse, 1998; Sala and Folco, 2001).

2.3.3 Cyclooxygenase inhibitors

For centuries it has been known to man that fever can be suppressed by ingesting an extract from willow bark, which since has been shown to contain high amounts of salicylates. In 1971, it was shown that the mechanism of action of salicylates and other non-steroidal anti-inflammatory drugs (NSAIDs) is their inhibitory action on the prostaglandin producing COX-enzyme. Twenty years later it became evident that there are two COX enzymes, COX-1 and -2, and drugs with selectivity towards the former are associated with increased adverse effects, mainly gastrointestinal ulcerations (Hawkey, 1999; Laine *et al.*, 1999; Warner *et al.*, 1999). Since the discovery of the inflammation induced COX-2 in 1991 (Xie *et al.*, 1991) and the structure of its gene in 1994 (Appleby *et al.*, 1994), there has been an ever-increasing tendency to produce anti-inflammatory drugs that selectively inhibit this inflammation-associated enzyme. The rationale is to produce anti-inflammatory drugs that are targeted to the site of inflammation, suppress a process detrimental to the host while sparing the physiological functions of prostanoids. Recently, after the report that treatment with a selective COX-1 inhibitor (SC-560) was not associated with gastrointestinal damage (Wallace *et al.*, 2000) it was proposed that non-selective inhibition of both COX-enzymes is required for gastric ulceronigenic toxicity. A selection of anti-inflammatory drugs and their isoform selectivities is shown in Table 7.

Acetylsalicylic acid (aspirin, ASA) is the oldest commercial NSAID; it was launched in 1899 (Vane, 2000). Its mode of action is, however, different from the other NSAIDs. ASA covalently and irreversibly acetylates the COX-enzyme at a key position for substrate entry into the enzyme's catalytically active site. Acetylation of the amino acid serine at position 530 or 516 by ASA results in loss of PGH_2 synthesizing activity

of the COX-1 and COX-2 enzymes, respectively (Ferreira *et al.*, 1971; Roth *et al.*, 1975; Mancini *et al.*, 1994; Marnett *et al.*, 1999). Thus, synthesis of prostaglandins from AA is suppressed by treatment with ASA. Due to the relatively fast *de novo* protein synthesis of COX-2, and low turnover of the constitutive COX-1, the inhibitory effect of ASA on COX-1 is pronounced. ASA-acetylated COX-2 produces 15-(R)-hydroxyeicosatetraenoic acid (15-HETE) instead of PGH_2 from AA, and functions principally as a 15-lipoxygenase (Mancini *et al.*, 1994). Furthermore, 15-HETE is metabolised by the 5-LOX –enzymes into lipoxins, which have been suggested to have anti-inflammatory activities (Samuelsson *et al.*, 1987). It is also interesting to note that in the search for more and more COX-2 selective anti-inflammatory drugs even aspirin-like molecules, which covalently, irreversibly, and preferentially inactivate COX-2 have been developed (Kalgutkar *et al.*, 1998).

Table 7. Some inhibitors of cyclooxygenases and their isoform selectivities adapted from Warner *et al.* (1999).

	Selectivity (fold) COX-1 vs. COX-2		
	IC_{50} -ratio (COX-2/COX-1)	IC_{80} -ratio (COX-2/COX-1)	IC_{50} µM (COX-2)
Non-selective			
Acetylsalicylic acid	>100	>100	>100
Ketoprofen	61	22	2.9
Indomethacin	80	11	1.0
Ibuprofen	0.9	1.2	7.2
Selectivity for COX-2			
Celecoxib	0.7	0.21	0.83
Nimesulide	0.19	0.17	1.9
NS398	0.051	0.015	0.35
L745,337	<0.01	<0.01	8.6
Rofecoxib	0.013	<0.05	0.84

IC_{50}/IC_{80}, concentration causing 50/80% of maximal inhibition

Indomethacin is a widely used, potent, non-selective, and slowly reversible inhibitor of both COX-1 and COX-2 (Stanford *et al.*, 1977; Mitchell *et al.*, 1993). Like aspirin and other non-selective NSAIDs, treatment with indomethacin is associated with gastrointestinal bleeding and ulcerations (Warner *et al.*, 1999). It has been proposed that a part of indomethacin's adverse effect is unrelated to COX-inhibition, and that the ulcerogenic activity is associated with the combined disruptive action of indomethacin and bile on the mucosal barrier (Yamada *et al.*, 1993; Lichtenberger, 2001). Indomethacin has been shown to have anti-inflammatory radical scavenging properties *in vitro* (Prasad and Laxdal, 1994).

Nimesulide preferentially inhibits COX-2 over COX-1 in clinically used concentrations (Warner *et al.*, 1999). Nimesulide is a sulphonamide derivative, and has additional

mechanisms of action besides COX-inhibition (Singla *et al.*, 2000). For example, nimesulide is a steroid receptor agonist (Pelletier *et al.*, 1999), inhibits leukocyte functions and chemotaxis (Dallegri *et al.*, 1992; Dallegri *et al.*, 1995), inhibits the activity of complement (Auteri *et al.*, 1988), and functions as an antioxidant (Facino *et al.*, 1993). In accordance with the hypothesis on gastroduodenal toxicity and COX-unselectivity is the fact that treatment with nimesulide is associated with less gastroduodenal lesions as compared to non-selective COX-inhibitors (Warner *et al.*, 1999; Shah *et al.*, 2001).

A novel group of NSAIDs are the COX-2 selective coxibs, rofecoxib and celecoxib (Turini and DuBois, 2002). They are associated with good anti-inflammatory potential and low risk of gastroduodenal side effects (Warner *et al.*, 1999). However, similar to other NSAIDs they inhibit COX-2 activity and production of prostanoids relevant for perfusion maintenance in the kidney and cause fluid retention (Harris and Breyer, 2001).

2.4 Inflammatory bowel diseases

Inflammatory bowel diseases are chronic relapsing diseases of the gastrointestinal tract characterized by bowel wall inflammation, ulcerations, diarrhea, bloody stools, and abdominal pain (Fiocchi, 1998; Ghosh *et al.*, 2000; Hendrickson *et al.*, 2002). The incidence of IBD is higher (4-10 per 10,000) in westernized countries than in developing countries or in the East (Karlinger *et al.*, 2000; Hendrickson *et al.*, 2002). IBD is divided into two separate entities: Crohn's disease (CD) and ulcerative colitis (UC), both of which have distinct differences as explained in detail below and in Table 8. Diagnosis of IBD is based on clinical, endoscopic and histopathological findings. In some cases it may be almost impossible to separate the two disease entities.

No single etiological factor or triggering agent has been identified to cause IBD, thus they are designated as idiopathic diseases. Many factors such as genes and hereditary susceptibility, environmental factors (bacteria, nutrition), and host immune responses have been found to play a role in the pathogenesis of IBD (Fiocchi, 1998). The inflamed colon mucosa in IBD does harbor more bacteria than normal mucosa (Schultsz *et al.*, 1999; Swidsinski *et al.*, 2002) even though defensive responses (production of pro-inflammatory cytokines and other inflammatory mediators, increased proliferation etc.) of the inflamed bowel wall to bacterial agents and cytokines are enhanced (Schreiber *et al.*, 1998). Loss of tolerance to normal luminal contents has been implicated in the pathogenesis of IBD (Nagler-Anderson, 2001). This abnormal host response to bacteria leads to increased permeability, breakdown of the epithelial barrier function and subsequent potentiation of immune responses due to unregulated bacterial translocation in the gut (Linskens *et al.*, 2001). The factors predisposing to this disadvantageous activity of the intestinal mucosa in IBD may include hereditary defects in distinct cellular or humoral responses (Hendrickson

et al., 2002). In both forms of IBD, however, end-point mediators of inflammation are in common.

Dysregulation of host immune reactions, as well as pathological activation of the immune and non-immune systems are acknowledged as possible etiological or at least contributing factors to the perpetuated inflammatory response (Fiocchi, 1998). As pointed out previously, several pathways normally utilized in immune defense converge to promote intestinal inflammation in IBD. These contributing factors include cytokines, growth factors, eicosanoids, neuropeptides, reactive oxygen species, nitric oxide, proteolytic enzymes, antibodies, and autoantibodies (Fiocchi, 1998).

Table 8. Some differences between Crohn's disease and ulcerative colitis. Adapted from Fiocchi (1998), Ghosh *et al.* (2000), and Hendrickson *et al.* (2002).

	Crohn's disease	**Ulcerative colitis**
Location of lesions	Can occur throughout the GI-tract, "skip" lesions	Colonic involvement
Rectal involvement	Infrequent	Frequent
Perianal disease and fistulas	Frequent	Rare
Lesions	Transmural	Limited to mucosa and submucosa
Immunological activation	T_H1 subtype dominant	T_H2 subtype dominant
Cytokine profile	TNF-α, IFN-γ, IL-1, T_H1-profile	IL-4, IL-5, IL-6, T_H2-profile
Humoral	Increase in IgG_2	Increase in IgG_1, ANCA-associated
Cell mediated	+, T-cell infiltration	+, neutrophil infiltration
Environmental factors	Smoking harmful	Smoking beneficial
Genetic factors	Partly different between UC and CD, familial aggregation in both	

ANCA, anti-neutrophil cytoplasmic antibodies; GI-tract, gastrointestinal tract; IFN-γ, interferon- gamma; IgG, immunoglobulin G; IL-1, interleukin-1; T_H1, T-helper subtype 1 -cells; TNF-α, tumor necrosis factor alpha

2.4.1 Nitric oxide

The initial finding of increased nitrite in rectal dialysates from patients with active UC was published in 1986 (Roediger *et al.*, 1986). Increased production of NO in IBD was then reported in 1993, and was measured as increased nitrite and nitrate concentrations in plasma and increased NOS-activity in inflamed colon mucosa of IBD patients (Boughton-Smith *et al.*, 1993a; Boughton-Smith *et al.*, 1993b; Guslandi,

1993; Middleton *et al.*, 1993; Tran *et al.*, 1993). This was followed by the finding that colon luminal gas in UC contained increased NO-concentrations (Lundberg *et al.*, 1994). It is now known that to a great extent the induction of iNOS activity is responsible for this excessive NO production in the inflamed gut (Ribbons *et al.*, 1995; Singer *et al.*, 1996). In inflammation the mucosal NO-production is 1000-10,000 times greater than the normal, physiological NO production (Lundberg *et al.*, 1994); the inflammatory concentrations of NO range from nanomolar to micromolar. Increased production of NO is not specific for IBD. Rather, it is an unspecific inflammatory response triggered and sustained by injury, cytokines, and microbial exposure (Kroncke *et al.*, 1998). In colitis, iNOS has been localized to the epithelium (Godkin *et al.*, 1996; Singer *et al.*, 1996; Dijkstra *et al.*, 1998), to infiltrated or resident cells of the *lamina propria* (Kimura *et al.*, 1998), and to fibroblasts (Ikeda *et al.*, 1997). The expression of iNOS was localized to smooth muscle cells and to the muscular layer, when colitis was associated with toxic dilatation of the colon (Mourelle *et al.*, 1995). It has also been shown that the pattern of iNOS expression may vary according to the stage of inflammation (Vento *et al.*, 2001). In UC, iNOS activity and expression are quite strictly associated with active inflammation (Kimura *et al.*, 1997; Rachmilewitz *et al.*, 1998), in CD this association is less clear (Tran *et al.*, 1993; Kimura *et al.*, 1997). Increased iNOS is found in the cells of inflamed areas together with 3-nitrotyrosine staining suggestive of increased nitrosative stress, RNS and peroxynitrite formation (Singer *et al.*, 1996; Dijkstra *et al.*, 1998; Kimura *et al.*, 1998).

One of the many features of IBD is reduced apoptosis of T-cells (Bu *et al.*, 2001) and increased apoptosis of epithelial cells (Strater *et al.*, 1997) of the intestinal mucosa. NO is a modulator of apoptosis (Chung *et al.*, 2001). As for many other NO-regulated processes also the effects on apoptosis are dose dependent. In low concentrations NO inhibits apoptosis, but increases it at high concentrations supposedly through formation of peroxynitrite (Kim *et al.*, 1999). A pathologic feature of the T-cells in CD mucosa is their resistance to NO-induced apoptosis (Ina *et al.*, 1999). This lack of response may reflect some pathognomonic, supposedly of genetic origin, feature of CD.

Evidence has accumulated which shows that iNOS-derived massive NO production and especially its oxidative metabolism in colitis are detrimental to the host (Guslandi, 1998; McCafferty, 2000). The beneficial effects of NO-supplementation were challenged in a recent clinical trial with enteric release glyceryl trinitrate treatment in CD. In that study no advantageous effects were found on the clinical parameters after a 12-week treatment (Hawkes *et al.*, 2001). However, even though the increased NO concentrations may initially have served an antimicrobial, anti-inflammatory, or homeostatic purpose (Kubes and McCafferty, 2000) it seems feasible to assume that there is lack of appropriate control in the chronically inflamed tissue for suppressing the undesired effects of NO (Whittle, 1997).

2.4.2 Eicosanoids

Increased production of prostaglandins was implicated in the pathogenesis of IBD, especially UC, as early as 1975 (Gould, 1975; Gould *et al.*, 1977; Whittle and Vane, 1987). It was found that disease activity correlated well with the production of PGE_2 from the mucosa (Smith *et al.*, 1979), however attempts to treat colitis with the NSAID, flurbiprofen, failed (Rampton and Sladen, 1981a); even disease relapses were seen during treatment (Rampton and Sladen, 1981b). This led the investigators to conclude that NSAID-therapy was of little or no benefit in UC, and that prostaglandins possibly had a protective role in colon inflammation. However, also treatment with prostaglandin analogs produced either relapses or severe diarrhea (Goldin and Rachmilewitz, 1983; Faich *et al.*, 1991). A role for increased leukotriene synthesis in the pathogenesis of UC was then suggested (Boughton-Smith *et al.*, 1983), and a selective 5-LOX inhibitor was tested in UC-patients with initial promising results (Laursen *et al.*, 1990). In a clinical trial thereafter, selective 5-LOX inhibition was found to be no better than placebo for treating patients with UC (Hawkey *et al.*, 1997). Similarly, increased concentrations of thromboxanes in UC and CD inspired further studies (Ligumsky *et al.*, 1981; Hawkey *et al.*, 1983), and the effects of a combined thromboxane receptor antagonist and inhibitor of thromboxane synthase, ridogrel, were beneficial in experimental models and pilot studies (Tozaki *et al.*, 1999; Carty *et al.*, 2000). However, no difference in efficacy when compared with placebo was found in clinical trials (Carty *et al.*, 2001; Tytgat *et al.*, 2002).

Since the characterization of the inducible COX in 1991 (see 2.3.1), it is now hypothesized that in the gastrointestinal tract prostaglandins are produced for physiological purposes by constitutive COX-1 activity, and the COX-2 activity and expression is induced in inflammation. Induction of COX-2 in IBD has been mainly localized into inflamed mucosa and particularly to epithelial and immune cells (Hendel and Nielsen, 1997; Singer *et al.*, 1998). Activated eosinophils and macrophages (Raab *et al.*, 1995) as well as myenteric neurons (Roberts *et al.*, 2001a) also contribute to the production of prostaglandins in colitis. Recently, in contrast to these findings COX-1 expressing mononuclear cells in the *lamina propria* were identified as the major source of prostaglandins in inflamed mucosa. Their amount exceeded the COX-2 positive cells twofold even during active inflammation (Cosme *et al.*, 2000).

In addition to increased production of prostanoids, elevated concentrations of 15-HETE have also been measured in both the mucus and mucosa in UC (Zijlstra and Wilson, 1991; Zijlstra *et al.*, 1992). In an experimental colitis model 15-HETE was shown to possess mild anti-inflammatory activity (van Dijk *et al.*, 1993). There is also some data from clinical trials on the beneficial efficacy of polyunsaturated fatty acid supplementation, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in IBD (Grimminger *et al.*, 1993; Shoda *et al.*, 1995; Almallah *et al.*, 2000a; Almallah *et al.*, 2000b).

The role of COX-2 and eicosanoids in gut inflammation is complex, and the hypothesis of beneficial COX-1 activity and detrimental COX-2 activity seems oversimplified. Moreover, as chronic inflammation is a premalignant state, and NSAID treatment has preventive chemotherapeutic effects on colon cancer and premalignant states, COX-2 selective inhibitors may prove to be effective in retarding malignant change in inflammation associated tumorigenesis.

2.4.3 Pro-inflammatory cytokines

According to the widely accepted hypothesis on IBD pathology, there are different cytokine profiles for UC and CD (MacDonald *et al.*, 2000). They are characterized by activation of distinct T-helper cell subtypes. For CD there is evidence of a T_H1 cell predominance associated with the production of IFN-γ and TNF-α (Romagnani *et al.*, 1997) (Table 9). In UC, there is not so strong experimental evidence of a T-cell dominance even though T_H2 cells and associated cytokines (e.g. IL-4, IL-5) may be involved (Hendrickson *et al.*, 2002) (Table 9). In CD, treatment with the TNF-α binding monoclonal antibody, infliximab, has proven beneficial in active disease refractory to steroids or other conventional treatment (Targan *et al.*, 1997; Monteleone and MacDonald, 2000). As yet, no direct anti-cytokine therapy has been adopted for the treatment of UC.

Table 9. Effects of some relevant cytokines in IBD.

Cytokine	Produced by	Function
IL-1β	Macrophages, neutrophils	Activation of immune cells
IL-2	Activated T-cells, T_H1	T-cell proliferation, epithelial restitution
IL-4	T_H2 cells, mast cells	Lymphocyte function activation, macrophage function depression
IL-5	T_H2 cells, mast cells, eosinophils	Eosinophil differentiation, inhibition of apoptosis, etc.
IL-6	Macrophages, epithelial cells, endothelial cells T_H2 cells, etc.	Lymphocyte activation, inhibition of T-cell apoptosis
IL-7	Epithelial cells	Regulation of lymphocyte proliferation, activation of monocytes and macrophages
IL-8	Macrophages, epithelial cells, fibroblasts	Chemoattraction, activation of neutrophils
IL-10	T-cells, B-cells, activated monocytes	Inhibition of cytokine production
IL-12	Activated macrophages, B-cells	T_H1 differentiation, IFN-γ production
IL-18	Macrophages, epithelial cells, etc.	Promotion of T_H1 differentiation, IFN-γ production
IFN-γ	T_H1 cells, natural killer cells	Activation of macrophages, inhibition of T_H2 cells
TNF-α	Macrophages	Activation of immune and inflammatory cells

IFN-γ, interferon-gamma; IL, interleukin; T_H1, T-helper subtype 1 -cells; TNF-α, tumor necrosis factor alpha

Despite the suggested differences in cytokine profiles between UC and CD, in active inflammation there is an increased production of inflammation promoting pleiotropic

cytokines, e.g. IL-1β, IL-6, INF-γ, and TNF-α, in both disease states suggestive of a non-IBD specific event, but rather an inflammation reflecting response (Reimund *et al.*, 1996a; Reimund *et al.*, 1996b; Fiocchi, 1998; Ishiguro, 1999).

2.4.4 Current therapy

The therapy of IBD first evolved as an empirical anti-inflammatory and antibiotic treatment with the anti-rheumatic, sulfasalazine (SASP). The anti-inflammatory moiety of SASP, 5-aminosalicylic acid is devoid of the adverse effects associated with SASP (skin rash, hepatitis, agranulocytosis, sperm abnormalities) and its antibiotic and carrier moiety, sulfapyridine. 5-ASA is currently used to treat mild to moderate IBD and also as the most common maintenance therapy. It exerts its effects locally within the inflamed mucosa (Hanauer and Dassopoulos, 2001).

Oral, rectal and systemic steroids are used for treatment of exacerbations of IBD (Hanauer and Baert, 1995; Parkes and Jewell, 2001). Steroids have broad spectrum, nonspecific anti-inflammatory actions, and their continued administration is limited by dose- and duration-dependent side effects.

Immunomodulatory therapies such as azathioprine, its metabolite 6-mercapto-purine, cyclosporin, tacrolimus, methotrexate, mycophenolate, and anti-cytokine –therapy with infliximab (anti-TNF-α) counteract the pathologic activation of T-cells and the production of cytokines and cytokine actions (Hanauer and Dassopoulos, 2001). Despite these various treatment strategies and their combination therapies, inflammation in IBD may persist or relapse. Even if remission is induced, sometimes the drugs' adverse effects are so severe that only surgical removal of the inflamed bowel is left as an option. In UC, proctocolectomy is regarded as a curative treatment because of the disease's limited nature to the colon and rectum (Hanauer and Baert, 1995). After a successful operation and ileoanal-anastomosis, these patients can live a relatively normal life. However in CD several surgical approaches and bowel resections may be required to induce a quiescent state (Parkes and Jewell, 2001). Since inflammation in CD may occur in any part of the GI-tract, curative surgical treatment using bowel resections is virtually impossible.

There is growing need for additional options in the treatment repertoire in IBD (Hanauer and Dassopoulos, 2001). Novel approaches include antagonism or inhibition of cytokine actions or transcription factor activity, suppression of adhesion molecule expression, reconstitution of nonpathogenic intestinal bacteria, promotion of epithelial repair, anti-oxidative therapies and also inhibition of inflammation specific enzymes e.g. COX-2 and iNOS (Blumberg and Strober, 2001).

Even if the NOS-, COX-, and cytokine-pathways in inflammation are regarded as promising targets for therapy, the currently used drugs are not without effect on these parameters. In *in vitro* studies aminosalicylates have been shown to downregulate

transcription and production of iNOS (Kennedy *et al.*, 1999) and cytokines (Rachmilewitz *et al.*, 1992), and to inhibit COX (Warner *et al.*, 1999) and 5-LOX (Peskar *et al.*, 1987) –enzymes. Aminosalicylates have also anti-oxidantive functions as scavengers of reactive oxygen species (Greenfield *et al.*, 1993). The additional beneficial actions of aminosalicylates on inflammation and the fact that these drugs are quite well tolerated by patients may extend and bring new rationale to their use as primary medical care in IBD; in introducing and maintaining remission.

Glucocorticoids are potent suppressors of inducible genes in inflammation (Barnes, 1995). In active IBD, iNOS expression is not suppressed by glucocorticoid treatment (Leonard *et al.*, 1998), even though profound effects on COX-2 expression are to be found (Barnes, 1995). Immunosuppressive drugs, aimed at suppressing T-cell functions, downregulate the production of T-cell -derived cytokines and thus reduce the iNOS and COX-2 transcription promoting stimuli. As pointed out previously, glucocorticoids and immunosuppressive drugs have numerous adverse effects such as: immunecompromisation of the patient, bone marrow suppression, and inceased susceptibility for cancer as well as the classic adverse effects of glucocorticoids (Barnes, 1995). Glucocorticoids are effective in the treatment of moderate to severe active disease, they are less efficacious as a maintenance therapy (Hanauer and Dassopoulos, 2001).

Immunosuppressive therapy (cyclosporine, tacrolimus, methotrexate, azathioprine) potently downregulates T-cell functions, suppresses cytokine production and release (Sands, 2000). As these pro-inflammatory cytokines activate the transcription of iNOS and COX-2 genes, immunosuppressive therapy may also reduce NO and prostanoid production. These drugs have been shown to directly inhibit iNOS via inhibition on the level of protein synthesis or post-translationally (Dusting *et al.*, 1999).

2.4.5 Novel therapies

Currently cytokine-oriented therapy of IBD is limited to treatment of CD with infliximab or etanercept, antibodies that bind TNF-α and thus limit its activity (Bell and Kamm, 2000). According to the present European guidelines on the use of anti-tumor necrosis factor agents the efficacy of infliximab in inducing remission is 33%, and improvement is found in 81% of patients with refractory CD (Schreiber *et al.*, 2001). In most cases remission lasts for up to 8-12 weeks. If the first infusion of infliximab was ineffective, a second infusion of infliximab (usually after a 4 week interval) provides benefits in only a small portion of patients. The usefulness of anti-TNF-α therapy in UC is questionable since no clear beneficial effect has been seen (Schreiber *et al.*, 2001).
Therapy of IBD with IL-10 has also been evaluated in clinical trials (van Deventer *et al.*, 1997; Fedorak *et al.*, 2000; Schreiber *et al.*, 2000). IL-10 is an anti-inflammatory cytokine, which suppresses the formation of inflammatory cytokines such as IL-1,

IL-6, and TNF-α (Narula *et al.*, 1998). It has been shown to have therapeutic efficacy in clinical trials in CD (Fedorak *et al.*, 2000; Sands, 2000). In studies done on UC-patients, no clear improvement in disease activity was found in response to IL-10 administration (Sands, 2000).

Other therapies currently under study, mainly for the steroid dependent patients, include thalidomide, heparin, mycophenolate mofetil, anti-intercellular adhesion molecule-1, and anti-oxidant oriented therapies (Sands, 2000). Diet, or the use of probiotics or prebiotics to attain favorable/tolerable intestinal microbiota may also provide additional help for some patients (Campieri and Gionchetti, 1999; Shanahan, 2001).

2.5 Experimental models of inflammatory bowel diseases

For studying the effects of experimental treatments *in vivo*, numerous experimental models of colitis have been developed (Table 10) (Elson *et al.*, 1995; Sartor, 1997; Elson *et al.*, 1998; Strober *et al.*, 2002). All these models mimic some known features of human IBD, nevertheless the ideal experimental model as well as treatment have yet not been found due to the unknown etiology of IBD. These experimental models can be divided into chemically-induced, immune reconstitution associated, and spontaneously occurring in genetically susceptible strains or species. Due to their low costs and ease of establishment, the chemically induced models have been the most widely used for evaluation of novel drug treatments.

2.5.1 2,4,6-Trinitrobenzenesulfonic acid (TNBS)–induced colitis

The 2,4,6-trinitrobenzenesulfonic acid (TNBS, Figure 3) in ethanol -induced colitis is a widely used and reproducible model of both acute and chronic colitis (Neurath *et al.*, 2000; Strober *et al.*, 2002). It utilizes many aspects implicated in the pathogenesis of inflammatory bowel disease, such as initiation of inflammation by mucosal damage, activation of the immune system, increased nitrosative and oxidative stress.

When TNBS-solution is administered to the colon, the ethanol component disrupts the intestinal epithelial barrier facilitating penetration of the hapten, TNBS (Morris *et al.*, 1989). TNBS then couples trinitrophenyl groups to endogenous proteins, which in turn initiate immunologic activation (Cavani *et al.*, 1995). TNBS modifies host cell protein lysine residues rendering these cells prone to recognition and lysis by cytotoxic T-cells and macrophages (Kunin and Gallily, 1983). The cellular enzymes may reduce TNBS to a nitro radical anion with concomitant formation of a superoxide radical thereby providing an additional mechanism for its inflammation inducing potential (Chamulitrat and Spitzer, 1997). Another contributing factor to TNBS toxicity is the formation of the sulfite radical, a mild oxidant and precursor of the highly cytotoxic sulfate and sulfiteperoxyl radicals (Chamulitrat, 1999).

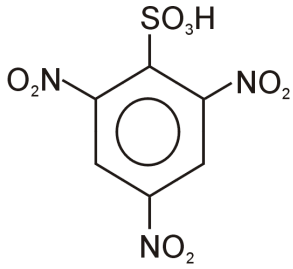

Figure 3. Chemical structure of 2,4,6-trinitrobenzenesulfonic acid.

In a colon carcinoma cell line, TNBS increased the production of COX-2 derived PGE_2, PGI_2 and LTB_4 suggesting that TNBS acts directly and locally on epithelial cells (Stratton *et al.*, 1996). The epithelial cell activation significantly contributes to the formation of eicosanoids by a mechanism unrelated to immune activation in TNBS-induced colitis. Colonocytes possess nitroreductase activity and enhance the production of TNBS-nitro radical anions, which catalyze the formation of superoxide radical anion from molecular oxygen (Grisham *et al.*, 1991; Chamulitrat and Spitzer, 1997). Superoxide and its metabolite hydrogen peroxide in turn activate NF-kappaB and induce iNOS (Adcock *et al.*, 1994) and COX-2 (von Knethen *et al.*, 1999) expression and activity.

In TNBS-induced inflammation increased mucosal damage, ulcerations, and edema are limited to the distal colon, the place of the initial topical insult (Morris *et al.*, 1989; Rachmilewitz *et al.*, 1989). Colitis is associated with fasting and diarrhea (Morris *et al.*, 1989). Dysfunctional changes in motility (alterations in contractility and neuronal function) are found in the inflamed and uninflamed colon and ileum (Jacobson *et al.*, 1997; Aube *et al.*, 1999; Hosseini *et al.*, 1999). In the acute stage of inflammation there is marked infiltration of granulocytes and increased production of cytokines IL-1β, IL-6, and TNF-α (Sun *et al.*, 2001). The chronic lesions in TNBS-induced colitis are dominated by lymphocytes and macrophages, and there is increased production of cytokines e.g. IFN-γ, IL-1, IL-6, IL-12 and TNF-α (Elson *et al.*, 1996; Sun *et al.*, 2001). This pattern is suggestive of T_H1 type activation. Increased production of various inflammatory mediators, e.g. PGE_2, PGI_2, TXB_2, LTB_4, and LTC_4 has been reported in TNBS-colitis (Kim and Berstad, 1992).

Table 10. Some different experimental approaches of inflammatory bowel disease. Adapted from Kim and Berstad (1992), Elson *et al.* (1995), Dieleman *et al.* (1997), Sartor (1997), Elson *et al.* (1998), and Strober *et al.* (2002).

Model		Mechanism	Prominent features	Animal	Acute/chronic	Pathology
Induced (chemical irritants, drugs, bacterial products etc.)	Intracolonic	Acetic acid		Mouse/Rat/ Rabbit/ Guinea pig	Acute	Mucosal to transmural, dose dependent
		Chemotactic peptide, formyl-methionyl-leucyl-phenylalanine, fMLP		Rat/Rabbit	Acute	
		Phorbolmyristate acetate, PMA	Protein kinase activation	Rabbit	Acute	
		Ethanol >30%		Rat	Acute	
		Peroxynitrite		Rat	1-7 days	
		Ozone water		Rat	ad 1 week	Microscopic colitis
		Iodoacetamidine		Rat	ad 3 weeks	Transmural
		Butyric acid		Mouse	5 hours	
		2,4,6- trinitrobenzene-sulfonic acid (TNBS), 2,4-dinitrobenzene-sulfonic acid (DNBS)	T_H1 dominant immune activation	Rat/Mouse/ Rabbit	Acute and chronic	Transmural, granulomas induction also by intramural injection
		Oxazolone	T_H2 dominant, IL-4↑, IL-5↑	Rat/Mouse	< 2 weeks	Mucosal, similar to UC Skin sensitization prior to induction, SJL/J strain prone
	Oral	Carrageenan	Epithelial injury	Mouse/ Guinea pig/ Rabbit	3-6 weeks	Mucosal, cecal involvement mainly
		Dextran sodium sulfate	Toxic, immune activation	Mouse/Rat	Acute and chronic	Similar to UC
		Amylopectin sulfate		Rabbit	Chronic	See carrageenan-induced model
		Formalin/ immune complex	Complement activation, IL-1↑	Rat/Rabbit	Acute < 5 days	Mainly mucosal oral+systemic injection
		Cyclosporin A	Graft vs. host disease	Mouse		Requires bone marrow transfer
	Intramural injection	Peptidoglycan polysaccharide, PG-PS	IL-1↑, IL-6↑, T-cell mediated	Lewis strain rats	3-4 weeks	Granulomas, spontaneous relapse
	Intraperitoneal injection	Alkylating agents, mitomycin-C	Bone marrow suppression and immunosuppression	Rat	1 week	Mucosal
	Rectal inoculation with *Chlamydia Trachomatis*	Lymphogranuloma venerum	T_H1 dominant	Monkey	Acute and chronic	Rectal mucosal inflammation
	Subcutaneous	Indomethacin	Epithelial injury (requires bile?)	Rat/Dog/ Rabbit	Acute and chronic	Small intestine, cecum, transmural
		Free radical initiator (2-amidinopropane)				

Table 10. Continued. Some different experimental approaches of inflammatory bowel disease.

Model		Mechanism	Some main features	Animal	Acute/chronic	Pathology
Immune reconstitution		$CD4^+CD45RB^{hi}$ → scid or Rag -/-	IL-12↑, T_H1 dominant	Mouse	5-8 weeks after transfer	Mucosal
		Bone marrow transfer/ Immune suppression → CD3ε26		Mouse	Chronic	Transmural
Genetic, spontaneous	Naturally occurring			Cotton top tamarin	Acute and chronic	Mucosal
		In juvenile individuals		Rhesus macaques	Chronic	Mucosal
		C3HH-HeJBir	T_H1 dominant	Mouse	at 3 weeks of age	Transmural
		SAMP1/Yit	T_H1 dominant	Mouse	Chronic	Transmural
	Knockout	IL-2 or IL-2receptor	IFN-γ↑	Mouse	At 10 weeks	Mucosal/submucosal
		IL-10	T_H1 dominant, IFN-γ↑, IL-1↑, IL-6↑	Mouse	At 4-8 weeks	Transmural
		Double: IL-10/ iNOS	Similar to IL-10 knockout	Mouse	Chronic	Role of iNOS?
		$TCR\alpha^-$ or β^- or β^- x δ^-	T_H2 dominant, IL-4↑	Mouse	At 12-16 weeks	Mucosal
		$G_{\alpha i2}$	T_H1 involvement	Mouse	At 8-13 weeks, neoplasms	Mucosal
		TGF-β1	T_H1 dominant	Mouse	Chronic, neoplasms	Transmural
		Double: Gpx1/ Gpx2		Mouse	At 15 days	Mucosal
		Mdr1a	Intestinal epithelial dysfunction	Mouse		Mucosal
		WASP	T_H2 dominant, deficient lymphocyte activation	Mouse	At 4 months	Mucosal
		CRF2-4	Unresponsiveness to IL-10, T_H1 dominant	Mouse	At 12 weeks	Mucosal
		Stat3	T_H1 dominant	Mouse	In aged	Mucosal
		TNFΔARE	TNF-α↑	Mouse	Chronic	Transmural
	Transgenic, T_H1 models	Stat4	Stat4↑	Mouse	Acute and chronic	Transmural
		HLAB27 /β_2m	IFN-γ↑	Rat	Acute and chronic	Mucosal
		IL-7	IL-7↑	Mouse	At 4-12 weeks	Mucosal
Genetic, increased susceptibility	Susceptible to induction	TGF-α knockout	DSS	Mouse		
		EGF-receptor mutation	DSS	Mouse		
		Mutated TGF-β receptor	DSS	Mouse		
		ITF knockout	DSS, impared mucosal healing	Mouse		
		IRE1β knockout	DSS	Mouse		
		NF-kappaB p50 knockout	*Helicobacter hepaticus*	Mouse		

CRF2-4, class II cytokine receptor family orphan receptor; DSS, dextran sulfate sodium; EGF, epidermal growth factor; GPX, gluthatione peroxidase; HLA, human leukocyte antigen; IFN-γ, interferon-gamma**;** IL, interleukin; IRE, endoplasmic reticulum stress signal response gene; ITF, intestinal trefoil factor; MDR, multi drug resistance; TCR, T-cell receptor; TGF, transforming growth factor; T_H1, T-helper subtype 1 cells, TNFΔARE, TNF mRNA AU-rich elements; STAT, signal transducer and activator of transcription; WASP, Wiskott Aldrich syndrome protein

Histologically the transmural lesions (Morris *et al.*, 1989) are similar to the damage seen in CD. Similar to active human IBD, in TNBS-colitis there is increased expression of the inflammatory enzymes iNOS (Miampamba and Sharkey, 1999) and COX-2 (Nakase *et al.*, 2001), increased production of peroxynitrite, and increased epithelial cell death (Yamada *et al.*, 1992; Yue *et al.*, 2001). Intestinal permeability is increased (Gardiner *et al.*, 1995), and bacterial endotoxins find their way to the systemic circulation (Brand *et al.*, 1994; Gardiner *et al.*, 1995; Neilly *et al.*, 1995). After resolution, colitis can be reactivated by intravenous TNBS (Appleyard and Wallace, 1995). Inflammation is ameliorated by introducing prehaptenated proteins orally prior to induction of colitis (Elson *et al.*, 1996) and by treatment with a neutralizing antibody to IL-12 (Neurath *et al.*, 1995). The precise pattern of the inflammatory response is dependent on the animal genotype, for example in BALB/c mice, the chronic stage is T_H2 dominant and the lesions representative of UC (Dohi *et al.*, 1999). When compared to Sprague-Dawley rats, for Wistar-strain rats the time-span of the inflammatory response is usually shorter, being 6 weeks and 1 week, respectively (Morris *et al.*, 1989; Moreels *et al.*, 2001).

2.5.2 Nitric oxide-related treatment of experimental colitis

In TNBS-colitis the production of NO and the activity of iNOS are increased and associated with increased production of peroxynitrite and cell damage just like in the human disease (see 2.2). Since NO-metabolism serves as a common end-point effector in inflammation, its manipulation has been targeted in the treatment of colitis. The earlier unavailability of selective inhibitors of iNOS has ment that ambiguous results have been obtained. This has been overcome by administering non-selective inhibitors at different time points after the induction of inflammation. The rationale behind this is inhibition of constitutive enzymes by pretreatment or concomitant drug administration to colitis induction. Because the induction of iNOS requires *de novo* synthesis of protein, delayed administration of a non-selective inhibitor would simulate selective iNOS inhibition. By this rationale and the use of a non-selective NOS-inhibitor, L-NAME, it was shown, that delayed inhibition of NOS has a beneficial action on colitis (Kiss *et al.*, 1997).

Effects of some NOS-inhibitors in experimental models of colitis are shown in Table 11. It has been speculated that also the route of drug administration plays a major role in determining a beneficial or detrimental effect. There are data suggesting that systemic administration of iNOS inhibitors or topical administration of a nonselective inhibitor at time of most iNOS activity show beneficial effects (Table 11). These drug effects have been studied in only a limited number of models, and their effects need to be evaluated in human tissues. In conclusion, nitric oxide and its metabolites have been shown to be mediators of inflammation by use of these models of colitis. Manipulation of the L-arginine-nitric oxide –pathway, and specifically the suppression of iNOS, are likely to be of therapeutic value in the treatment of IBD.

Table 11. Treatment of experimental colitis with nitric oxide synthase inhibition.

Drug	Route of administration	Beginning of treatment	Daily dose	Model of colitis	Duration of experiment	Effect	Detailed effects	Reference
L-NIL	s.c.	Overt disease	10 mg/kg x2	HLA-B27 rats	3 weeks	Detrimental	Despite IFN-γ↓ and TNF-α↓ aggravation of colitis and IL-1β↑	(Blanchard *et al.*, 2001)
	p.o.	Active severe disease	60 mg/kg	Spontaneous colitis in monkey	10 days	No effect	Diarrhea -, inflammation index -	(Ribbons *et al.*, 1997)
	i.p.	At time of induction	10 mg/kg x4	TNBS, rats	24 hours	Beneficial	Increase in normal epithelial cells, decrease in nitrotyrosine positive and apoptotic cells. MPO-activity↓	(Yue *et al.*, 2001)
	p.o.	Minimal or absent colitis	25 mg/kg	SCID mice	4 weeks	No effect	Increased VCAM-1 -, macroscopic inflammation -	(Kawachi *et al.*, 1999)
L-NNA	i.v.	At time of induction	10 mg/kg	TNBS, rats	1 week	Detrimental	Body weight loss↑, macroscopic inflammation↑, MPO-activity↑, lipid peroxidation↑	(Yamaguchi *et al.*, 2001)
	i.p.	24 hours after induction	25 mg/kg x3	Acetic acid, rats	2 days	Detrimental	IL-6↑, macroscopic inflammation↑	(Dobosz *et al.*, 1996)
Aminogua-nidine	i.v.	At time of induction	10 mg/kg	TNBS, rats	1 week	Beneficial	Body weight loss↓, macroscopic inflammation↓, MPO-activity↓,lipid peroxidation↓, colon edema↓	(Yamaguchi *et al.*, 2001)
	p.o.	3 days prior to induction	200 mg/kg	TNBS, rats	1 week	Detrimental	MPO-activity↑, TNF-α↑, NF-kappa-B activity↑	(Dikopoulos *et al.*, 2001)
	p.o.	1 day prior to induction	~20-300 mg/kg (50-2500 mg/l)	TNBS rats	1 week	No effect	Body weight loss -, bowel wall citrulline or arginine concentrations -, inflammation -	(Armstrong *et al.*, 2000)
	p.o.	Simultaneously with DSS	400 mg/kg	DSS rats	1 week	No effect/ detrimental	Portal venous blood white cells↑ and lipid peroxidation↑, macroscopic inflammation -	(Yoshida *et al.*, 2000)
	p.o.	1 week after induction	~0,2 mg/kg (1.5 µmol/kg)	TNBS rats	2 weeks	Beneficial	MPO-activity↓, macroscopic and microscopic inflammation↓, epithelial cell proliferation↑	(Nakamura *et al.*, 1999)
	p.o.	Signs of overt disease	~7 mg/kg (52 µmol/kg)	HLA-B27 rats	3 weeks	Beneficial	MPO-activity↓, increased colon permeability↓, increased mucosal thickness↓	(Aiko *et al.*, 1998)
	p.o.	Active severe disease	60 mg/kg	Spontaneous colitis in monkey	10 days	No effect	Diarrhea -, inflammation index -	(Ribbons *et al.*, 1997)
	p.o.	3 days prior to induction	~2 mg/kg (15 µmol/kg)	Peptidoglycan polysaccharide, rats	3 weeks	Beneficial	MPO-activity↓, macroscopic inflammation↓, inflammatory edema↓, maintenance of normal crypt structure	(Grisham *et al.*, 1994)
	p.o.	3 days prior to induction	~5 mg/kg (10 mg/l)	TNBS, rats	24 hours	Beneficial	Inhibition of inflammatory colonic dilatation	(Mourelle *et al.*, 1996)
	i.p.	4-6 weeks after DSS treatment	~500 mg/kg (10 mg/mouse)	DSS, BALB/c mice	6 days	Beneficial	Histological score↓, colon length↑	(Obermeier *et al.*, 1999)
Mercapto-ethylguani-dine	i.v.	1 day prior to induction	10 mg/kg x2	TNBS, rats	4 days	Beneficial	Body weight loss ↓, diarrhea↓, MPO-activity↓, amelioration of disrupted colonic structures, iNOS and nitrotyrosine immunoreactivity↓	(Zingarelli *et al.*, 1998)
ONO-1714	i.p.	At time of induction	0.03-0.3 mg/kg	DSS, BALB/c mice	1 week	Beneficial/ detrimental?	MPO-activity↓, lipid peroxidation↓, IL-4↓, IL-10↓, IFN-γ↓, inflammation severity↓, nitrotyrosine immunoreactivity↓	(Naito *et al.*, 2001)

Table 11. Continued. Treatment of experimental colitis with nitric oxide synthase inhibition.

Drug	Route of administration	Beginning of treatment	Daily dose	Model of colitis	Duration of experiment	Effect	Detailed effects	Reference
L-NMMA	i.p.	From time of induction for 4 days	50 mg/kg	TNBS, rats	2 weeks	Detrimental	Colonic damage score↑	(Hosoi *et al.*, 2001)
	i.p.	From day 4 from induction	50 mg/kg	TNBS, rats	2 weeks	No effect	Colonic damage score -	(Hosoi *et al.*, 2001)
L-NAME	p.o.	1 day prior to induction	~34 mg/kg (500mg/l)	TNBS, rats	1 week	No effect	Body weight loss -, bowel wall citrulline or arginine concentrations -, inflammation -	(Armstrong *et al.*, 2000)
	p.o.	Simultaneously with DSS	100 mg/kg	DSS, rats	1 week	Detrimental	Lipid peroxidation↑, macroscopic inflammation -	(Yoshida *et al.*, 2000)
	p.o.	Signs of overt disease	~13 mg/kg (45 µmol/kg)	HLA-B27 rats	2 weeks	Beneficial/ detrimental	MPO-activity↓, increased mucosal thickness↓, body weight loss↑	(Aiko *et al.*, 1998)
	p.o.	3 days prior to induction	~4 mg/kg (15 µmol/kg)	Peptidoglycan polysaccharide, rats	3 weeks	Beneficial	MPO-activity↓, macroscopic inflammation↓, maintenance of normal crypt structure	(Grisham *et al.*, 1994)
	i.p.	3 days prior to induction	10 mg/kg x2	TNBS, rats	24 hours	Beneficial	Inhibition of inflammatory colonic dilatation	(Mourelle *et al.*, 1996)
	p.o.	2 days prior to induction	~25 mg/kg (100 µmol/l)	TNBS, rats	72 hours	Detrimental	Lesion area↑, iNOS activity↑	(Kiss *et al.*, 1997)
	p.o.	6 hours after induction	~25 mg/kg (100 µmol/l)	TNBS, rats	72 hours	Beneficial	Lesion area↓, iNOS activity↓, MPO-activity↓	(Kiss *et al.*, 1997)
	s.c. pumps	5 days prior to induction	40 mg/kg (1.667 mg/kg/h)	TNBS, rats	3 days	Detrimental	MPO-activity↓, ulcer index↑, lesion area↑	(Pfeiffer and Qiu, 1995)
	s.c.	1 day prior to and 3-7 days after induction	100 mg/kg	TNBS, rats	3 to 7 days	Detrimental	MPO-activity↑, ulcer index↑, lesion area↑	(Pfeiffer and Qiu, 1995)
	p.o.	At time of induction	500 mg/l	TNBS, rats	8 days	Beneficial	Macroscopic inflammation↓, colonic weight↓, body weight loss↓	(Neilly *et al.*, 1996)
	enema	24 hours after linduction	1 000 mg/l	TNBS, rats	8 days	Beneficial	Macroscopic inflammation↓, colonic weight↓, body weight loss↓	(Neilly *et al.*, 1996)
	p.o.	At time of induction	~30 mg/kg (100 mg/l)	TNBS, rats	1 week	Beneficial	Colonic lesions↓, colonic weight↓, MPO-activity↓	(Rachmilewitz *et al.*, 1995b)
	p.o.	At time of induction	~30 mg/kg (100 mg/l)	Acetic acid (capsaicin pre-treatment), rats	24 hours	Beneficial	Colonic lesions↓, colonic weight↓	(Rachmilewitz *et al.*, 1995b)
	p.o.	At time of induction	~30 mg/kg (100 mg/l)	Iodoacetamidine, rats	1 week	Beneficial	Colonic lesions↓, colonic weight↓, PGE_2↓	(Rachmilewitz *et al.*, 1995a)
	p.o.	At time of induction	~30-90 mg/kg (100-300 mg/l)	Iodoacetamidine, rats	1-3 days	Beneficial	Colonic lesions↓, colonic weight↓, MPO-activity↓	(Rachmilewitz *et al.*, 1995a)
	p.o.	At time of induction	30 mg/kg	TNBS, rats	6 days	Beneficial	MPO-activity↓, tissue damage↓	(Hogaboam *et al.*, 1995)
	p.o.	At time of induction	500 mg/l	TNBS, rats	8 days	Beneficial	Lipid peroxidation↓, macroscopic inflammation -, CuZn-SOD –activity↑	(Seven *et al.*, 2000)

Table 11. Continued. Treatment of experimental colitis with nitric oxide synthase inhibition or a nitric oxide-donor (NO-mesalamine).

Drug	Route of administration	Beginning of treatment	Daily dose	Model of colitis	Duration of experiment	Effect	Detailed effects	Reference
1400W	i.p.	5 days after induction	0.4-2 mg/kg	TNBS, rats	15 days	Beneficial	MPO-activity↓, macroscopic and histological inflammation↓	(Menchen *et al.*, 2001)
	s.c. pumps	At time of induction	240 mg/kg	DSS, mice	1 week	Beneficial	Disease activity↓, histological inflammation↓, MPO-activity↓, colon length↑	(Krieglstein *et al.*, 2001)
	s.c. pumps	At time of induction	240 mg/kg	DSS, $p47^{phox}$-/- mice	1 week	Beneficial	Disease activity↓, histological inflammation↓, MPO-activity↓, colon length↑	(Krieglstein *et al.*, 2001)
ITU	s.c.	At time of induction	50 mg/kg	TNBS, rats	1 week	Beneficial	Body weight loss↓, colon damage↓, colon weight↓	(Southey *et al.*, 1997)
NOR3	p.o.	Simultaneously with DSS	100 mg/kg	DSS, rats	1 week	Detrimental	Portal venous blood white cells↑, lipid peroxidation↑, macroscopic inflammation ↑/-	(Yoshida *et al.*, 2000)
NO-mesalamine	Intracolonic			TNBS, rats	1 week	Better than mesalamine		(Wallace *et al.*, 1999)
iNOS deficiency				TNBS, mice	2 days	Detrimental		(McCafferty *et al.*, 1999)
				TNBS, mice	2 weeks	No effect		(McCafferty *et al.*, 1999)
				TNBS, mice		Beneficial	Resistance to lethality, reduced damage,	(Zingarelli *et al.*, 1999b)
				DSS, mice	42 days	Beneficial	Macroscopic damage↓, diarrhea↓, submucosal thickness↓, resistance to DSS injury	(Hokari *et al.*, 2001)
				Acetic acid, mice	1 week	Detrimental	Macroscopic damage↑ (at 1 day-1 week), delayed decline in MPO activity, delayed healing	(McCafferty *et al.*, 1997)
				DSS, mice	1 week	Beneficial	Disease activity↓, body weight loss↓, rectal bleeding↓, histological damage↓, ulcerations↓	(Krieglstein *et al.*, 2001)

↑/↓, increase/decrease, 1400W, N-[3-(aminomethyl)benzyl]acetamidine; DSS, dextran sulfate sodium; HLA, human leukocyte antigen; IFN-γ, interferon-gamma; ITU, ONO-1714, NOR3 experimental inhibitors of NOS; i.p., intraperitoneal; L-NAME, N^G-nitro-L-arginine methyl esther; L-NIL, N-iminoethyl-L-lysine; L-NNA, N^G-nitro-L-arginine; L-NMMA, N^G-nitro-monomethyl-L-arginine; MPO, myeloperoxidase; $p47^{phox}$, a component of phagocyte oxidase complex; p.o., peroral; VCAM vascular cell adhesion molecule; s.c., subcutaneous; SOD, superoxide dismutase; TNBS, 2,4,6-trinitrobenzenesulfonic acid

Changes in NO-metabolism and/or availability are achieved also by indirect means. For example, treatment of colitis with a superoxide dismutase mimicking drug increases the availability and putative anti-inflammatory actions of increased NO while inhibiting NO reactivity by decreasing O_2^- and the production of toxic $ONOO^-$ (Cuzzocrea *et al.*, 2001a). Similarly, treatment with anti-oxidants has been reported to suppress experimental colitis (Cuzzocrea *et al.*, 2000). It has also been shown that inhibition of NF-kappaB activation by oligonucleotides (Neurath *et al.*, 1996), or inhibition of the degradation of the endogenous NF-kappaB inhibitor, I-kappaB, ameliorates colitis (Conner *et al.*, 1997; Cuzzocrea *et al.*, 2001c). NF-kappaB is activated in experimental colitis (Neurath *et al.*, 1996; Marrero *et al.*, 2000) as well as in human IBD (Schreiber *et al.*, 1998), where it is associated with active inflammation (Ardite *et al.*, 1998; Egan and Sandborn, 1998). Less severe colitis has been reported in poly(adenosinediphosphate-ribose)synthetase (PARS)-deficient mice (Zingarelli *et al.*, 1999a) as well as in iNOS deficient mice (Zingarelli *et al.*, 1999b). PARS is activated in response to cell and tissue injury by e.g. $ONOO^-$. The activation of PARS, however, depletes the cell of its energy stores thus contributing to increased apoptosis and tissue destruction (Szabo *et al.*, 1996).

In experimental colitis models inhibition of iNOS-derived NO production and $ONOO^-$ formation results in the development of less severe forms of disease. Results with iNOS inhibitors such as aminoguanidine cannot be readily interpreted, since these inhibitors possess iNOS-unrelated effects (see 2.2.1). However, the recent literature and experimental data point to the fact that inhibition of increased NO synthesis may provide benefit in IBD.

2.5.3 Eicosanoid-related treatment of experimental colitis

The effects on experimental models of colitis of COX-inhibitors or E-series prostaglandin supplementation are shown in Table 12. Drugs with selectivity for the inducible isoform, COX-2 have been shown to have beneficial effects, while nonselective inhibitors like indomethacin or naproxen have exacerbated the inflammatory lesions in both acute and chronic models. However, supplementation with analogs of E-series prostaglandins such as enprostil, misoprostol or alprostadil has had consistently beneficial effects in different models of experimental colitis. COX-2 has been shown to have anti-inflammatory effects and to play a crucial role in resolution of inflammation (Morteau *et al.*, 2000; Halter *et al.*, 2001).

Table 12. Treatment of experimental colitis with cyclooxygenase inhibitors or prostaglandin analogs.

Drug	Route of administration	Beginning of treatment	Daily dose	Model of colitis	Duration of experiment	Effect Beneficial/ Detrimental	Detailed effects	Reference
Celecoxib	p.o.	At time of induction	5 mg/kg x2	DNBS, rats	4 days	Beneficial	Macroscopic and histologic damage↓, colon edema↓, body weight loss↓, survival↑, TNF-α↓, IL-1β↓, MPO-activity↓, lipid peroxidation↓, P-selectin and ICAM-1 expression↓	(Cuzzocrea *et al.*, 2001b)
	p.o.	At time of induction	10 mg/kg	TNBS, rats	3 hours	Detrimental	MPO-activity↑, PGD_2↓	(Ajuebor *et al.*, 2000)
SC-236	i.p.	1 hour prior to and 2 hours after induction	2.5 mg/kg	TNBS, rats	24 hours	Beneficial	MPO↓, inflammatory edema↓, body weight loss↓	(Guo *et al.*, 2001)
	p.o.	Single treatment at time of induction	6 mg/kg, single dose	Iodoacetamidine, rats	3 days	Beneficial	Lesion area↓, colon weight↓, MPO-activity↓, NOS-activity↓	(Karmeli *et al.*, 2000)
Nimesulide	p.o.	At time of induction and 6 hours thereafter	10 mg/kg x2	Acetic acid, rats	24 hours	Beneficial	Lesion area↓, colon weight↓, MPO-activity↓, NOS-activity↓	(Karmeli *et al.*, 2000)
	p.o.	At time of induction	10 mg/kg x 2	Iodoacetamidine, rats	3 days	Beneficial	Lesion area↓, colon weight↓, MPO-activity↓, NOS-activity↓, TNF-α↓, IL-1β↓, histological inflammation↓	(Karmeli *et al.*, 2000)
	s.c.	4 days prior to induction	25 mg/kg	TNBS, rats	24 hours	Beneficial	Lesion area↓, MPO-activity↓, inflammatory edema↓, body weight loss↓	(Guo *et al.*, 2001)
	p.o.	1 day prior to induction	5 mg/kg	DSS, rats	2 days	Beneficial	DNA damage↓, apoptosis↑ No neutrophil infiltration in this model!	(Tardieu *et al.*, 2000)
NS-398	p.o.	1 hour prior to induction	1-100 mg/kg	TNBS, rats	2 days	No effect	No COX-2 expression/PGE_2 formation in inflamed colon in this model!	(Lesch *et al.*, 1999)
SC-58125	p.o.	1 hour prior to induction	1-100 mg/kg	TNBS, rats	2 days	No effect	No COX-2 expression/PGE_2 formation in inflamed colon in this model!	(Lesch *et al.*, 1999)
PD-138387	p.o.	1 hour prior to induction	1-100 mg/kg	TNBS, rats	2 days	No effect	No COX-2 expression/PGE_2 formation in inflamed colon in this model!	(Lesch *et al.*, 1999)
Acetylsalicylic acid	p.o.	2 weeks after induction	100 mg/kg	TNBS, rats	7 days	No effect	Macroscopic damage -	(Boughton-Smith *et al.*, 1988)
Indomethacin	i.m.	5 days pre-treatment only	2.5 mg/kg	Acetic acid, rats	2 days	Detrimental	Macroscopic damage↑, MPO-activity -, fluid absorbtion↓	(Empey *et al.*, 1992)
	s.c.	At time of induction	2 mg/kg	Peptidoglycan polysaccharide, rats	7 days	Detrimental	IL-1α↑, MPO-activity↑	(Kandil *et al.*, 1999)
	s.c.	4 days prior to induction	2.5 mg/kg	TNBS, rats	24 hours	Beneficial	MPO-activity↓, colonic edema↓, body weight loss↓, lesion area -	(Guo *et al.*, 2001)
	i.p.	24 hours after induction	5 mg/kg	Acetic acid, rats	5 days	Detrimental	Lesion area↑	(LeDuc *et al.*, 1993)
	s.c.	3 hours prior to induction	0.1-1 mg/kg x2	TNBS, rats	7 days	Detrimental	MPO↑, mortality↑, colon macroscopic damage↑	(Wallace *et al.*, 1992)
	p.o.	At time of induction	10 mg/kg	TNBS, rats	3 hours	Detrimental	MPO↑, PGD_2↓	(Ajuebor *et al.*, 2000)

Table 12. Continued. Treatment of experimental colitis with cyclooxygenase inhibitors or prostaglandin analogs.

Drug	Route of adminis-tration	Beginning of treatment	Daily dose	Model of colitis	Duration of experiment	Effect Beneficial/ Detrimental	Detailed effects	Reference
Indomethacin	i.m.	From day 7 to day 21 from induction	1 mg/kg	TNBS, rats	21 days	No effect	Macroscopic and histologic damage -	(Vilaseca *et al.*, 1990)
	p.o.	2 hours prior to induction	10 mg/kg x2	Formalin-immunecomplex, rabbits	2 days	No effect	Inflammatory cell index -, exudate -, edema -, necrosis -	(Schumert *et al.*, 1988)
	i.m.	1 hour prior to induction	2 mg/kg	TNBS, rats	3 days	No effect	Macroscopic damage -	(Hoshino *et al.*, 1992)
	i.m.	From day 4 after induction	2 mg/kg	TNBS, rats	14 days	No effect	Macroscopic damage -	(Hoshino *et al.*, 1992)
	p.o.	24 hours after induction	2.25 mg/kg	Acetic acid, rats	6 days	Detrimental	MPO-activity↑	(Fitzpatrick *et al.*, 1990)
	p.o.	2 hours prior to induction	2.25 mg/kg	Acetic acid, rats	24 hours	No effect	Macroscopic damage -	(Fitzpatrick *et al.*, 1990)
	p.o.	2 weeks after induction	0.5 mg/kg	TNBS, rats	7 days	No effect	Macroscopic damage -	(Boughton-Smith *et al.*, 1988)
	p.o.	1 hour priot to induction	10 mg/kg	Iodoacetamide, rats	3 days	Detrimental	Lesion area↑, MPO activity↑, edema↓	(Satoh *et al.*, 1997)
Indomethacin (I) + misoprostole (M)	p.o.+ i.r.	5 days (I) + 30 min (M) prior to induction only	2.5 mg/kg + 100 µg/kg	Acetic acid, rats	2 days	Beneficial	Macroscopic ulcerations↓	(Empey *et al.*, 1992)
	s.c.	At time of induction	2 mg/kg + 90 µg/kg	Peptidoglycan polysaccharide, rats	7 days	Beneficial	Indomethacin induced IL-1α↓ and MPO-activity↓	(Kandil *et al.*, 1999)
Naproxenl	s.c.	3 hours prior to induction	5 mg/kg x2	TNBS, rats	7 days	Detrimental	MPO-activity↑, mortality↑, colon macroscopic damage↑	(Wallace *et al.*, 1992)
	p.o.	3 hours prior to induction	5 mg/kg x2	TNBS, rats	7 days	Detrimental	Mortality↑	(Reuter *et al.*, 1996)
Diclofenac	p.o.	3 hours prior to induction	10 mg/kg x2	TNBS, rats	3 days	Detrimental	Macroscopic and histologic damage↑, MPO -	(Reuter *et al.*, 1996)
	p.o.	3 hours prior to induction	10 mg/kg x2	TNBS, rats	7 days	Detrimental	Mortality↑	(Reuter *et al.*, 1996)
Nabumetone	p.o.	3 hours prior to induction	75 mg/kg x2	TNBS, rats	3 days	Detrimental	Macroscopic and histologic damage↑, MPO -	(Reuter *et al.*, 1996)
	p.o.	3 hours prior to induction	25 mg/kg x2	TNBS, rats	7 days	Detrimental	Mortality↑	(Reuter *et al.*, 1996)
L745,337	p..o.	3 hours prior to induction	5 mg/kg x2	TNBS, rats	3 days	Detrimental	Macroscopic and histologic damage↑, MPO -	(Reuter *et al.*, 1996)
	p.o.	3 hours prior to induction	5 mg/kg x2	TNBS, rats	7 days	Detrimental	Mortality↑	(Reuter *et al.*, 1996)
Etodolac	p.o.	3 hours prior to induction	10 mg/kg x2	TNBS, rats	7 days	Detrimental	Mortality↑	(Reuter *et al.*, 1996)

Table 12. Continued. Treatment of experimental colitis with cyclooxygenase inhibitors or prostaglandin analogs.

Drug	Route of adminis-tration	Beginning of treatment	Daily dose	Model of colitis	Duration of experiment	Effect Beneficial/ Detrimental	Detailed effects	Reference
Enprostil (PGE_2)	i.r.	1 week after DSS	~50-250 µg/kg (10 µg or 50 µg)	DSS, rats	1 week	Beneficial	Macroscopic and histologic lesions↓, MPO↓, IL-1β↓	(Sasaki *et al.*, 2000)
Alprostadil (PGE_1)	i.p.	12 hours after induction	~10 µg/kg (2 µg)	Acetic acid, rats	3-10 days	Beneficial at 3 days	Macroscopic and histologic damage↓	(Terzioglu *et al.*, 1997)
16,16-dimethyl PGE_2	i.r.	20 minutes prior to induction	2000 µg/kg	Butyric acid, mice	5 hours	Beneficial	Edema↓, MPO-activity↓, macroscopic inflammation↓	(McCafferty and Zeitlin, 1992)
	s.c.	2 hours prior to induction	100 µg/kg	Formalin-immunecomplex, rabbits	2 days	Beneficial	Inflammatory cell index↓, necrosis↓, exudate ↓, edema↓	(Schumert *et al.*, 1988)
	i.r.	45 min prior to or 24 h after induction	20 mg/kg	TNBS, rats	3 days	Beneficial	Macroscopic inflammation↓, MPO-activity↓	(Allgayer *et al.*, 1989)
	i.r.	20 min prior to induction	0.2-20 µg/kg	Ethanol, rats	10 minutes	Beneficial	Macroscopic and histological inflammation↓, cell disruption↓	(Wallace *et al.*, 1985)
Misoprostol	s.c.	At time of induction	90 µg/kg	Peptidoglycan polysaccharide, rats	7 days	Beneficial	IL-1α↓, MPO-activity↓	(Kandil *et al.*, 1999)
	i.r.	30 minutes prior to induction	100 µg/kg	Acetic acid, rats	2 days	Beneficial	Colonic ulcerations↓, histological damage↓, restores mucosal fluid absorption	(Fedorak *et al.*, 1990)
	i.r.	30 minutes prior to induction	100 µg/kg	Acetic acid, rats	2 days	Beneficial	Macroscopic injury↓, MPO-activity↓	(Empey *et al.*, 1992)
COX-2 cDNA phosphotioated oligonucleotides	i.p.	1 hour pretreatment	3 mg/kg	TNBS, rats	7 days	Beneficial	MPO activity↓, COX-2 expression↓, PGE_2 ↓	(Khan *et al.*, 2001)
COX-1 -/-				DSS, mice	5 days	Exacerbation		(Morteau *et al.*, 2000)
COX-2 -/-				DSS, mice	5 days	Exacerbation		(Morteau *et al.*, 2000)

↑/↓, increase/decrease; -, no effect; COX, cyclooxygenase; DNBS, dinitrobenzenesulfonic acid; DSS, dextran sulfate sodium; ICAM, intercellular adhesion molecule; IL, interleukin; i.m., intramuscular; i.p., intraperitoneal; i.r., intrarectal; MPO, myeloperoxidase; NOS, nitric oxide synthase; PGE_2 prostaglandin E_2; p.o., peroral; s.c., subcutaneous; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNF-α, tumor necrosis factor alpha

In colitis, the increased production of PGE_2 and PGD_2 is related to COX-2 induction and to anti-inflammatory effects (Ajuebor *et al.*, 2000). Prostaglandin D_2 and its metabolites PGJ_2, 12-delta-PGJ_2 and 15-deoxy-delta12,14-PGJ_2 are produced in high amounts by mast cells, and also by enterocytes in inflammation (Longo *et al.*, 1998). They have been suggested to have both anti- and pro-inflammatory effects. In TNBS-induced colitis administration of PGD_2 and its receptor agonist inhibited, while its metabolite increased infitration of neutrophil granulocytes (Ajuebor *et al.*, 2000). The production of PGD_2 in this TNBS-model of colitis was inhibited by celecoxib, a COX-2 selective NSAID (Ajuebor *et al.*, 2000). In concert with that study showing aggravation of inflammation by PGD_2 metabolites, 15-deoxy-delta12,14-PGJ_2 has been shown to induce production of IL-8, a potent pro-inflammatory cytokine in human T-cells (Harris *et al.*, 2002). However, PGJ_2 has also been shown to have anti-inflammatory properties and to act as an endogenous agonist for peroxisome proliferator activated receptors (PPAR), mainly for PPAR-γ (Clark *et al.*, 2000). In experimental colitis as well as in human disease beneficial anti-inflammatory effects with PPAR-γ agonists have been reported (Su *et al.*, 1999; Lewis *et al.*, 2001). It is interesting that some older NSAIDs, e.g. naproxen and indomethacin, can function as activators of PPAR-γ and may thereby extend their therapeutic benefits (Jaradat *et al.*, 2001).

During inflammation the production of PGI_2 is co-induced with PGE_2 (Ligumsky *et al.*, 1981; Brock *et al.*, 1999). In PGI_2-receptor deficient mice carrageenan-induced inflammatory edema was suppressed in a manner similar to indomethacin-treated wild-type mice suggesting that PGI_2 signalling increases inflammatory swelling (Ueno *et al.*, 2000). PGI_2 receptors are also involved in pain perception (Murata *et al.*, 1997). Increased production of PGI_2 has been associated with UC and CD, and shown to be inhibited by treatment with steroids and sulfasalazine (Ligumsky *et al.*, 1981).

During inflammation, AA is also metabolized by the 5-lipoxygenase (5-LOX) pathway leading to the synthesis of leukotrienes (see 2.3.2). LTB_4 is a very potent inducer of chemotaxis and an activator of neutrophil granulocytes (Yokomizo *et al.*, 2001). The therapeutic potential of inhibition of 5-LOX and leukotriene synthesis was challenged in a multicenter study with 183 patients suffering from UC (Roberts *et al.*, 1997). Despite a marked suppression of colonic LTB_4 no beneficial effect was seen. A similar lack of effect has also been seen in TNBS-induced experimental colitis in the rat (Holma *et al.*, 2001).

Plasma concentrations and mucosal production of PGE_2, and the inflammation in colitis are in direct correlation with each other (Wiercinska-Drapalo *et al.*, 1999a). Measurement of plasma PGE_2 has in fact been suggested to have a role as a surrogate marker of active disease (Wiercinska-Drapalo *et al.*, 1999b). PGE_2 has been shown to activate the T-helper 2 subtype and to suppress the T-helper 1 subtype T-cells (Snijdewint *et al.*, 1993; Katamura *et al.*, 1995). In UC, there is a

predominance of a T-helper 2 subtype activation and an increase in type 2 cytokines, so by this rationale the inhibition of PGE_2 production by e.g. NSAIDs could alleviate disease. On the other hand, PGE_2 may inhibit the production of the pro-inflammatory cytokine, TNF-α (Kunkel *et al.*, 1988). An increase in PGE_2 would thus lead to beneficial anti-inflammatory effects through this mechanism. Treatment of experimental colitis with PGE-analogues, such as misoprostole, has shown promising results (Table 12). It has also been shown that inhibition of increased mucosal thromboxane synthesis or antagonism of the thromboxane effects in TNBS-induced rat colitis increases PGE_2 concentrations and thereby exerts anti-inflammatory effects (Vilaseca *et al.*, 1990; Taniguchi *et al.*, 1997).

Coupled to the release of AA from a membrane phospholipid backbone and PLA_2 activity is the formation of platelet activating factor (PAF). The synthesis of PAF from the remaining phospholipid (phosphatidylcholine) precursor after AA discharge is catalysed by a specific acetyltransferase. In addition to activation of thrombocyte aggregation, PAF functions as a potent chemotactic agent, it increases the accumulation and activation of neutrophil granulocytes, and modulates cytokine synthesis during inflammation (Prescott *et al.*, 2000). The concentrations of PAF in inflamed mucosa of IBD patients (Wardle *et al.*, 1996) and in experimental colitis are increased (Longo *et al.*, 1994). One novel and attractive approach to treating IBD is through PAF antagonism. Beneficial effects in experimental colitis models utilizing this anti-inflammatory principle have been attained (Will *et al.*, 1991; Meenan *et al.*, 1996). A combination releasing 5-ASA and a PAF antagonist in the colon was found to have beneficial effects in TNBS-colitis (Galvez *et al.*, 2000).

Other metabolites of AA have also been implicated in IBD. For example, 15-hydroxy-eicosatetraenoic acid (15-HETE) was identified as the main eicosanoid formed by the colon mucosa (Zijlstra *et al.*, 1992). Its synthesis correlated positively with the severity of colon inflammation suggesting a role for 15-lipoxygenase activity in IBD. Moreover, the expression of 12-LOX was induced in both UC and CD colitic mucosa and was undetectable in normal colon mucosa (Shannon *et al.*, 1993). The role of 12-HETE has been studied in cancer, where it is associated with an increased metastatic potential of cancer cells (Silletti *et al.*, 1994; Yamamoto *et al.*, 1997).

Continuing inflammation in IBD predisposes to premalignant or malignant changes in the colon. NSAIDs reduce the incidence of colon cancer, and COX-2 is implicated in colon carcinogenesis (Prescott and Fitzpatrick, 2000). Treatment with a NO-releasing NSAIDs (nitrosulindac) potentiated the anti-proliferative effect of the parent compound (sulindac) on colon cancer cells (Lavagna *et al.*, 2001).

Colon epithelial cells undergo apoptosis in response to bacterial invasion (Kim *et al.*, 1998). In the TNBS-model of experimental colitis increased epithelial cell apoptosis is associated with peroxynitrite-induced cell damage and increased expression and activity of iNOS (Yue *et al.*, 2001). Treatment with a NO-releasing mesalamine has

been shown to protect epithelial cells from cytokine-induced apoptosis (Fiorucci *et al.*, 2001) and to provide an enhanced anti-inflammatory effect in the TNBS-model of colitis in rats (Wallace *et al.*, 1999). These anti-apoptotic effects of NO on normal epithelial cells are mediated by S-nitrosation and inactivation of caspase. This effect of NO may pose a threat of increased risk for carcinogenesis when considering treatment with NO-donors. Inhibition of apoptosis may allow survival of cells with genetic alterations i.e. putative cancer cells (Jaiswal *et al.*, 2001). In contrast to these findings, the NO-releasing NSAIDs, as pointed out previously, have been shown to inhibit tumor cell proliferation and to decrease preneoplastic changes in a rat model of colon cancer (Bak *et al.*, 1998) by a yet unidentified mechanism. Further studies will undoubtedly shed light on the role of NO-NSAIDs in inflammation and carcinogenesis.

There is an increasing trend to produce chimeras where a novel acting compound is combined to a conventionally used drug in order to achieve additive or even synergistic effects. Good examples of this are the above-mentioned NO-releasing NSAID and 5-ASA – PAF antagonist combinations. There is an increasing body of evidence stating that inhibition of the rate-limiting enzyme in the formation of prostanoids may not be the most efficient anti-inflammatory strategy in the gut. Perhaps downstream enzyme inhibition from COX, selective prostanoid/ eicosanoid receptor antagonism or agonism, or a combination of multiple inhibitory/ activating molecules will provide the desired beneficial effects in the future; adverse effects aside. Moreover, the multifactorial pathogenesis of IBD that has eluded scientist and clinicians does not provide a solid basis for drug design. The disheartening clinical trials so far on manipulation of eicosanoid actions may still hold back the acceptance of drugs aimed at a prostanoid-eicosanoid-system for colitis patients.

3 AIMS OF THE STUDY

The aim of this thesis was to investigate the roles of the inflammation associated inducible enzymes, iNOS and COX-2 in colitis. Their contribution was assessed by using NOS- and COX- enzyme inhibitors with different selectivities for the constitutive and inducible isoforms in an experimentally-induced model of colitis in the rat. The role of iNOS was further investigated in mucosal samples from patients with ulcerative colitis.

The detailed aims were as following:

1. To characterize a suitable, affordable, and reproducible experimental model for studying the role of NOS and COX –pathways in colitis and to find appropriate parameters of inflammation that could be used to quantitate the effects of drug treatments (Study I).

2. To investigate the contributions of the COX-enzymes in gut inflammation by using inhibitors with different selectivities towards the two COX-isoforms in the TNBS-induced experimental model of colitis (Study II).

3. To compare the effects of selective iNOS-inhibition and non-selective NOS-inhibition in the TNBS-model of colitis in order to clarify the role of iNOS in this model (Study III).

4. To extend the findings of Study III and to investigate further the anti-inflammatory effects of selective iNOS inhibition in human tissue and disease (Study IV).

4 MATERIALS AND METHODS

The exact methods are to be found in the original Studies I-IV.

4.1 Experimental setups

4.1.1 Acute TNBS-colitis in the rat

2,4,6-Trinitrobenzenesulfonic acid (TNBS) was diluted with 50% ethanol to obtain a final solution containing 60 mg/ml or 120 mg/ml TNBS. The male Wistar rats were anesthetized with halothane, and the TNBS-solution (1 ml/kg body weight) was administered rectally via a polypropene catheter in the distal colon seven centimeters proximally from the anus. The catheter was carefully withdrawn and the rat was left in a supine position to recover from anesthesia. The rats were admitted normally to laboratory food and water before and after induction of colitis.

The rats were decapitated, blood was collected and the distal colon was removed, weighed, and photographed. Blood was collected into heparinised tubes, and was immediately centrifuged, and plasma was collected. Three samples were cut from the distal part of the colon, and mucosa was scraped off from the remaining tissue for myeloperoxidase measurement. In Study I, samples of spleen were also taken. All samples were stored in a deep freezer (at –70 ^{o}C) before measurements.

4.1.2 Incubation of human colon samples

Colon mucosal samples were collected from patients with UC undergoing elective colectomy at the Helsinki University Central Hospital. Samples of mucosa with or without macroscopic inflammation were excised from the removed colon, washed in ice-cold physiological saline solution and dissected with a 4 mm diameter cuvette into samples for incubation. These samples were then washed in phosphate buffered saline (PBS) and incubated for 5 hours with or without the drugs under study.

4.2 Measurements and methods

4.2.1 Colon damaged area

For quantitation of colon inflammation in Study I, five independent assessors viewed the pictures of distal colons and used a visual analog scale to estimate inflammatory damage.

A new method for damaged area evaluation was developed for Studies II and III. The photographs of the distal colon were digitized using a Canoscan slide-digitizer (Canon Inc., Tokyo, Japan). The digitized images were then analyzed on a computer using the built-in properties of the commercial Corel Draw 7.0 program (Corel Corporation, Ottawa, Canada). The scanned pictures were split into hue-saturation-

brightness channels, from which the latter was used for further evaluation. Damaged area was defined as that with a densitometric value below 126 (hemorrhagic, red); likewise, densitometric values of 126 and above (healthy, pale) designated the healthy area. The damaged area divided by the total area of the colon sample was used to score inflammatory damage.

4.2.2 Myeloperoxidase activity

Measurement of myeloperoxidase activity was carried out using a method described by Pfeiffer & Qiu (1995). The scraped mucosa was weighed, and homogenized in ice-cold potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The homogenates then underwent three freeze-thaw cycles and were centrifuged at 40,000 g for 15 minutes thereafter. The supernatant was then used for further analysis. An aliquot of the supernatant was mixed with sample buffer containing o-dianisidine and H_2O_2. The enzymatic reaction was followed with a spectrophotometer at 480 nm for 2 minutes. Linear reaction kinetics were obtained by dilution of sample. The change in absorbance in one minute is directly proportional to the myeloperoxidase activity of the sample. One unit of activity was defined as that degrading 1 µmol of hydrogen peroxide in one minute.

4.2.3 Prostaglandin E_2 metabolite in plasma

The measurement of a metabolite of PGE_2, 13,14-dihydro-15-keto-prostaglandin-E_2 (PGEM), was carried out by radioimmunoassay as described by Mucha and Losonczy (1990).

4.2.4 Nitrate and nitrite in plasma

Nitrate and nitrate were measured using a modified Griess reaction (Green *et al.*, 1982). Proteins were precipitated in diluted serum samples with $ZnSO_4$. Since the Griess reaction detects only nitrite, nitrate was reduced to nitrite enzymatically by adding nitrate reductase and cofactors (Moshage *et al.*, 1995).

4.2.5 Nitric oxide synthase activity

Nitric oxide synthase activity was assessed by measuring the conversion of [^{14}C]arginine into [^{14}C]citrulline as described by Salter *et al.* (1991) and Moilanen *et al.* (1998). Ice-cold 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid buffer (pH 7.2) containing 200 mM sucrose, 0.1 mM ethylenediaminetetraacetate, 5 mM dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor and 1 µg/ml pepstatin A was added to the tissue samples. Samples were homogenized on ice and centrifuged at 10,000 g for 30 minutes. Supernatants were treated with an equal volume of cation exchange resin (Dowex-50W, sodium form) to remove endogenous arginine. The NOS activity in the supernatants (cytosol + microsomes) were

measured by the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline at 37 ^{o}C for 10 minutes in 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid buffer (pH 7.2) containing 10 µM tetrahydrobiopterin, 2.5 mM dithiothreitol, 400 U/ml calmodulin, 250 µM $CaCl_2$, 0.5 mg/ml bovine serum albumin, 125 µM reduced nicotineadeninedinucleotidephosphate, 10 µM arginine, 100 µM citrulline, 60 mM valine (to inhibit arginase) and 0.33 µCi/ml L-[U-^{14}C]arginine.

The total NOS activity was determined from the difference between the L-[U-^{14}C]-citrulline generated in control samples and samples containing 1 mM L-NMMA; the activity of the calcium-dependent NOS activity was determined from the difference between control samples and samples containing 1 mM of the calcium chelator, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate, and the activity of calcium-independent NOS was determined from the difference between samples containing 1 mM calcium chelator and those with 1 mM L-NMMA. Protein content of the tissue supernatant was measured by Coomassie Brilliant Blue assay (Bio-Rad, Hercules, CA, USA) using bovine albumin as a standard.

4.2.6 Western Blot

The expression of iNOS and COX-2 proteins in rat colon and colon mucosa from UC-patients was measured by Western Blot as described by Kosonen *et al.* (1998). Colon samples were weighed and 6 ml per mg of sample of ice-cold extraction buffer (10 mM Tris-base, 5 mM ethylenediaminetetraacetate, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethyl sulfonyl fluoride, 2 mM Na-orthovanadate, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1.25 mM NaF, 1 mM Na-pyrophosphate, 10 mM n-octyl-β-D-glucopyranoside) was added. Samples were homogenized using an Ultra Turrax T25 homogenizer (Janke & Kunkel GmbH, Staufen, Germany). Following extraction samples were centrifuged and the resulting supernatant boiled for 5 minutes in sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% 2-mercaptoethanol) and stored at -20 °C until analyzed. An aliquot of supernatant was used to determine protein by the Coomassie blue method (Bradford, 1976). Protein samples (20 µg) were separated by electrophoresis on polyacrylamide gel and were transferred to a nitrocellulose membrane. The desired proteins were detected using specific primary antibodies and their respective horseradish peroxidase conjugated secondary antibodies using the enhanced chemiluminescence technique. The primary antibodies were: iNOS rabbit polyclonal antibody (M-19) and COX-2 goat poyclonal antibody (N-20) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), nitrityrosine rabbit polyclonal antibody from Upstate Biotechnology (Lake Placid, NY, USA).

4.2.7 Reverse transcriptase-polymerase chain reaction

Colon samples (approximately 30 mg) were frozen and homogenized. The samples were lysed and purified using QIAshredder™ (QIAGEN Inc., Santa Clarita, CA, USA). Thereafter RNA was extracted using RNeasy® kit for isolation of total RNA (Qiagen Inc., Santa Clarita, CA, USA). Synthesis of cDNA from mRNA and subsequent amplification of cDNA by PCR was performed with GeneAmp® Thermostable rTth Reverse Transcriptase RNA PCR Kit (Perkin-Elmer; Roche Molecular Systems Inc., Branchburg, NJ, USA). First strand cDNA was synthesised using sequence specific downstream primer for rat iNOS or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as reference. The upstream primer was added into the reaction mixture at the beginning of PCR amplification. PCR conditions were denaturation, annealing and extension at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min, respectively, and 25 cycles for both iNOS and GAPDH. The Mg^{2+} concentration in the reaction mixture was 1.5 mM. Primers for amplification of iNOS were 5'-CAAGCTGTATGTGACTCCATCGAC-3' (sense) and 5'-AGATGAGCTCAT-CCAGAGTGAGCTG-3' (antisense) resulting a 346 bp product. Primers for amplification of GAPDH were 5'-CGGTGTCAACGGATTTGGCCGTAT-3' (sense) and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (antisense) resulting a 306 bp product. Products were analyzed on 1.5% agarose gel containing ethidium bromide and visualized in ultraviolet light.

4.2.8 Enzyme linked immunosorbent assay

The concentrations of IL-1β, IL-6 and TNF-α in incubation medium were analysed by enzyme linked immunosorbent assay (ELISA) using reagents from the Central Laboratory of the Netherlands Red Cross (CLB). IL-1Ra was analyzed by ELISA using reagents from R&D Systems, USA (Quantikine®).

4.2.9 Immunohistochemistry

Mucosal samples were embedded in Tissue Tek O. C. T. Compound (Sakura Finetek Inc., Zoeterwoude, Netherlands). Cryostat sections were air dried, and fixed in ice cold methanol. Endogenous peroxidase activity was quenched with subsequent incubation in PBS containing 0.3% hydrogen peroxide. The sections were then washed three times with ice cold PBS, incubated in normal blocking serum, washed and incubated overnight with the primary antibody. Thereafter the sections were washed and incubated with the secondary antibody. The Vectastain ABC Elite-kit (Vector Laboratories, Burlingame, CA, USA) was used for staining the sections. Monoclonal anti-iNOS (Transduction Laboratories, Franklin Lakes, NJ, USA) and polyclonal anti-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY, USA) were used as primary antibodies. Respective non-immune serum was used instead of primary or secondary antibody as negative controls. Rabbit/mouse IgG of the same

subtype as the primary antibodies but with an irrelevant specificity was used as an additional negative control. The sections were counterstained with hematoxylin.

4.2.10 Ethics

Studies I-III were approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Finland. Study IV was approved by the Ethics Committee of Helsinki University Central Hospital, Second Department of Surgery, Helsinki, Finland.

4.2.11 Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test. P values of <0.05 were considered significant.

4.2.12 Drugs

The drugs used in Studies II-IV are shown in Figures 4 and 5. In Study II a preferential inhibitor of COX-2 over COX-1, nimesulide, a non-selective COX-inhibitor, indomethacin, and a preferential COX-1 over COX-2 inhibitor acetylsalicylic acid were used. In this study a corticosteroid, dexamethasone, served as control. In Studies III-IV a non-selective NOS-inhibitor, L-NAME, and a selective iNOS-inhibitor, 1400W were used. In Study IV also an NO-donor, SNAP, was used.

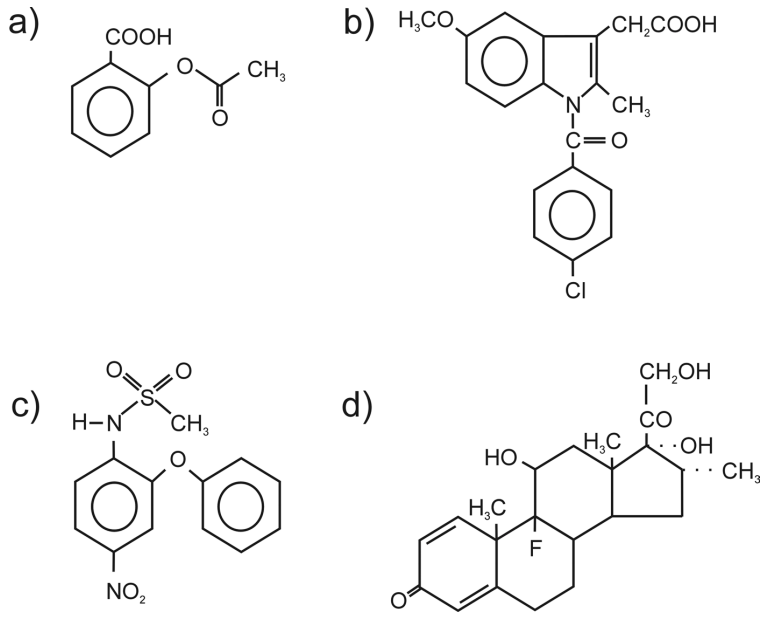

Figure 4. Structures of the test drugs in Study II. a) Acetylsalicylic acid, b) indomethacin, c) nimesulide, and d) dexamethasone.

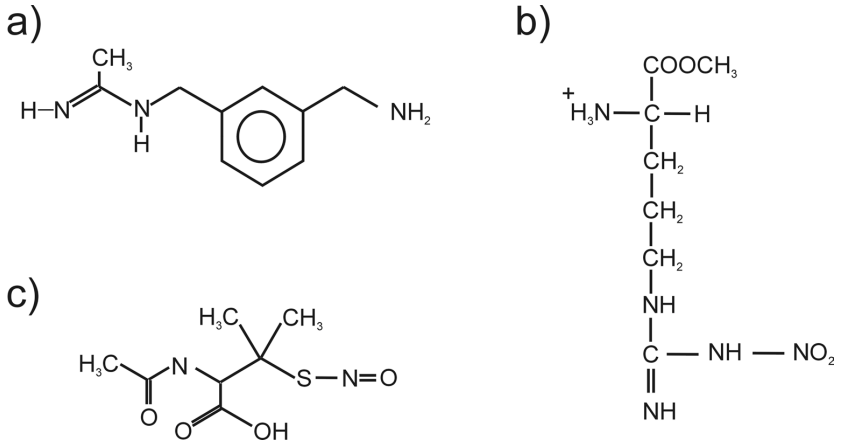

Figure 5. Structures of the test drugs in Studies III and IV. The nitric oxide synthase inhibitors a) N-[3-(aminomethyl)benzyl]acetamidine, 1400W and b) N^G-nitro-L-arginine methyl esther, L-NAME, and the nitric oxide donor c) S-nitroso-N-acetyl-penicillamine, SNAP.

5 SUMMARY OF RESULTS

The results of the Studies (I-IV) are collectively represented in Table 13.

5.1 TNBS-induced colitis (I-III)

5.1.1 Induction of iNOS and COX-2 (I-III)

A model of 2,4,6-trinitrobenzenesulfonic acid (TNBS) -induced acute colitis in the rat was characterized in Study I. TNBS induced severe macroscopic inflammation in the colon as early as 6 hours after induction attaining a sustained peak level at 24 hours. Infiltration of neutrophil granulocytes was measured as myeloperoxidase (MPO) acitivity. Neutrophil infiltration into the inflamed colon mucosa was enhanced at 6-24 hours after TNBS and returned to the pre-treatment levels in 48 hours. Both macroscopic inflammation and inflammatory cell infiltration were associated with increased inflammatory edema that showed a sustained elevation similar to macroscopic lesions. These increases were coupled to increases in PGE_2 production, which was measured as plasma concentrations of its stable metabolite 13,14-dihydro-15-keto-PGE_2, PGEM. At 6 to 24 hours from the induction of colitis elevated plasma PGEM concentrations were found (Figure 6).

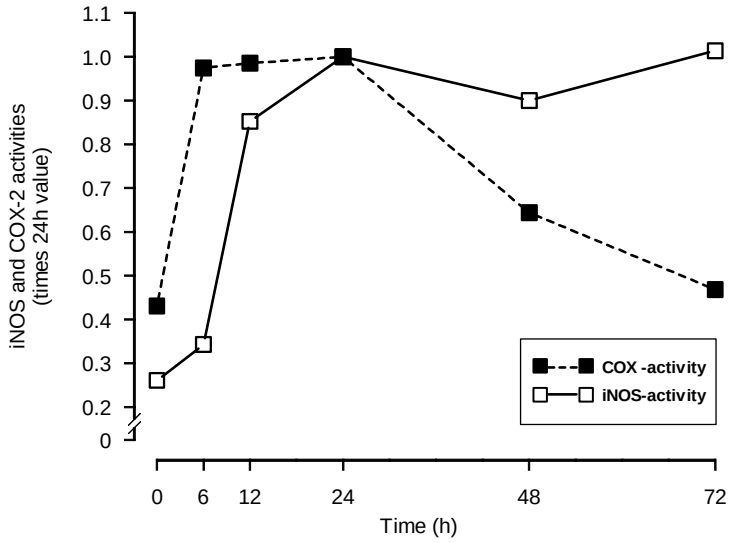

Figure 6. Time dependency of the activities of COX-2 and iNOS measured as plasma concentrations of a metabolite of prostaglandin E_2 and by the iNOS-activity assay, respectively.
COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase

Table 13. Summary of results. Studies I-IV. Values in parentheses designate change from the respective control.

STUDY	Effects/treatments	Inflammatory edema	Neutrophil infiltration	Macroscopic damage
I	TNBS-induced acute colitis	↑	↑	↑
II	ASA	n.e.	n.e.	n.e.
	Nimesulide	↓ (37%)	↓ (72%)	n.e.
	Indomethacin	↓ (69%)	n.e.	n.e.
	Dexamethasone	↓ (48%)	↓ (54%)	n.e.
III	1400W	↓ (95%)	↓ (63%)	↓ (26%)
	L-NAME	n.e.	n.e.	n.e.

STUDY	Effects/treatments		IL-1β	IL-1Ra	IL-6	TNF-α
IV	Incubation of UC-mucosa		↑	↑	↑	↑
	Inflamed mucosa	1400W	n.e.	n.e.	↓ (27%)	↓ (66%)
		L-NAME	n.e.	n.e.	n.e.	n.e.
		SNAP	↑ (175%)	↓ (34%)	↑ (52%)	↑ (62%)
	Uninflamed	1400W	n.e.	not done	n.e.	↓ (69%)
		L-NAME	n.e.	not done	n.e.	n.e.
		SNAP	n.e.	not done	↓ (48%)	n.e.

1400W, N-3-aminomethyl-benzylacetamidine; ASA, acetylsalicylic acid; COX, cyclooxygenase; IL-1β, Interleukin-1 beta; IL-1Ra, IL-1 receptor antagonist; iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine-methyl esther; n.e., no effect; SNAP, S-nitroso-N-acetyl-penicillamine; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNF-α, tumor necrosis factor alpha; UC, ulcerative colitis

The activity of the nitric oxide producing inducible enzyme, iNOS, showed a more sustained, but delayed onset response as compared to the production of PGE_2 (Figure 6). In order to compare the systemic and local inflammatory responses, iNOS activity was measured also in the spleen (a homing site for lymphocytes). A very low activation of iNOS as compared with local iNOS activation in the distal colon was seen in the spleen suggesting that the TNBS-insult produces a local rather than a systemic acute response, at least in the sense of nitric oxide production. This effect was also demonstrated in plasma nitrite and nitrate concentrations that remained unchanged throughout the study period. The study also identified neutrophil infitration and prostaglandin E_2 production as transient responses, while iNOS activity, macroscopic inflammation, and inflammatory tissue edema showed a more sustained course during the 72 hour study period.

5.1.2 Inhibition of cyclooxygenase (II)

The expression of the inducible cyclooxygenase, COX-2 was found to be increased in the distal colon at 6 to 24 hours after induction of colitis with TNBS. The time course of COX-2 protein expression was consistent with the previously measured plasma concentrations of PGEM. A novel method was introduced to measure the ratio of damaged to total mucosa instead of the previous more subjective evaluation by independent assessors that was used in Study I. The chemical composition of drugs used in Study II are shown in Figure 4.

Treatment with a preferentially COX-2 over COX-1 inhibiting NSAID, nimesulide, suppressed the increase in plasma PGEM, decreased formation of inflammatory edema and reduced infiltration of neutrophil granulocytes into the inflamed colon mucosa. Nimesulide inhibited COX-2 levels in the distal colon mucosa. Because nimesulide failed to down-regulate COX-2 expression in murine macrophages in cell culture, we concluded that its *in vivo* effect is due to reduced inflammatory area in the distal colon. However, nimesulide failed to show inhibitory action in macroscopic lesions in the gut as measured by the computerized method.

The anti-inflammatory actions of nimesulide in this model were compared to two other NSAIDs: to a relatively COX-1 selective inhibitor, acetylsalicylic acid (ASA), and to a non-selective COX –inhibitor, indomethacin. Indomethacin inhibited COX-2 activity (as measured by plasma PGEM concentrations) and the formation of inflammatory edema, but failed to suppress infiltration of neutrophil granulocytes or macroscopic damage. Treatment with ASA did not have any effects on these parameters measured. In this model, the actions of nimesulide were similar to those of dexamethasone treatment. The anti-inflammatory, synthetic glucocorticoid, dexamethasone exerts its effects via different mechanisms of action including suppression of COX-2 expression (as shown in Study II with J774 macrophages) and thereby reduces the formation of prostaglandins.

5.1.3 Inhibition of nitric oxide synthase (III)

The model of TNBS-induced acute colitis in the rat was evaluated in respect to the expression of iNOS. In Study I it was reported that in this model a local increase in iNOS activity is present. The time dependence of iNOS expression was demonstrated at mRNA and protein levels in the distal colon after TNBS enema. A marked increase in iNOS protein and mRNA expression was already found 6 hours after induction. Expression of iNOS mRNA declined to the baseline levels after 24 hours, and also iNOS protein levels decreased to some extent. However, in concert with previously reported iNOS activity there was still a significantly increased amount of iNOS protein present at the inflammatory focus at 72 hours after TNBS enema. The drugs used in this study are shown in Figure 5.

In the 24-hour acute model of colitis, subcutaneous administration of a highly selective iNOS inhibitor, N-[3-(aminomethyl)benzyl]acetamidine (1400W) attenuated the infiltration of inflammatory neutrophil granulocytes, inhibited the formation of inflammatory edema and prevented development of macroscopic ulcerative damage. Treatment of colitis with a preferential inhibitor of constitutive NOS, N-nitro-L-arginine-methyl-esther (L-NAME) showed no effects in this model. As found in Study I, no increases were seen in plasma nitrite and nitrate –concentrations after induction of colitis TNBS-enema. Thus the effectiveness of the studied drugs could not be evaluated by measuring the concentrations of these NO-metabolites in Study III.

5.2 Selective inhibition of mucosal iNOS in ulcerative colitis (IV)

The beneficial effect with selective inhibition of iNOS was further evaluated in human colon mucosal tissue from patients with active colitis. Tissue samples from seven patients undergoing elective proctocolectomy at the Helsinki University Central Hospital, II Department of Surgery, were obtained for the experiments. Uninflamed mucosal tissue from the same patient's colon served as a control.

The expression of iNOS protein was first determined in these samples using Western Blot. In inflamed mucosa a tenfold increase in iNOS expression was found as compared to uninflamed tissue samples. Using immunohistochemistry on samples from two patients, the expression of iNOS was localized to cells in the *lamina propria*. In some areas immunopositive staining was seen also in the apical microvillous border of epithelial cells. As reported in the literature iNOS activity in inflammation is coupled to the formation of reactive nitrogen species (RNS) (Coleman, 2001). iNOS-derived NO rapidly reacts with tissue superoxide to form the cytotoxic peroxynitrite anion. In Study IV nitrotyrosine was used as a marker of peroxynitrite formation, even though some nitrotyrosine is also formed in physiological processes unrelated to peroxynitrite. The total expression of nitrotyrosine showed an increasing tendency, and the expression of a tyrosine nitrated 25kDa protein was significantly enhanced in

the inflamed tissue. Expression of nitrotyrosine was co-localized with iNOS in cells of the *lamina propria*.

The effects of the iNOS inhibitor, 1400W were compared to those of a cNOS inhibitor, L-NAME and those of an NO-donor, S-nitroso-N-acetyl-penicillamine (SNAP). Both the inflamed and uninflamed mucosa was incubated in tissue culture for five hours in the absence or presence of the drugs studied. The accumulation of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α and of the anti-inflammatory cytokine IL-1Ra in the culture medium was quantitated. Both the inflamed and uninflamed mucosa produced detectable amounts of the proinflammatory cytokines, but the levels of nitrite, a stable end-product of NO metabolism, were undetectable.

In the inflamed mucosa, selective inhibition of iNOS with 1400W inhibited the accumulation of TNF-α and IL-6, while cNOS inhibition with L-NAME was ineffective. Supplementation of exogenous NO by SNAP produced significant increases in pro-inflammatory cytokine accumulation. As further evidence of a pro-inflammatory effect of exogenous NO in the inflamed mucosa, the concentrations of the anti-inflammatory cytokine IL-1Ra were inhibited with SNAP treatment. In the uninflamed mucosa, selective iNOS inhibition reduced the release of TNF-α, whereas inhibition of cNOS had no effect on pro-inflammatory cytokine concentrations. Treatment of the uninflamed mucosal samples with the NO-donor, SNAP, decreased the levels of IL-6 thus evidencing a dual role for NO depending on the activity of inflammation.

6 DISCUSSION

6.1 Methodological considerations

6.1.1 TNBS-induced experimental rat colitis

The TNBS-model of colitis is a widely used experimental animal model of human IBD. It features several important pathogenetic characteristics of these chronic relapsing diseases. The TNBS-model was set up for the present experiments based on the literature stating its usefulness for pharmacological studies. In the beginning, the acute model was characterized for iNOS and COX-2 expression and other parameters of inflammation needed later on in the experiments. The dose of TNBS needs to be adjusted for the rat strain used and the time course of inflammation should be evaluated before starting work with this model. Even though the TNBS model of experimental colitis is technically easy to perform, a highly routined inductee is required for reproducible results. For example, it is not uncommon that a fecal concrement blocks catheter entry and even distribution of TNBS in the colon leading to an unevenly localized inflammatory response. Fasting the rats, on the other hand may result in a less severe inflammation and increase aggression in animals. To avoid these problems in the Studies I-III the same person always routinely iduced colitis.

In Studies II and III only a piece from the distal colon was taken as a sample for measuring COX-2 or iNOS activity or expression. During interpretation of the results, however, it was found out that the whole distal colon mucosa should be sampled, not just merely one piece. Since the whole mucosa of the distal colon was scraped for the measurement of MPO-activity, these results more adequately represent the state of the distal colon, only 1.5 cm of the distal part was excluded. However for studying iNOS activity (Study I), COX-2 protein expression (Study II) and iNOS mRNA and protein expression (Study III) only samples from the distal colon were used. In these studies care was taken to ascertain even distribution of TNBS in the colon by slowly pulling the catheter outwards when injecting TNBS.

The present model of TNBS-induced colitis is characterized by the general features of acute inflammation, and thus provides information on common acute inflammatory mechanisms of the gut rather than on any specific disease. As compared to vehicle treatment, administration of TNBS induces a more severe and sustained inflammation associated with concomitant activation of nitric oxide and prostaglandin production. This acute model is suited to studying preventive effects of drugs while

the delayed, chronic model is better adapted for studies on healing or resolution of inflammation.

After the acute 24-48 hour phase, TNBS-colitis develops into a chronic phase that lasts up to eight weeks (depending on the animal and strain)(Morris *et al.*, 1989). This chronic phase is characterized by activation of the immune system and cell mediated adaptive immunity (Wirtz and Neurath, 2000). It is also associated with increased mucosal infiltration of T-helper lymphocytes and production of cytokines (e.g. IFN-γ, TNF-α, IL-2 and IL-12) (Strober *et al.*, 1998; Sun *et al.*, 2001). The chronic stage has distinct similarities with human CD: transmural lesions, formation of granulomas, and the overall cytokine profile (Morris *et al.*, 1989). An interesting feature of TNBS-colitis is that it can be reactivated by intravenous injections of TNBS suggesting a delayed hypersensitivity reaction (Appleyard and Wallace, 1995).

The TNBS model of colitis has many attractive features for studying human IBD. However, many beneficial drug effects initially found by using this model have turned out to be ineffective in subsequent clinical studies. Thus, the model can only provide a basis for preliminary testing and direction for drug development. The method of induction is easy, but quite ingenious in its principle in initiating disease by mimicking and utilizing known factors (e.g. the role of bacteria, epithelial damage and dysfunction, and immune activation) of IBD pathogenesis. It is a well characterized animal model, and as such the obtained results are to be considered to be reliable.

6.1.2 Incubation of colitic mucosa

Material for Study IV was obtained from patients undergoing elective proctocolectomy. These patients had previously been diagnosed with UC. All patients were treated with oral steroids. The resected bowel might have suffered from ischemia during its surgical removal. Nevertheless, reperfusion injury was avoided since the tissue was removed from the body.

Prior to incubation the mucosal samples were quickly transferred to the laboratory in ice-cold saline and were washed. In incubation, no external stimulus was added, only the spontaneous release of inflammatory mediators was assessed in the presence or absence of drugs. When it is being incubated the mucosal sample is of course without blood supply, but this setup permits study with experimental drugs on human tissues, and in human disease relevant to therapy. The results obtained are to be considered as a total sum of the interplay of all the mediators and cellular components. The patients were on steroid treatment that has various anti-

inflammatory actions at cellular level. Their other medications may also play some role in the results obtained from this particular setup.

In conclusion, *in vitro* incubation of colon tissue derived from patients with UC is a simple method that utilizes inflamed human tissue in pharmacological studies on novel treatment approaches for inflammatory bowel disease with experimental drugs.

6.2 Factors contributing to induction of iNOS and COX-2 in TNBS-colitis

The two components in the TNBS-solution, 2,4,6-trinitrobenzenesulfonic acid and ethanol both contribute to tissue injury in colitis. As evidenced in Study I, treatment with ethanol alone induces a less severe inflammatory response associated with low iNOS activity as compared to the lesions caused by its combination with TNBS. The TNBS-induced damage and iNOS activation lasted throughout the study period although some resolution was evident at 72 hours. The acute effect of TNBS and the lesser action of ethanol alone suggest that increased iNOS activity is a non-specific response to acute tissue injury and is related to the severity of the inflammatory response. Furthermore, these TNBS-induced changes were in direct correlation with the formation of inflammatory edema.

Activation of COX-2 in this model was evaluated by measuring plasma concentrations of a stable metabolite of PGE_2, PGEM. Plasma PGEM increased concomitantly with mucosal MPO-activity, a measure of neutrophil granulocyte infiltration. Both significantly increased for 6-12 hours and declined to almost their baseline levels 48 to 72 hours after induction of colitis. As found in Study II, also COX-2 expression showed a similar time-course. These results suggest that COX-2–derived PGE_2 contributes to the acute phase of inflammation (*ad* 24 hours) characterized by neutrophil infiltration, but is not as important thereafter in the perpetuation of inflammatory damage.

TNBS has been reported to induce cell lysis, increase oxidative and nitrosative stress, modify cell surface proteins, and initiate immunologic activation (see 2.5.1). These factors contribute to the prolonged and accentuated effects seen after induction of colitis with TNBS. After the inflammatory insult and cell damage, the body responds with activation of the acute inflammation cascade. In active inflammation cell destruction and increased permeability allow bacteria to enter the *lamina propria*. Pro-inflammatory cytokines and bacterial antigens induce and drive

the transcription of both COX-2 and iNOS. In the 24 hour acute model of TNBS-colitis increased levels of IL-1β, IL-6, and TNF-α have been reported (Sun *et al.*, 2001). These cytokines exert their activities through activation of the transcription factor NF-kappaB, which also has been shown to be induced in experimental (Neurath *et al.*, 1996) and IBD-colitis (Schreiber *et al.*, 1998).

In the acute TNBS-induced model of colitis induction of iNOS and COX-2 occurs in response to cellular injury and immune activation. After a rapid induction of COX-2 expression and activity associated with neutrophil granulocyte infiltration, a more persistent induction of iNOS expression and activity is seen that correlates well with macroscopic inflammation. These results are in concert with earlier published observations on human inflammatory bowel disease (Hendel and Nielsen, 1997; Kimura *et al.*, 1997) suggesting a further verification of the model in studying the roles of iNOS and COX-2 in gut inflammation.

Inflammation increases intestinal permeability and may thereby permit bacteria or bacterial antigens to enter the *lamina propria* and systemic circulation (Neilly *et al.*, 1995). This low-grade endotoxemia may explain the induction of splenic iNOS activity as found in Study I. TNBS-induced colitis has not been associated with hepatobiliary abnormalities even though they are quite frequently found in human disease especially in UC (Hendrickson *et al.*, 2002). In experimental models of IBD, various changes in sites unaffected by the initial insult have been reported. Some studies have reported alterations and disturbances in myoelectric activity and adrenergic nerve function in both the inflamed and the uninflamed colon (Jacobson *et al.*, 1997) and in the uninflamed ileum (Aube *et al.*, 1999). Also alterations in the expression of electrolyte transporter proteins in the uninflamed ileum have been found (Khan and Ali, 1999).

6.3 Effects of COX-2 inhibition in colitis

As found in Study II the expression of COX-2 was increased and was associated with inflammatory edema and neutrophil infiltration into the inflamed colon, and with plasma PGEM concentrations. However, even if marked suppression in these parameters was attained with preferential COX-2 inhibition (nimesulide, and also dexamethasone) and with non-selective COX-1/-2 inhibition (indomethacin) no beneficial effect was found in evaluation of macroscopic damage of the inflamed colon.

A relatively low dose of ASA (10 mg/kg) was used in this study in order to inhibit only COX-1, and failed to provide anti-inflammatory benefit. Since prostaglandin production in this model was associated with COX-2 expression and activity, the lack of effect on COX-2 by ASA may explain its poor anti-inflammatory action. Deacetylation or decomposition of ASA was not evaluated, but the salicylate concentrations formed were most likely too low to cause any significant inhibition of COX-2.

In the literature, the general assumption of a straightforward detrimental role for COX-2 activity and prostaglandin production has been challenged in colitis (Felder *et al.*, 2000). There are reports of acute exacerbations of IBD in patients treated with inhibitors of COX (Kaufmann and Taubin, 1987; Aabakken and Osnes, 1989). The NSAIDs have also been associated with colitis in otherwise healthy individuals (Faucheron and Parc, 1996). Selective inhibitors of COX-2 as well as conventional NSAIDs have been reported to aggravate inflammation or show no beneficial effect in some experimental models of IBD (Table 12). Recent studies have identified an anti-inflammatory role for COX-2 derived prostaglandins and PGD_2 as the major anti-inflammatory prostaglandin that is produced through this pathway (see 2.5.3). On the other hand, there are a number of studies showing a beneficial effect on colitis by NSAIDs of various selectivities towards COX-2 (Tardieu *et al.*, 2000; Cuzzocrea *et al.*, 2001b). The favorable effects of these drugs can also be attributed to their COX-2 unrelated actions.

6.4 Effects of iNOS inhibition in colitis

In Study III, a beneficial effect of selective iNOS inhibition on experimental TNBS-induced colitis was found. This finding is in concert with previous observations using relatively less iNOS selective agents, which also have additional mechanisms of action besides iNOS inhibition (see 2.2.1). Thus it is proposed that in acute inflammation of the gut iNOS activity has a pro-inflammatory action. The same highly selective iNOS inhibitor was concomitantly used in a more chronic model of colitis, and also showed beneficial effects on inflammation in that model (Menchen *et al.*, 2001).

Since iNOS is preferentially expressed in areas of inflammation, it presents a highly focused approach to treating inflammation. Even in IBD patients treated with glucocorticosteroids, there is sustained expression of iNOS associated with macroscopic inflammation (Leonard *et al.*, 1998). In patients, whose disease is still

uncontrolled receiving steroid therapy, selective iNOS inhibitors may provide additional benefits.

Analogous to prostanoids, NO may also have anti-infammatory effects. This is demonstrated by the findings that iNOS deficient mice are susceptible to more severe inflammatory damage induced with TNBS enema than their wild type littermates (McCafferty *et al.*, 1997), and IL-10/iNOS –double deficient mice develop spontaneous colitis (McCafferty *et al.*, 1999). In these studies iNOS deficiency does not provide additional protection from colitis as would be expected if iNOS activity was totally pro-inflammatory. In light of these results, NO has anti-inflammatory actions, but high concentrations of iNOS-derived NO and its oxidative, toxic metabolites augment the inflammatory response (Zingarelli *et al.*, 1999b; Hokari *et al.*, 2001).

6.5 Interplay of prostaglandins and nitric oxide in colitis

It has been unambiguously shown *in vitro* that there are complex interplay and feedback mechanisms between prostaglandin production and nitric oxide synthesis. NO has been shown to activate COX enzymes (Salvemini *et al.*, 1993; Maccarrone *et al.*, 1997) and prostaglandins (PGE-analogs) have been shown to activate NOS (Dieter *et al.*, 1999). In Study I, the time-courses of iNOS activity and plasma PGEM suggest that since the increase in PGE_2 is more acute, it may drive the activation of NOS in this model. iNOS activity remained at a relatively high level, but PGEM concentrations had a more transient course demonstrating that iNOS-derived NO production is not capable of maintaining PGE_2 production or that PGE_2 is derived from infiltrating inflammatory cells and that NO inhibits this infiltration.

NO has been shown to inhibit the COX-enzymes representing an additional mechanism for NO's anti-inflammatory effects (Swierkosz *et al.*, 1995; Tanaka *et al.*, 2001). However, as some prostaglandins have protective effects on the gastrointestinal mucosa, inhibition of their production by NO may increase inflammatory damage. On the other hand, in other models NO activates COX-enzymes and increases production of prostaglandins (Salvemini *et al.*, 1993; Goodwin *et al.*, 1999; Devaux *et al.*, 2001). Peroxynitrite has been shown to increase and activate the cyclooxygenase activity of COX-enzymes *in vitro* (Goodwin *et al.*, 1999). It may serve as a substrate for the enzyme's peroxidase activity leading to enhanced production of prostaglandin endoperoxides (PGG_2 and PGH_2) (Landino *et al.*, 1996) demonstrating a physiological rather than toxic effector function for peroxynitrite.

Prostagladins E_2 and J_2 have been shown to inhibit NOS activity and expression in different experimental setups (Harbrecht *et al.*, 1997; D'Acquisto *et al.*, 1998). Also overexpression of COX-2 in intestinal epithelial cells inhibits iNOS induction in response to pro-inflammatory cytokines (Kobayashi *et al.*, 2001). In contrast, inhibition of COX-activity reduces iNOS expression and activity in murine macrophages (Aeberhard *et al.*, 1995; Posadas *et al.*, 2000) demonstrating NOS-activation by prostaglandins. Furthermore, PGE_2 increases LPS-stimulated NO production (Dieter *et al.*, 1999) and enhances NF-kappaB activity (Poligone and Baldwin, 2001). The exact mechanism and relevance of these modulations remain to be elucidated and seem to exhibit significant variation depending on the experimental setup (Weinberg, 2000). From Study I it is concluded that the increased production of PGE_2 preceding the induction of iNOS activity suggests activation of NOS by prostaglandins *in vivo*. The increased NO produced by induced iNOS activity subsequently inhibits COX and PGE_2 production as measured in plasma as PGEM.

Since the expression and activity of both iNOS and COX-2 is induced by the same pro-inflammatory agents and is associated with inflammatory conditions, it has been proposed that inhibition of both iNOS and COX-2 would provide the most potent anti-inflammatory effect (Weinberg, 2000).

6.6 Selective inhibition of iNOS in ulcerative colitis mucosa

Study IV concentrated on the anti-cytokine activities of selective iNOS inhibition and showed that inhibition of iNOS activity in UC may beneficially modulate the pro-inflammatory cytokine profile in both inflamed and uninflamed colon mucosa.

NO has both anti-inflammatory and pro-inflammatory effects. The latter is mainly due to the detrimental oxidative metabolism of NO in inflammation while the former is mediated via specific intracellular pathways, namely the formation of cGMP and direct feedback inhibition of enzyme activity or transcription factor activation (see below). As a radical species, NO reacts with other free radicals and thus exerts anti-inflammatory radical quenching activity. However, under oxidative stress as frequently found in inflammation other nitrogen oxides such as N_2O_3, and peroxynitrite anion are rapidly formed from NO. This detrimental metabolism of NO can be limited through selective inhibition of iNOS, which in addition to producing high concentrations of NO has been shown to produce superoxide and to contribute to oxidative stress (see 2.2).

Expression of iNOS and 3-nitrotyrosine co-localized to cells in the *lamina propria*. Incubation of inflamed mucosa with a highly selective iNOS inhibitor decreased pro-

inflammatory cytokine release and synthesis supporting the hypothesis that in active inflammation iNOS activity is detrimental. The NO-donor, SNAP increased the release of IL-1β, IL-6, and TNF-α. The results suggest that both iNOS-derived and exogenous NO may have pro-inflammatory effects in UC. In uninflamed tissue selective iNOS inhibition suppressed the release of TNF-α, while SNAP decreased the release of IL-6 in the incubation medium. Inactive mucosa may therefore benefit from NO supplementation, but selective inhibition of iNOS activity also seems to provide some anti-inflammatory advantages in quiescent inflammation.

The mechanisms underlying these effects of NO and NO-derived species on cytokine production in inflammation are intricate and equivocal. One of many explanations, which is highly attractive but hypothetical is the finding that NO and reactive oxygen species directly activate $p21^{ras}$ (Connelly *et al.*, 2001). This G-protein in turn activates kinases involved in NF-kappaB activation such as the I-kappaB-kinase, which phoshorylates and inactivates the inhibitor of NF-kappaB activation (I-kappaB) (Lander *et al.*, 1995b). Under different conditions, for example in inactive disease as shown in Study IV, the anti-inflammatory effects of exogenous NO are prominent. NO inhibits I-kappa-B and the DNA-binding of NF-kappaB; NO acts as an antioxidant and quenches reactive oxygen species thereby limiting their $p21^{ras}$-activating potential and NF-kappaB driven cytokine synthesis (Figure 7).

In Study IV, a beneficial effect was seen on the accumulation of TNF-α with the selective inhibitor of iNOS, 1400W. This result agrees with previous findings, where another selective iNOS inhibitor, ITU, was reported to inhibit TNF-α release from rat peritoneal macrophages (Southey *et al.*, 1997). This pro-inflammatory effect of NO may be mediated through increased cGMP since NO enhances TNF-α release form monocyte macrophages and neutrophils by an effect mimicked by cyclic GMP (Gong *et al.*, 1990; Eigler *et al.*, 1993; Van Dervort *et al.*, 1994; Deakin *et al.*, 1995; Marcinkiewicz *et al.*, 1995) and associated with a decrease in cAMP (Wang *et al.*, 1997).

Interactions between cytokines modulate their own release and that of each other. TNF-α increases its own and IL-6 release, the effects seen in inflamed mucosa treated with the NO-donor (enhanced release of both mediators) or iNOS (suppression of release of both cytokines) may be partly explained by this mechanism (Strong *et al.*, 1998).

The inhibitory effect of NO on IL-6 production as observed in uninflamed mucosa after treatment with the NO-donor SNAP in Study IV has also been found in murine

macrophages (Deakin *et al.*, 1995) and enterocytes (Meyer *et al.*, 1995), and also in human chondrocytes (Henrotin *et al.*, 1998). However, the exact mechanism behind this downregulation is unclear. It has been shown that NO through S-nitrosylation inhibits the c-jun N-terminal kinase, which in human monocytes has been shown to increase IL-6 release (Tuyt *et al.*, 1999). In the uninflamed mucosa of Study IV, the effects of exogenous NO may be mediated through similar mechanisms.

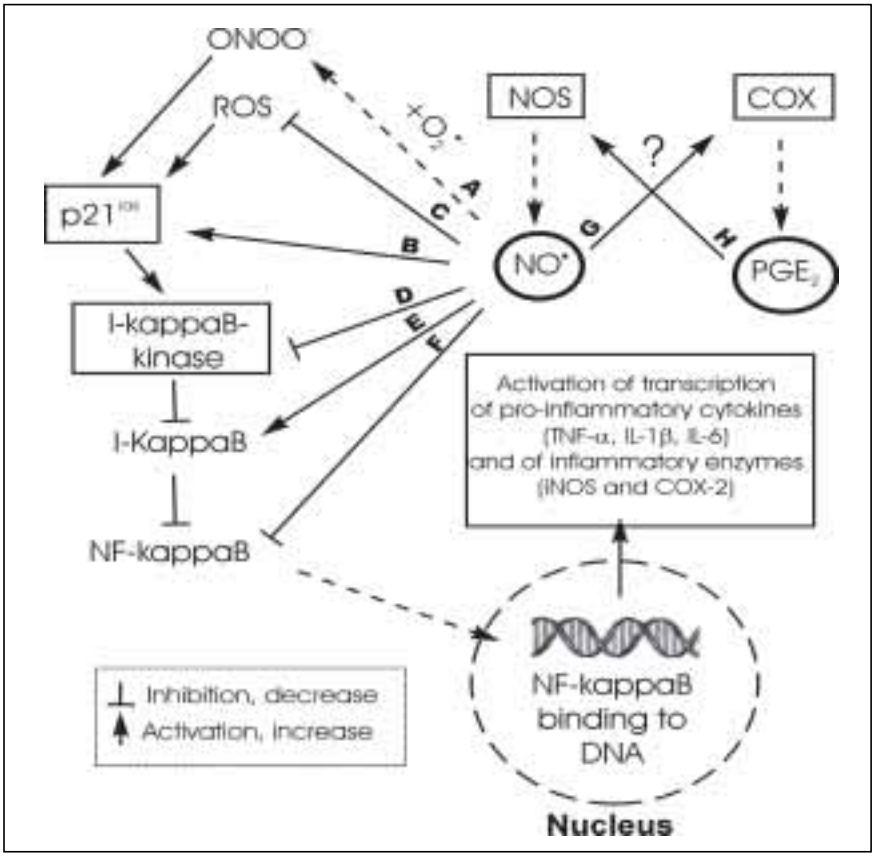

Figure 7. Biphasic regulation of inflammation by nitric oxide (Connelly *et al.*, 2001).
COX-2, cyclooxygenase-2; I-kappaB, inhibitor of NF-kappaB; iNOS, inducible nitric oxide synthase; NF-kappa-B, nuclear factor kappaB; NO, nitric oxide; $ONOO^-$, peroxynitrite anion; $p21^{ras}$, a G-protein; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; TNF-α; tumor necrosis factor alpha. See below for an explanation of the letters.

Explanation of the letters in Figre 7.

A Nitric oxide rapidly reacts with the superoxide anion to produce peroxynitrite anion (Pryor and Squadrito, 1995). Peroxynitrite has been shown to activate the oxygen sensing G-protein $p21^{ras}$ (Spear *et al.*, 1998)

B NO reacts directly with the oxygen sensing, $p21^{ras}$ and modifies this protein rendering it constitutively active thereby exerting an NF-kappaB activating cascade (Lander *et al.*, 1995a)

C NO as a radical reacts with e.g. lipid peroxides and reactive oxygen species. This has been proposed as the anti-oxidative effect of NO. Thus NO quenches ROS, which act as activators of $p21^{ras}$ (Lander *et al.*, 1995b)

D High concentrations of NO inhibit the activation of I-kappaB kinase (IKK), an inhibitor of I-kappaB's NF-kappaB inhibiting activity. Active IKK phosphorylates I-kappaB leading to release of I-kappaB from the NF-kappaB dimer and subsequent activation of NF-kappaB (Umansky *et al.*, 1998). By this mechanism NO inhibits NF-kappaB activation

E NO induces and stabilizes I-kappaB (Peng *et al.*, 1995) and thus enhances its inhibitory effect on NF-kappaB. I-kappaB is an inhibitor of NF-kappaB activity, and is associated with the NF-kappaB dimer preventing its translocation and transcriptional activity (Baldwin, Jr., 1996)

F NO has been shown to directly nitrosylate cysteine 62 in the p50-subunit of NF-kappaB dimer. This nitrosylation results in inhibition of NF-kappaB DNA-binding activity (Matthews *et al.*, 1996)

G and **H** Conflicting results exist on the regulation of COX and NOS activites by NO and PGE_2, respectively. This was discussed in more detail in section 6.5

7 SUMMARY AND CONCLUSIONS

This thesis addressed the roles of the inducible enzymes, iNOS and COX-2 in colitis. The contribution of these enzymes to inflammation was evaluated with the use of pharmacologic agents having different inhibitory selectivities for the inducible and constitutive isoforms of NOS or COX.

I TNBS-induced colitis in the rat was characterized by induction of iNOS and COX activity and was associated at 24 hours after induction with increased macroscopic inflammation, edema, and infiltration of inflammatory cells.

II Increased PGE_2 formation was coupled to the local induction of COX-2 protein in the inflamed colon mucosa in TNBS-colitis. Inhibition of COX-2 activity or expression reduced inflammatory edema and neutrophil granulocyte infiltration, but not macroscopic inflammation in the inflamed distal colon.

III The expression of iNOS was increased in the inflamed colon. Selective inhibition of iNOS activity reduced edema formation, neutrophil granulocyte infiltration, and macroscopic inflammation in the distal inflamed colon.

IV The expression of iNOS was increased in the inflamed mucosa of UC as compared with the uninflamed control mucosa. Selective inhibition of iNOS suppressed the release of pro-inflammatory cytokines providing additional proof of a detrimental role for iNOS activity in UC.

The activities of iNOS and COX-2 contributed to inflammatory cell infiltration and formation of edema. The macroscopic severity of inflammation was associated with iNOS activity, and was suppressed by selective inhibition of this enzyme. Increased iNOS expression was also found in the inflamed mucosa of patients with UC. Selective inhibition of iNOS *in vitro* suppressed the release of pro-inflammatory cytokines by the inflamed mucosa providing additional proof of a detrimental, inflammation promoting role for iNOS activity in these patients.

These results suggest that in inflammatory bowel disease a beneficial therapeutic effect is to be obtained with selective inhibition of iNOS. Inhibition of iNOS is a novel anti-inflammatory mechanism which attacks the inflammation associated NO producing enzyme. In chronic diseases iNOS produces reactive nitrogen oxides that increase host tissue damage and contribute to perpetuation of immune activation and inflammation. Further studies with highly selective iNOS inhibitors are warranted.

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