UNIVERSITY OF CALGARY

Epithelial Dysfunction Following a Bout of Colitis

by

Samuel Asfaha

A DISSERTATION SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GASTROINTESTINAL SCIENCES

CALGARY, ALBERTA

OCTOBER, 2000

Education is what remains after one has forgotten everything he learned in school.

Albert Einstein (1879-1955)

Abstract

Intestinal motility, epithelial permeability, electrolyte and fluid secretion, mucus release, and rapid epithelial turnover provide mechanisms of intestinal defence. Alterations in this defence system may increase susceptibility to infection or inflammation. Inflammatory bowel disease is often characterized by periods of remission and relapse. In addition, many post-infectious irritable bowel syndrome patients report symptoms of abdominal pain and diarrhea well after infection. Given that epithelial function (i.e., permeability, electrolyte secretion and mucus release) plays a critical role in mucosal defence, we investigated whether a bout of colitis could cause long-term changes in epithelial function that increase the susceptibility to infection/inflammation.

Six weeks after the induction of colitis in the rat, the colon appeared macroscopically and histologically normal, and no changes in epithelial permeability or mucus secretion were detectable. Despite the absence of damage, the colonic epithelium was hypo-responsive to several secretagogues with respect to electrolyte and fluid secretion *in vitro* and *in vivo*. Small intestinal, but not extra-intestinal, inflammation also caused similar epithelial dysfunction. This epithelial dysfunction was at least partially mediated by nitric oxide (NO) derived from the inducible nitric oxide synthase (iNOS) enzyme. Altered epithelial function was also associated with increased colonic bacterial colonization, bacterial translocation and increased epithelial cell proliferation.

These results demonstrate that intestinal inflammation results in prolonged impairment of colonic epithelial secretion. This may contribute to increases in bacterial

load and bacterial translocation. Epithelial dysfunction could underlie an increased propensity for further inflammation or infection.

In another study, the role of NO, a free radical proposed to contribute to tissue injury, was examined. The results of this study demonstrated that NOS inhibitors do not ameliorate damage in an experimental model of colitis. This may be due to other free radicals such as hydroxyl radical being produced in greater amounts concomitantly with a reduction in NO production. Thus, NO may be beneficial in preventing tissue injury as a free radical scavenger, rather than harmful as a source of toxic nitrogen-derived species. Examining the oxidative state of the tissue may be essential in predicting whether inhibitors of NOS are beneficial or harmful in inflammation.

Acknowledgments

Over the last 4 years there are a number of people, without whom my experiences as a graduate student would not have been so enjoyable. First, and foremost, I would like to express my gratitude and appreciation to my supervisor, Dr. John L. Wallace, for his encouragement, kindness, generosity and inspiring enthusiasm. As a mentor, he has provided me with an opportunity to experience a fanstastic journey in the field of scientific research. His support, selfless generosity, and most importantly friendship will never be forgotten.

I would also like to express my sincere gratitude to all the people in the Wallace Lab for making work a gratifying experience. I have enjoyed coming to the lab everyday to work with friends. To students, post-doctoral fellows and technicians, I would like to express my thanks for advice, support and motivation. Additional thanks are also sent to Webb McKnight, Michael Dicay and Kevin Chapman for their technical assistance and memorable experiences.

To my supervisory committee – Drs. Wallace MacNaughton, Keith Sharkey and Mark Swain – I would like to express my appreciation and thanks for all their guidance and encouragement.

Finally, I would like to thank my parents and my brother for their encouragement, love and support over the course of my studies. Your guidance and generosity will never will be forgotten.

Table of Contents

List of Figures and Tables

List of Abbreviations and Symbols

~.....approximately

©.....copyright

μEq.....micro-equivalents

°C.....degree(s) Celsius

AG.....aminoguanidine

ANOVA.....analysis of variance

cAMP.....cyclic adenosine-3',5'-monophosphate

CFU.....colony forming units

Cr.....chromium

DNA.....deoxyribonucleic acid

cDNA.....complementary DNA

EDTA.....ethylenediaminetetraacetic acid

ELISA.....enzyme-linked immunosorbent assay

E. coli.....*Escherichia coli*

GAPDH.....glyceraldehydes 3-phosphate dehydrogenase

i.p..... intraperitoneal

IL..... interleukin

IFN..... interferon

LPS..... lipopolysaccaride

LT.....leukotriene

kDa.....**kilodalton**

L-NAME.....**N^G-nitro-L-arginine methyl ester**

L-NIL..... **L-N^6-iminoethyllysine**

mM.....**millimolar**

MPO.....**myeloperoxidase**

MW.....**molecular weight**

7-NI.....**7-nitroindazole**

NF-κB.....**nuclear factor-κB**

NO.....**nitric oxide**

NOS.....**nitric oxide synthase**

PAF..... **platelet activating factor**

PG.....**prostaglandin**

RNA.....**ribonucleic acid**

mRNA.....**messenger RNA**

RT-PCR..... **reverse transcriptase**

polymerase chain reaction

s.c..... **subcutaneous**

SOD.....**superoxide dismutase**

SNP..... **sodium nitroprusside**

SNAP.....**S-nitrosoacetylpenacillamine**

TNF.....**tumor necrosis factor**

All measurements are expressed using Système International units and prefixes.

Chapter 1

GENERAL INTRODUCTION

1.1. Inflammatory Bowel Disease

Ulcerative colitis (UC) and Crohn's disease are inflammatory disorders of the GI tract collectively known as inflammatory bowel disease (IBD). The incidence of these diseases is 2-10 per 100,000 for UC and 1-6 per 100,000 for Crohn's disease (1). The peak age of onset for both diseases is between 15 and 25 years, and the prevalence of disease for UC is 35-100 per 100,000 while that for Crohn's disease is 10-100 per 100,000 (1). The main symptoms of UC are bloody diarrhea, fever, malaise and weight loss. The symptoms of Crohn's disease patients include diarrhea, abdominal pain and weight loss. However, in UC the inflammatory response is confined to the mucosa and submucosa, whereas in Crohn's disease there is inflammation extending from the mucosa to serosa (i.e., transmural) (1). These two diseases are also differentiated by the fact that UC only occurs in the colon, while Crohn's disease can occur anywhere in the GI tract. Curiously, cigarette smoke appears to have a beneficial effect on UC, but a harmful effect on Crohn's disease.

The pathogenesis of both of these disorders is not fully understood. What triggers the initial inflammatory response and why it goes unchecked for long periods of time is unknown. At least three hypotheses have been proposed for what the trigger may be. The first hypothesis is that the trigger is a microbial pathogen. One of the most studied candidate pathogens is *Mycobacterium paratuberculosis*. In cattle, this bacterium

produces symptoms and histological features similar to those of Crohn's disease (i.e., transmural, granulomatous inflammation) (1). However, researchers have been unable to isolate the bacterium from many patients with IBD. As a result, this bacterium has fallen out of favour by many researchers as the cause of IBD. A second hypothesis is that the trigger is a common dietary antigen or non-pathogenic microbial agent to which patients mount an abnormal immune response. Thus, the failure to suppress the inflammatory response results in uncontrolled inflammation and subsequent tissue injury. The third theory is that an antibody directed against dietary or luminal antigens cross-reacts with similar antigens expressed on the patient's own cells (1). In this manner, IBD would result from an inflammatory response akin to autoimmunity.

5-aminosalicylic acid (5-ASA) and sulfasalazine, which is metabolized by intestinal bacteria to form sulfapyridine and 5-ASA, are the most common treatment regimens used for IBD (1). The mechanism through which these drugs reduce inflammation is unknown. Corticosteriods such as hydrocortisone and prednisone are also commonly employed in the treatment of IBD. These drugs are quite effective in reducing the inflammatory response. However, side effects of these treatments include Cushing's syndrome, attributed to increased androgens and mineralcorticoids, as well as osteoporosis, obesity and acne. Immunosuppressives such as 6-mercaptopurine and methotrexate are also used as the primary therapy for fistule in Crohn's disease, but side effects of these drugs include leukopenia and development of allergies (1). Finally, antibiotics have been suggested to have some beneficial effects on the clinical outcome of IBD. Metronidazole is probably the best known antibiotic used for this purpose. Once again, however, side effects such as pseudomembranous colitis pose a risk to patients.

To date, studies examining the pathogenesis of IBD have implicated a number of inflammatory mediators and exogenous factors. For example, IL-1, IL-8, TNF-α, and IFN-γ are just a few of the many cytokines that have been proposed to contribute to the tissue injury observed during colonic inflammation. Several investigators have demonstrated that oxidative stress and free radical (ie. reactive nitrogen species, reactive oxygen species) generation are key to the production of tissue injury in these conditions. The focus of the following project was the role of nitric oxide (NO) and epithelial cells in colonic inflammation. In addition, given the role of epithelial cells in mucosal defense, the experiments conducted in this project also have considerable relevance to another condition known as irritable bowel syndrome (IBS). Irritable bowel syndrome (IBS) is the most common digestive disorder encountered by gastroenterologists, representing up to 50% of outpatient referrals. Symptoms of IBS often include passage of mucus, fecal urgency, bloating and feeling of abdominal distension. As in the case of IBD, the pathogenesis of IBS is not well understood. However, it is believed that a subset of IBS patients present their symptoms following a bout of infection or inflammation (2-5). As a result, a goal of this project was to examine epithelial function following an inflammatory episode. Since an extensive background on nitric oxide is provided in the latter half of the introduction, in the next section an overview of epithelial function in inflammation followed by a brief outline of what is known about the role of NO in IBD will be provided.

1.2. The Epithelial Barrier

The epithelial layer consists of several types of cells, each with different functions. These cells include absorptive columnar cells, intraepithelial lymphocytes, enteroendocrine cells and goblet cells. Each of these cell types contributes to the barrier properties. Given the high levels of bacteria in the lumen of the intestine, it is critical that the host be protected from potentially harmful factors. Controlled sampling of the luminal contents is necessary for the immune system to be prepared to fight infection. In addition, the intestinal epithelium must not be completely impermeable, as the absorption of nutrients (ie. carbohydrates, fat, and proteins) and water is critical to the survival of the organism. The epithelium serves as the first line of defense against luminal antigens and toxins, while also allowing for absorption of water and nutrients.

Epithelial barrier function is regulated by the structural elements of the epithelium. Tight junctions between epithelial cells serve as critical gateways for the passage of ions, water, and large molecules. Transcellular transport of ions, proteins, and fluid is further controlled by specific membrane channels and transporters. Under normal conditions the epithelium is relatively impermeable to potentially toxic elements in the lumen. However, epithelial permeability may be altered by numerous factors, including cytokines and autocoids, as well as immune cells such as macrophages and neutrophils. Kubes and co-workers (6) demonstrated that the inhibition of nitric oxide synthase with L-nitroarginine methyl ester (L-NAME) increased epithelial permeability as detected by increased blood-to-lumen clearance of ^{51}Cr-EDTA (6,7). Several investigators have shown that cytokines such as interferon-γ (IFN-γ), IL-4 and IL-13 can increase

paracellular permeability across epithelial cell layers (8,9). In contrast, IL-10 attenuates IFN-γ-induced increases in permeability of T_{84} epithelial cell monolayers (10). Neutrophils that migrate across the intestinal epithelium can also increase epithelial permeability (11,12). This increased epithelial permeability has been attributed to neutrophil-derived oxidants, as the changes in permeability were reversed by treatment with glutathione peroxidase, an iron chelator, or a hydroxyl radical scavenger (12). Wallace *et al.* (13) have shown that increased colonic permeability associated with colitis was largely caused by neutrophil transmigration. The neutrophil transmigration was prevented by administration of a monoclonal antibody directed against the leukocyte adhesion molecule CD-18. Moreover, acute inflammation caused by infection with *Nippostrongylus brasilensis* has been shown to increase intestinal epithelial permeability. Thus, inflammation can result in elevated epithelial permeability through the release of mediators capable of affecting epithelial barrier function and through the transepithelial migration of leukocytes.

Another important aspect of mucosal defence in the intestine is that of antigen sampling M cells and secretory immunoglobulin A (IgA). The intestinal epithelium is faced with the continuous challenge of transporting antigen samples across the mucosa without compromising its intregity and protective functions. Specialized epithelial M cells in the follicle-associated epithelium carry out this function by transporting antigens from the lumen to the basolateral surface of the epithelium where antigen-presenting cells such as B-cells are activated (14). In addition, IgA serves a protective function through its ability to inhibit the absorption of soluble and particulate antigens (15). This is accomplished by forming immune complexes within the intestinal lumen that neutralize

biologically active antigens and interfere with their adherence to epithelial cells. Furthermore, phagocytic cells including macrophages, neutrophils and eosinophils express surface receptors for IgA (16). This is of critical importance for the removal of antigens that cross the mucosa, as it allows for antibody-dependent cell mediated antibacterial activity to occur (17).

1.3. Epithelial Ion and Fluid Secretion

Regulation of intestinal epithelial ion transport occurs through ion channels specific for sodium, chloride, potassium, and bicarbonate ions. Absorption occurs primarily in the crypt region of the epithelial lining whereas secretion takes place in the surface epithelia. A schematic representation of the various channels and ion transporters in the colonic epithelium is provided in figure 1.3.

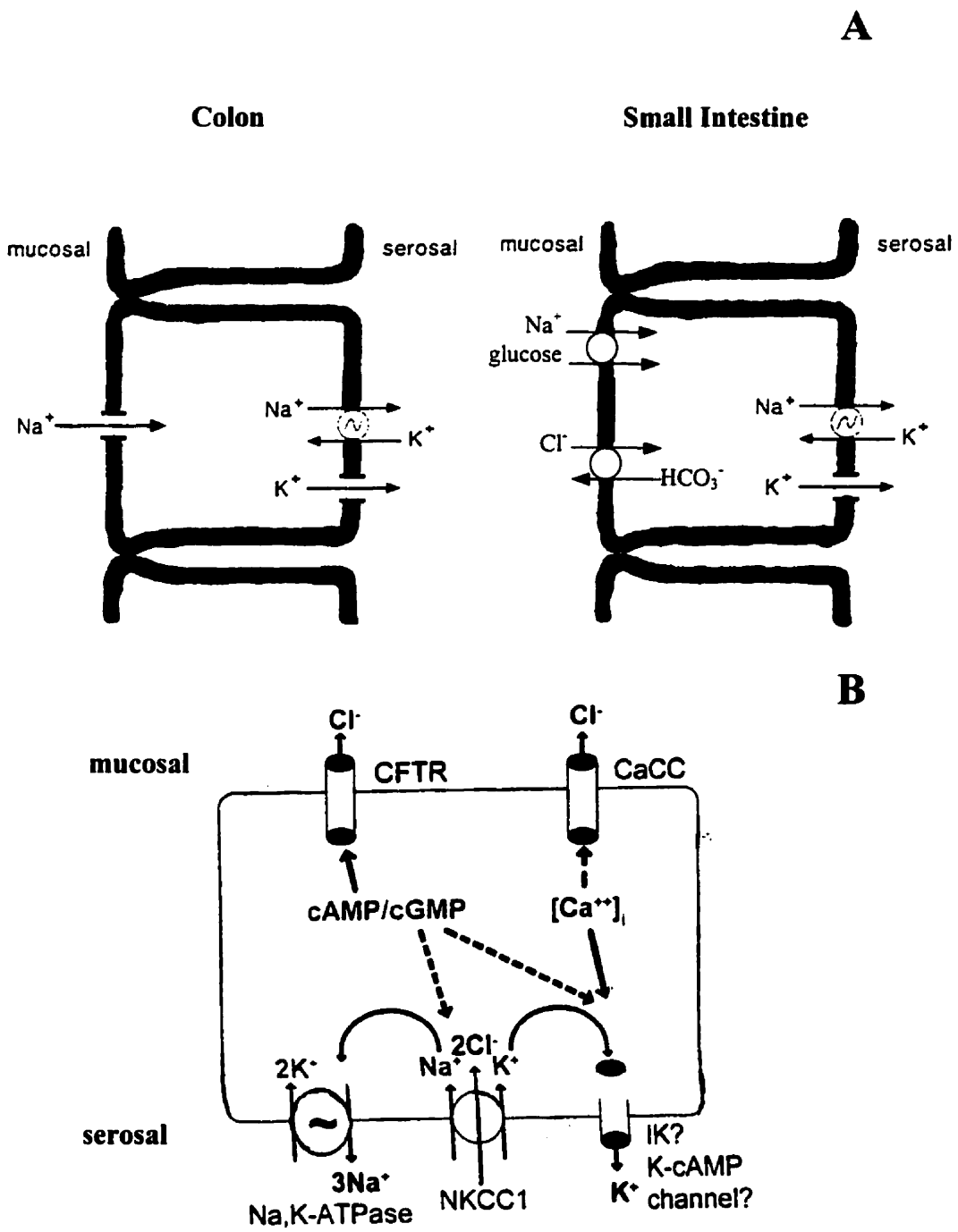

Figure 1.3. Illustration of (A) Na^+ absorption in the colon versus small intestine and (B) electrogenic Cl^- secretion in the colon and small intestine. Figure was modified from Barrett et al., (18)

The colon does not have the same capacity as the small intestine to absorb nutrients such as carbohydrates, amino acids, and fat. The apically located Na^+-glucose co-transporter of the small intestine is absent in the colon, and the colonocyte has an amiloride-sensitive Na^+ channel on the apical membrane. The colon has an increased capacity to absorb fluid compared to the small intestine. In addition to the trancellular transport of ions, the small and large intestines allow for the passive movement of water and ions through the tight junctions between cells. However, the tight junctions in the colon are less permeable to paracellular transport than the tight junctions in the small intestine. It appears that most of the passive paracellular movement of water and electrolytes occurs in the crypt region (19,20).

As illustrated in figure 1.3, intestinal electrolyte transport is regulated through a number of different pathways by a variety of mediators. These mediators include cytokines, eicosanoids, autocoids, and neurotransmitters. For instance, enteroendocrine cells are important regulators of epithelial function through their release of hormones (i.e., such as CCK, somatostatin) and neurotransmitters such as 5-hydroxytryptamine (5-HT). The hormones released are important in the digestive and absorptive functions of the gut by affecting perstalsis (21). However, the neurotransmitter 5-HT also plays a critical role in the mucosal defence system as a consequence of its effects on epithelial transport and motility. Stimulation of enteroendocrine release of 5-HT results in stimulation of epithelial anion secretion. This effect has been attributed to direct effects on epithelial cells through $5\text{-}HT_2$ receptors and indirect effects through $5\text{-}HT_3$ receptors on submucosal neurons (22). In instances of mechanical stimulation of the intestine,

enteroendocrine cells also serve to coordinate the simultaneous stimulation of epithelial secretion and muscle contractility that flushes away luminal contents (22).

Exogenous factors such as bacterial toxins can also alter fluid and electrolyte transport. For example, enterotoxin *Escherichia coli* STa and *Clostridium difficile* toxin A can increase Cl^- secretion (23,24). Infection with *Vibrio cholera* results in cholera toxin release that stimulates chloride secretion in a cAMP-, prostaglandin- and 5-HT-dependent manner (25). Short chain fatty acids (SCFA), which are a major respiratory fuel for colonic epithelial cells, can also stimulate water and sodium absorption primarily in the proximal colon (26). A more detailed discussion of inflammatory factors affecting electrolyte transport is provided in section 1.5.

1.4. Mucus Secretion

The mucous layer consists of water, electrolytes, secreted proteins, immunoglobulins and mucins. This gel layer provides a chemical and physical barrier against luminal bacteria and toxins. Goblet cells are the main source of mucin secretion in the intestine. Goblet cells release mucin in response to cholinergic stimulation (27) intestinal anaphylaxis (28), or chemical irritants (29). In addition, atropine-sensitive and capsaicin-sensitive pathways of mucin secretion (30) have indicated a role for neural regulation of mucus secretion. However, VIP, cAMP, 5-HT and adrenergic agonists have no effect on intestinal mucus secretion (29). During bouts of intestinal inflammation, mediators such as prostaglandin E_2, histamine, IL-1β and nitric oxide have been shown to stimulate mucus discharge from goblet cells (31-33). Indeed, goblet cell depletion is a

characteristic feature of ulcerative colitis. This has been attributed to hypersensitivity of goblet cells to secretory agonists (32). In contrast, patients with Crohn's disease have been reported to have a layer of mucus of normal thickness and not to exhibit goblet cell depletion (32). On the other hand, in IBS mucus secretion is increased, with mucus discharge being reported to be in up to 50% of IBS patients (34).

1.5. Effects of Inflammation on Intestinal Function

Many of the mediators that can affect epithelial function have been shown to be elevated in colonic tissue in IBD. With so many mediators with varying effects on epithelial permeability, ion transport and mucus secretion the regulation of the epithelium is complex. Therefore, with respect to disease, it is important to understand what the effects of inflammation on epithelial function are. This is illustrated by the fact that inflammation can have many long-term effects on other intestinal functions, such as smooth muscle contractility and enteric nerve function.

Changes in smooth muscle contractility may be clinically important, because it is believed that changes in colonic motor function contribute to pain and diarrhea. Vermillion *et al.* (35) demonstrated that *Trichinella spiralis* infection of the rat results in hypercontractility of smooth muscle *in vitro*. Marzio *et al.* (36) further demonstrated that this altered smooth muscle function was not only characteristic of inflamed areas of the intestine, but also the non-inflamed regions. Similarly, inflammation of the colon caused an attenuated contractile response to carbachol and substance P in longitudinal muscle (37). This smooth muscle hypo-responsiveness was observed regardless of the method

used to induce colitis (ie. TNBS, acetic acid, *T. spiralis*, mitomycin C) (37,38). Similarly, studies of IBD have revealed marked changes in both longitudinal and circular muscle function (37). Longitudinal muscle from Crohn's disease patients displayed increased maximum contraction to carbachol but not histamine (39). Conversely, there was no change in the maximum contraction of circular muscle to carbachol, but a 2.5-fold increase in the maximum response to histamine (39).

Several studies have further demonstrated that intestinal inflammation alters nerve function. Swain *et al.* (40) reported that *T. spiralis* infection of the rat results in suppression of norepinephrine release from the myenteric plexus that persists for up to 100 days (40). The reduction of norepinephrine release was attenuated by betamethasone, suggesting that the altered nerve function was an inflammation-dependent effect (40). Furthermore, in the colon, TNBS and *T. spiralis* infection caused suppressed noradrenaline release from the myenteric plexus (41). As with smooth muscle, altered nerve function was observed in non-inflamed sites of the large and small bowel (41). In a subsequent study, the same group demonstrated that this change in nerve function occurred via an IL-1-mediated, steroid-sensitive and T-cell-independent pathway (42).

Inflammatory mediators have also been shown to affect epithelial ion transport. Chang *et al.* (43) showed that IL-1 and IL-3 stimulate chloride secretion in the chicken intestine. In the rabbit ileum, IL-1 was shown to stimulate an increase in short circuit current and this was attributed to inhibition of Na^+ and Cl^- absorption (44). These effects were due to the release of PGE_2 (43,44). Similarly, TNF-α has been shown to stimulate chloride secretion in a PGE_2-dependent manner (45). In the human colon, IL-1β and

TNF-α, but not IFN-α, IFN-γ, IL-6 or IL-8, cause secretion (46). Other cytokines can down-regulate chloride secretion by epithelial cells. In T_{84} cell monolayers, IL-4 but not IL-13, attenuated forskolin- and carbachol-stimulated chloride secretion (47,48). IFN-γ, which increases epithelial permeability, has additionally been shown to attenuate the chloride secretory response to VIP, cholera toxin, forskolin, and carbachol (10,49).

Eicosanoids can also influence ion transport by the intestinal epithelium. Prostaglandins including PGE_2, PGE_1, and 6-keto-$PGF_{1\alpha}$ have been shown to cause chloride secretion through activation of cAMP-dependent pathways in the epithelial cell (50-52). On the other hand, PGD_2 has been shown to reverse the increase in chloride ion secretion elicited by PGE_2, forskolin and carbachol (50,53). Leukotrienes exert a net secretory effect on epithelial transport as the peptidoleukotrienes LTC_4, LTD_4 and LTE_4 cause an increase in short circuit current (54). Smith *et al.* (55) showed that in case of LTC_4, the effect observed was due to inhibition of Na^+ and Cl^- absorption, and stimulation of Cl^- secretion (55,56).

The release of histamine by mast cells is another important factor in the regulation of epithelial ion transport. Wang *et al* (57) reported that histamine acting at H_1-receptors caused chloride secretion in the colon in a prostaglandin-dependent manner. Ion transport alterations (i.e., chloride secretion, hypersecretory responses to inflammatory mediators such as substance P) due to intestinal anaphylaxis have been attributed to the release of histamine and 5-hydroxy-tryptamine (5-HT) from mast cells (58,59).

The neurotransmitters neuropeptide Y (NPY), acetylcholine, 5-HT and norepinephrine have also been shown to affect epithelial ion transport. For example, acetylcholine and cholinergic agonists such as carbachol cause chloride secretion (60)

whereas norepinephrine stimulates Na^+ and Cl^- absorption and inhibits Cl^- secretion (61). These effects occur through Ca^+-mediated pathways (62). Vasoactive intestinal peptide (VIP) also stimulates chloride secretion, but it is thought to act through a cAMP-dependent pathway (61,63). Neuropeptide Y (NPY), on the other hand, increases Na^+ and Cl^- absorption and reduces Cl^- secretion (64). In fact, NPY is thought to serve as an inhibitory neuromodulator on leukotriene-sensitive neurons that normally stimulate secretion in the colon (56). Hardcastle *et al.* (65) demonstrated that 5-HT stimulated chloride secretion and this effect was subsequently shown to be at least partially mediated by non-cholinergic nerves that release nitric oxide (65,66).

In summary, smooth muscle and nerve function are altered not only at sites of active inflammation, but also at non-inflamed regions of the intestine. Changes in contractility and nerve function persist well after the initial bout of inflammation. Therefore, it is possible that intestinal inflammation could also have long-term effects on the epithelium.

1.6. Nitric Oxide: Historical Perspective

The story of nitric oxide, as it pertains to medicine, began in 1847 when an Italian by the name of Ascanio Sobrero moved to Paris in order to work with the famous French chemist Theophile-Jules Pelouze. At the time, Sobrero's main projects included the production of explosives such as those derived from nitromannitol and nitroglycerin. However, nitroglycerin was formed in a highly exothermic reaction by nitration of glycerol with nitric and sulphuric acids. Consequently, detonation of nitroglycerin

occurred if the reaction wasn't cooled during its production. This effect had important repercussions for many individuals, including Sobrero himself, who fell victim to explosions in the lab. In fact, Ascanio Sobrero would later be ashamed to be known as the discoverer of a substance that would cause so much death and destruction. Nevertheless, if it were not for his discovery, and even more importantly his observation that nitroglycerin placed on the tongue caused headaches, the potential of nitrates for medicinal use may never have been realized.

In 1849, Constantin Herig, a physician with an interest in homeopathic remedies, tested nitroglycerin in healthy volunteers and noticed that headaches resulted "with precision". Being an advocate of the theory that "like cures like", he began to use nitroglycerin to treat headaches. By this time (1850), Alfred Nobel had arrived at the Pelouze laboratory in Paris. Nobel immediately recognized the potential of nitroglycerin as an explosive. When he returned home to Stockholm, he began the commercialization of nitroglycerin as the "Nobel patent detonator".

By the mid-19th century, British scientists had taken an interest in amyl nitrite and recognized it as a powerful vasodilator. In 1867, a pharmacologist named Lauder Brunton was the first to use amyl nitrite to relieve angina, while William Murrell, was the first physician to use nitroglycerin to treat angina nearly ten years later. Ironically, Alfred Nobel, who had become wealthy from his commercialization of nitroglycerin as an explosive, later refused to take nitroglycerin for the treatment of his own angina as he could not believe that something so destructive could be injected as a cure.

Despite the use of nitrate-containing compounds for the treatment of angina for nearly one and a half centuries, it would not be until the late 20th century that scientists

would discover the mechanism of their action. In 1977 Ferid Murad and co-workers discovered that nitric oxide was released from nitroglycerin, and observed that this had a relaxant effect on vascular smooth muscle (67). This was followed by the work of Robert Furchgott and John Zawadski, who recognized the importance of the endothelium for vasorelaxation (68). In 1980 they reported the existence of an "endothelial-derived relaxation factor" (EDRF). However, it would be nearly another decade before two groups would separately prove that EDRF was in fact nitric oxide. In 1987, through a series of classical experiments including oxyhemoglobin inhibition of endothelial and NO-elicited relaxation, NO activation of soluble guanylate cyclase, and superoxide dismutase augmentation of EDRF and NO relaxation, Ignarro *et al.* (69) and Moncada *et al.* (70) proved that EDRF was NO.

1.7.Sources of Nitric Oxide

Since the discovery that NO was produced endogenously, scientists began to search for the source of its production. In 1991, the first isoform of the enzyme nitric oxide synthase (NOS) was cloned from neurons (71). This isoform, known as Type I or neuronal NOS (nNOS), was later shown to be present in skeletal muscle, neutrophils, pancreatic islets, endometrium, and respiratory and gastrointestinal epithelial cells (72). Shortly thereafter, a second isoform of NOS named Type II or inducible NOS (iNOS) was described in macrophages and hepatocytes. In contrast to the first isoform, iNOS was inducible by lipopolysaccaride (LPS) and by certain cytokines. A third isoform was found in endothelial cells, and this enzyme was termed Type III or endothelial NOS (eNOS). It is now generally accepted that nNOS and eNOS are constitutive enzymes that

are Ca^{2+}-dependent and primarily expressed in neuronal and endothelial cells, respectively. On the other hand, iNOS is an inducible enzyme that is Ca^{2+}-independent and is primarily expressed in inflammatory cells such as macrophages and neutrophils. However, these divisions are not entirely accurate given that iNOS has been shown to be constitutively expressed in paranasal epithelial cells, whereas nNOS has been detected in skeletal muscle, and eNOS in neuronal cells of the brain (73-75).

1.8. Biosynthesis of NO

The production of NO by nitric oxide synthase occurs through a 5 electron transfer in which the substrate L-arginine undergoes a two-step oxidation of one of its guanidino nitrogens to form L-citrulline and NO. The complete reaction is illustrated in the reaction below (Figure 1).

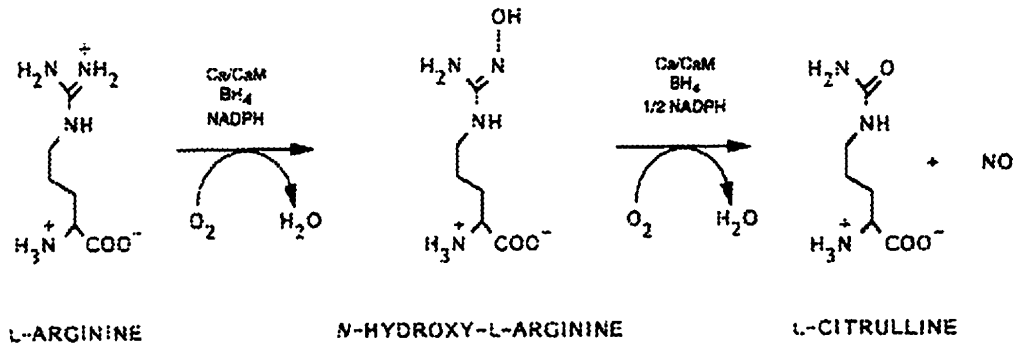

Figure 1.1. The biosynthesis of nitric oxide by nitric oxide synthase (NOS). NOS catalyzes a five-electron oxidation of a guanidino nitrogen of L-arginine to generate NO and L-citrulline. Both steps in the reaction are dependent on calcium and calmodulin (Ca/CaM), tetrahydrobiopterin (BH_4) and NADPH. Figure was taken from Bonner *et al.* (76).

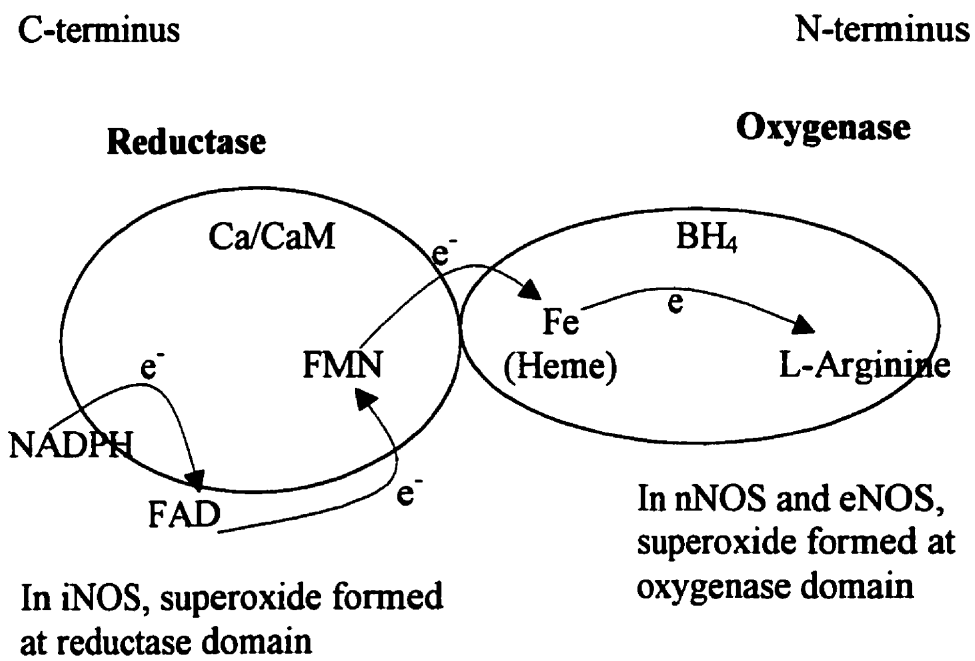

Figure 1.2. Schematic diagram illustrating the reductase and oxygenase domains of the NOS enzyme and the mechanism of electron transfer from NADPH to L-arginine.

The NOS enzymes function as homodimers that can be structurally and functionally divided into two major units; the first domain consists of a C-terminal reductase while the second consists of an N-terminal oxygenase domain (refer to Figure 1.2.). The reductase domain is homologous to cytochrome P_{450} reductase, and has binding sites for one molecule each of NADPH, FAD, and FMN. The oxygenase domain has binding sites for heme, tetrahydrobiopterin (BH_4), and L-arginine. Between these two domains lies the calmodulin binding domain. The formation of NO occurs by the transfer of electrons from NADPH to the flavins, and subsequently to heme and L-arginine. The reaction involves the initial hydroxylation of L-arginine to form N^G-hydroxy-L-arginine which in turn is further oxidized by NOS to form L-citrulline and NO.

In general, the three NOS isoforms are conserved, with the oxygenase and reductase domains being highly homologous. However, each isoform exhibits its own distinct features that are extremely important with respect to *in vivo* function. This will become evident as the functional aspects of each isoform are discussed later. Heme is essential for dimerization of all three NOS isoforms, and is necessary for catalyzing L-citrulline/NO production (77,78). BH_4 is also believed to be important in NOS dimerization; however, differences in the requirement for BH_4 exist among the isoforms. For example, BH_4 stabilizes nNOS and eNOS dimers once the dimers are formed, whereas in iNOS, BH_4 is important for dimerization, but not essential for stabilization of the dimer (77). L-arginine also appears to facilitate dimerization of iNOS, and to increase the rate of NADPH oxidation (77).

It was recently noted that under certain conditions nitric oxide synthase can form the free radical superoxide (O_2^-) (79,80). This is particularly important in inflammatory

conditions where NO and O_2^- can interact to form other potentially toxic molecules such as peroxynitrite ($OONO^-$). A more detailed description of the chemistry of NO is provided below. However, in this section a brief description of how O_2^- is produced by NOS is outlined. All three NOS isoforms are capable of producing O_2^- *in vitro* (81-83). Unlike the production of NO, the mechanism through which O_2^- is formed is variable among the three enzymes. In nNOS and eNOS, O_2^- is formed by NADPH oxidation and electron transfer to heme, followed by the reduction of molecular oxygen at the oxygenase domain (81,82). Although both nNOS and eNOS are theoretically capable of O_2^- production, it is generally thought that this only occurs under pathophysiological conditions of L-arginine depletion and/or BH_4 depletion (81,82). In iNOS, although oxygen is the final electron acceptor, the electron transfer occurs at the reductase domain of the enzyme (83). These biochemical differences are extremely important as they often determine what effect various drugs will have on enzyme activity, as well as what cellular factors can affect enzymatic function (81,84,85). For instance, because O_2^- production occurs at the oxygenase domain in nNOS and eNOS, L-arginine elicits an inhibitory effect on O_2^- formation (78,81,82). In the case of iNOS, however, O_2^- continues to be formed in presence of L-arginine (83). In fact, *in vitro* studies have demonstrated that even concentrations of L-arginine as high as 1 mM only partially inhibit O_2^- production by iNOS (83). Moreover, differences in mechanisms of O_2^- production suggest that so called "NOS inhibitors" that block NO production may also inhibit O_2^- formation in one isoform, but not necessarily the others.

1.9. Nitric Oxide Biochemistry

Nitric oxide is a diatomic free radical gas that is relatively unreactive, unless it undergoes one of several physiologically relevant redox reactions. These include oxidation to form nitrosonium (NO^+) or reduction to form nitroxyl ion (NO^-) (76). This poses a problem when attempting to understand the biochemistry of nitric oxide as the distinction among the various redox forms is often not made. As a result, reports of the biological actions of NO often confuse readers by clumping reactions attributable to all the redox forms of NO, and referring to them as those due to NO. For example, the reactions of NO with amines to form nitrosamines and with thiols to form nitrosothiols are thought to be due to NO. In fact, these substances react with the nitrosonium ion (NO^+) rather than with NO (86). On the other hand, nitrosylation is due to the reaction of NO with transition metals such as heme-iron (86,87). Thus, it is important to make a distinction among the various forms of nitrogen oxides, because in biological systems of varying redox status, different forms can be derived from various sources and have differing physiological consequences. When nitric oxide reacts with molecules such as oxygen, although the final product is nitrite, other nitrogen oxides are formed. These include NO_2 and N_2O_3, both of which are highly reactive molecules (86). N_2O_3 is a major source of NO^+ that can nitrosate proteins. N_2O_3 can also undergo oxidation reactions, while NO_2 can undergo oxidation and nitration reactions (86). These reactions can occur *in vivo* to allow for a variety of effects. Nitrosation reactions by NO^+ can influence cellular functions by causing conformational changes in proteins, or by such products

simply acting as carriers of NO^+ until a critical nitrosation can occur (88). In addition, antioxidant enzymes such as glutathione transferase and glutathione peroxidase can be inactivated through nitration (87).

NO can also undergo a one-electron reduction to form nitroxyl ion. This nitrogen oxide species is formed *in vivo* by one of three mechanisms. First, direct enzymatic production can occur by NOS. The NOS intermediate hydroxyl-L-arginine can be oxidized to form NO^-, or S-nitrosothiols can decompose to yield NO^- (89). NO^- is particularly important in inflammation during conditions of oxidative stress. This molecule reacts with thiols more rapidly than other reactive nitrogen species such as peroxynitrite. NO^- can also deplete intracellular GSH which renders cells susceptible to NO^--induced damage such as lipid peroxidation.

Finally, a discussion of nitric oxide chemistry would not be complete without mentioning peroxynitrite ($OONO^-$). Peroxynitrite is an important end-product of the reaction between NO and O_2^-. Additionally, it has been suggested that peroxynitrite can be formed by the reaction of NO^- with O_2. As a very powerful oxidant, peroxynitrite exhibits strong nitration and nitrosation of lipids, amino acids and DNA (86,87,90,91). Furthermore, peroxynitrite can become protonated to form peroxynitrous acid (ONOOH), which can subsequently undergo homolytic cleavage to form the potent oxidizing agents hydroxyl radical (HO•) and nitrogen dioxide (NO_2•) (92). Therefore, production of peroxynitrite and its by-products can have detrimental effects on a tissue through inactivation of metabolic enzymes and transport proteins (86,90,93).

Another important modification of proteins by $ONOO^-$ is nitration of aromatic amino acids. Beckman *et al.* (94) demonstrated that tyrosine nitration by $ONOO^-$

proceeds by way of nitronium ion (NO_2^+) formation, and that this reaction was catalyzed by transition metal ions. However, van der Vleit *et al.* (95) have shown that nitration of tyrosine by $ONOO^-$ may occur by formation of the intermediates tyrosyl and $NO_2\bullet$ radical. This type of amino acid modification can have significant effects on signaling pathways in the cell. For example, nitration of tyrosine may interfere with signaling pathways involving phosphorylation and dephosphorylation of tyrosine residues. Therefore, a variety of nitrogen oxide species are capable of evoking different physiological effects.

1.10. Nitric Oxide Physiology: Vascular, Renal & Neural Systems

Several of the roles of nitric oxide in the body have already been alluded to. Probably the most well known of these is the regulation of blood pressure through vasodilation. As already described, NO was first discovered through an examination of vasodilation of arterial vessels *in vitro*. Today, it is known that the eNOS isoform produces NO that diffuses to the adjacent smooth muscle cells of blood vessels where it activates soluble guanylate cyclase. This results in an increase in intracellular cGMP levels which then, through a series of steps including activation of protein kinase G-I (PKGI) and phosphorylation of Ca^{2+}-ATPase, results in reduced intracellular Ca^{2+} levels and relaxation of smooth muscle (96).

While the NO that regulates vascular tone is primarily derived from eNOS, there are exceptions. For instance, in the corpus cavernosum, nitrergic nerves that release NO derived from nNOS regulate penile erection (97). In the cerebral vasculature, nNOS-

derived NO coordinates vasodilation to ensure that blood flow is increased to the metabolically active areas of the brain (98). Although iNOS-derived NO does not generally contribute to vascular relaxation in normal tissues, under inflammatory conditions, induction of iNOS leads to high levels of NO, which can lead to profound vascular relaxation and significant hypotension (99).

In addition to its effects on the endothelium, NO inhibits platelet activation as well as platelet adhesion to endothelial matrix and collagen fibrils (100). Each of these effects entails the activation of soluble guanylate cyclase and elevation of intracellular cGMP levels (96). The inhibitory effects of NO on platelets is most likely due to the reduction of intracellular calcium levels, which in turn decreases the ability of platelets to aggregate. Moreover, NO inhibits the expression of P-selectin and the fibrinogen receptor (IIb/IIIa). This reduces the adherence of platelets to damaged vessel walls (98). These effects are attributed to eNOS-derived NO from both endothelial cells and the platelets themselves.

Similar to the mechanism through which NO relaxes vascular smooth muscle, NO can regulate bronchial smooth muscle tone. For example, NO can counteract the bronchoconstriction due to cholinergic stimulation (98).

In the central nervous system, NO plays an important role as a neurotransmitter that behaves as a long-lasting diffusible messenger. NO has also been implicated in long-term potentiation in long-term memory processes (101), and iNOS-derived NO has been implicated in the pathogenesis of cerebral ischemic injury (102).

NO can influence blood pressure through mechanisms other than direct relaxation of vascular smooth muscle. Renin released by the smooth muscle cells of the renal

afferent arterioles converts angiotensinogen I to angiotensin I. Angiotensin I, in turn, is converted by the enzyme angiotensin converting enzyme (ACE) to angiotensin II, a potent vasoconstrictor. As a result, factors such as prolonged low-salt intake or hypotension stimulate this pathway (103). NO influences this pathway by acting as the stimulator of renin release from the afferent arteriole smooth muscle cells (104). Therefore, renal eNOS- and nNOS-derived NO ultimately lead to an increase in blood pressure. However, this renal NO influence is probably only relevant under specific conditions such as hypotension.

1.11. Nitric Oxide and Disease

Although nitric oxide has many beneficial effects, elevated levels of NO have been implicated in the pathogenesis of several diseases. NO elicits most of its physiological effects (e.g., vasodilation, sphincter relaxation, peristalsis, epithelial secretion and absorption) through activation of soluble guanylate cyclase. However, as previously described, the various chemical forms of nitrogen oxide (NO^+, NO^-, N_2O_3) can react with enzymes, lipids, and DNA in a cGMP-independent manner. These reactions generally have beneficial effects during inflammatory conditions, since NO complexes with metals, thiols and amines to elicit bactericidal effects. NO plays a critical role in controlling the inflammatory response, such as through inhibitory effects on the adhesion of circulating cells to the vessel walls. This effect of NO is produced through inhibition of adhesion molecule expression on both the vessel wall and the circulating leukocytes (105).

When the inflammatory response goes unchecked and high levels of NO are present, it has been proposed that NO has cytostatic, cytotoxic and even carcinogenic effects. For example, DNA damage can occur by nitrosative deamination of purines and pyrimidines, inactivation of DNA repair enzymes, or DNA base modification that results in DNA strand breaks (87). Furthermore, NO inhibits the mitochondrial respiratory enzymes NADH:ubiquinone reductase (complex I), succinate:ubiquinone oxidoreductase (complex II), and aconitase (76). This has a cytotoxic effect due to inhibition of cellular respiration. NO has also been implicated in the pathogenesis of artherosclerosis. Upon reaction with superoxide to form $OONO^-$, lipid and low density lipoprotein (LDL) oxidation occurs quite readily. In addition, $OONO^-$ reacts with oxyhemoglobin and oxymyoglobin to form nitrate and ferric ion.

1.12. Nitric Oxide and Gastrointestinal Function

eNOS is expressed throughout the GI microvasculature, while nNOS is predominantly expressed in nonadrenergic, noncholinergic (NANC) nerves. iNOS is expressed in a number of resident immunocytes such as macrophages, neutrophils and mast cells, as well as in epithelial cells. Within the GI tract, NO has a wide variety of functions varying from influences on blood flow and acid secretion to effects on mucus and electrolyte secretion. In the next few paragraphs a brief overview of some of these effects is provided.

NO is an important regulator of blood flow in the stomach. NOS inhibitors reduce gastric blood flow in a manner reversible by concomitant administration of L-arginine (106,107). The hyperemic response to luminal irritants, which is an important

component of mucosal defense, is mediated by NO (108-110). NO decreases gastric acid secretion (111,112) and stimulates mucus release (113,114). These effects contribute to the protective actions of NO against gastric mucosal injury (115).

Throughout the intestinal tract, as a mediator of NANC nerves, nNOS derived NO also plays a critical role in the regulation of gastrointestinal motility. NO from NANC nerves serves as the main inhibitory neurotransmitter that stimulates relaxation of sphincters (116). In addition, as an important neurotransmitter in parastalsis, it allows for the relaxation of both circular muscle distal to and longitudinal muscle proximal to a bolus in the intestinal tract (116).

In the intestine, NO increases blood flow and contributes to the hyperemic response to irritants. The importance of NO in maintaining intestinal mucosal integrity is illustrated by the fact that inhibition of NOS results in a rapid increase in epithelial permeability (6,117). Intestinal secretion and absorption is also affected by NO, although the exact nature of this interaction is not fully understood. *In vitro* studies have demonstrated that NO donors elicit active ion secretion (118,119), whereas *in vivo*, NOS inhibitors cause fluid secretion, suggesting that NO has an absorptive effect (120,121). Further details of this controversy are provided in the paragraphs below.

1.13. NO and Electrolyte Transport

The ability of nitric oxide to modulate epithelial secretion is somewhat controversial. Several studies have demonstrated an intestinal secretory response to NO-releasing agents (118,119,122,123), while others have reported NO to have an anti-

secretory effect (120,124,125). The conflicting findings appear to be due to contrasting results from *in vivo* versus *in vitro* experiments. With the exception of one group demonstrating that NOS inhibitors elicit secretion in the mouse ileum, NOS inhibitors have been shown to have no effect on basal intestinal absorption and secretion *in vitro* (126). Similarly, addition of L-arginine to the rat or mouse ileum *in vitro* has been shown to have no effect (126,127). In contrast, NO-releasing compounds cause increased electrolyte secretion in the intestine through a prostaglandin- and neurally-mediated mechanism (118,119,122). Therefore, based on these observations, NO was suggested to have either no effect or a secretory effect on basal intestinal transport.

Treatment with a NOS inhibitor *in vivo* induces net fluid secretion in the rat small intestine in a manner reversible by L-arginine or an NO donor (120,128-132). In addition, the NO-donor sodium nitroprusside (SNP) has been shown to exert pro-absorptive effects *in vivo* (129,130). On the other hand, the effects of L-arginine have been less clear. Most investigators have reported L-arginine to have no effect (120,133), while some have reported a secretory (131,134) or absorptive (129,134) effect. Wapnir *et al.* (134) suggested that this discrepancy was due to the amount of L-arginine administered. Low concentrations of L-arginine appear to stimulate water and electrolyte absorption, while high concentrations cause secretion (134). Thus, in contrast to the conclusions derived from *in vitro* experiments, *in vivo* studies have generally suggested that NO exerts pro-absorptive effects on basal intestinal tranport. Consequently, the role of NO in the regulation of electrolyte transport is not well understood. Izzo *et al.* (126) have proposed the theory that in physiological conditions NO most likely has pro-absorptive effects, whereas in pathophysiological conditions such as IBD, high levels of

NO reverse fluid and electrolyte transport in favour of secretion, and resulting in secretory diarrhea (126).

1.14. Clinical Implications of Inflammation-Induced Alterations in Intestinal Function

The pathogenesis of IBS is not well understood. A number of mechanisms have been proposed to explain the altered motility and pain that characterize IBS (135). These include malabsorption of carbohydrates, food intolerance and defects in neural and hormonal activity (34). Bouts of infection and inflammation have also been suggested to be the underlying cause of IBS (135,136). Several investigators have reported that a subset of IBS patients present their symptoms following a bout of infection or inflammation (2-5). The incidence of post-infectious IBS has been reported to be as high as 30% (2,3,137). Thus, the symptoms of IBS may be related to the long-term effects of inflammation on intestinal nerve, muscle and epithelial function. As already described, inflammatory mediators can affect electrolyte transport, mucus secretion and permeability in the intestine. Bell *et al.*, (138) demonstrated that in a rat model of colitis, the colonic epithelium is hyporesponsive to a secretagogue. In addition, several investigators have shown that inflammatory mediators such as cytokines cause intestinal epithelial cells to become hypo-responsive (49,139). Therefore, it is possible that altered epithelial function could result in impaired mucosal defence. For example, Mishima *et al.* (140), showed that induction of iNOS in septicemia was associated with increased intestinal bacterial translocation. Furthermore, this phenomenon was absent in iNOS-deficient mice and prevented in wild-type mice by treatment with a iNOS-inhibitor

(140,141). Given these findings, it is possible that a bout of inflammation could alter epithelial function (i.e., permeability, electrolyte transport, mucus secretion) and thereby contribute either to the symptoms of IBS, or in the case of IBD, to relapse of disease.

1.15. Nitric Oxide in Intestinal Inflammation

NO is important in normal colonic function as a regulator of mucosal blood flow, mucus and electrolyte secretion, and leukocyte adhesion to the vascular endothelium. However, it has been suggested that in inflammatory conditions, NO may also contribute to tissue injury in IBD (86,142,143). Consistent with this hypothesis, several studies have reported a correlation between the extent of colonic injury and nitrate levels in colonic biopsies from patients with IBD (144-146). Likewise, in animal models of colitis, a correlation between nitric oxide synthase activity and colonic injury has been reported (143,147). These findings have led to the suggestion that inhibition of NO production might alleviate tissue injury in inflammatory states such as IBD. However, the results of studies conducted thus far have been contradictory and/or inconclusive. Morover, several studies have shown that the source of iNOS in IBD and experimental models of colitis includes a variety of cells including macrophages and neutrophils within the lamina propria, and epithelial cells (148-150). A comprehensive review of the literature encompassing this area is described in chapter 3.

1.16. Objectives

The core of this dissertation is comprised of three studies. Each study will be presented individually in chapters 3, 4 and 5. The main objectives and hypotheses for these studies were as follows:

1. To examine the role of nitric oxide derived from different NOS isoforms in an experimental model of colitis, as well as to assess the effects of NOS inhibitors on free radical production in the inflamed colon. The hypothesis was that iNOS-selective, but not non-selective NOS inhibitors would reduce the extent of colonic injury in an experimental model of colitis.

2. To examine the long-term effects of inflammation of colonic epithelial function (i.e., permeability and electrolyte transport) *in vitro*. Given the effects of inflammation on motility and enteric nerve function, our hypothesis was that epithelial function *in vitro* is altered following a bout of colitis.

3. To assess colonic epithelial function (i.e., permeability, fluid secretion, mucus secertion) *in vivo* following a bout of inflammation and to determine if alterations in epithelial function are associated with changes in barrier function. The hypothesis tested was that colonic epithelial function *in vivo* is altered following a bout of inflammation, and that this results in increased bacterial translocation across the mucosa.

Chapter 2

GENERAL METHODS AND MATERIALS

To examine the long-term effects of colonic inflammation on epithelial function, an experimental model of colitis in the rat was used. The same model of colitis was also used to investigate the role of nitric oxide in the pathogenesis of acute colitis. This chapter describes in detail the methods used to induce colitis and assess the extent of colonic injury and inflammation. In addition, two of the following chapters include experimental data on electrolyte transport and RT-PCR. Therefore, a description of how the Ussing chamber and mRNA studies were carried out is outlined in this section. Methods and materials pertinent to a specific study are detailed in the appropriate chapter.

2.1 Animals

Male Wistar or Hooded-Listar rats weighing 175-200 g were obtained from Charles River Laboratories (Montreal, QC, Canada) and were housed in transparent plastic cages in a room with a 12 h light/12 h dark cycle. The rats were fed standard laboratory chow and tap water *ad libitum*. All experiments were approved by the Animal Care Committee of the University of Calgary and were performed in accordance with the guidelines of the Canadian Council on Animal Care.

2.2 Induction of Colitis

Colitis was induced by intracolonic administration of 30 mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS) dissolved in 0.5 ml of 50% ethanol (vol/vol) as previously described (151). A plastic cannula was made with an infant feeding tube (5 Fr.; Mallinckrodt Medical, Inc., St. Louis, MO, USA) fitted over a blunt 18 gauge needle. The cannula was inserted rectally into the colon such that the tip was ~ 8 cm proximal to the anus. This was used to instill the TNBS/ethanol solution. Rats were conscious during the procedure and care was taken to minimize the level of discomfort and stress.

2.3 Assessment of Colonic Macroscopic Damage

To investigate the effects of inflammation on epithelial function, rats were sacrificed 6 weeks following the induction of colitis. In studies examining the role of NO in acute colitis, rats were sacrificed 7 days after the induction of colitis. In both cases, the distal colon was removed, opened by longitudinal incision along the mesenteric border, and pinned on a wax platform. Macroscopic damage was scored by an observer unaware of the treatment that each rat had received. The criteria used to assess the colonic injury has previously been described (152) and are outlined in Table 2.1. Briefly, the presence or absence of diarrhea (loose, watery stool), and scores for the presence or absence of adhesions between the colon and other organs was noted. In addition, colonic bowel thickness was measured and extent of ulceration assessed. A global colonic score

consisting of maximal bowel thickness (in mm), scores for ulceration, and presence of diarrhea and adhesions was then calculated (Table 2.1).

2.4 Measurement of Myeloperoxidase Activity

Samples of macroscopically inflamed tissue, or samples from the same sites in colons devoid of macroscopically visible inflammation, were taken for myeloperoxidase (MPO) activity. MPO is an enzyme found in the azurophilic granules of neutrophils and other cells of myeloid origin (153,154). The tissue samples were weighed, frozen on dry ice and stored at –20°C until assayed for MPO activity according to the technique described by Bradley *et al.* (153). Absorbance was measured at 450 nm every 30 seconds for 1.5 min using a Titertek plate scanner (Flow Laboratories, Mississauga, ON, Canada). Absorbances were then converted to units of activity per mg tissue where one unit was defined as that degrading one unit μmol of peroxide per min at 25°C (153).

2.5 Measurement of Electrolyte Transport

The colon was removed and gently washed of fecal contents. The colon was then stripped of its external muscle (to allow for better tissue oxygenation and drug absorption) as previously described (28). A segment of the mucosa from each rat, taken from macroscopically inflamed tissue or from the same site in colons devoid of macroscopic inflammation, were mounted in Leucite Ussing-type diffusion chambers (Navicyte Inc., Sparks, NV). The tissues were bathed in a modified Krebs buffer

containing (in mM) 115.0 NaCl, 2.0 KH_2PO_4, 2.4 $MgCl_2$, 1.3 $CaCl_2$, 8.0 KCl and 25.0 $NaHCO_3$. Serosal buffer contained 10 mM glucose, whereas the mucosal buffer contained 10 mM mannitol. The bathing solutions were maintained at 37°C and bubbled with 95% O_2-5% CO_2. After a 20 min equilibration period, the basal transport parameters of short circuit current (I_{sc}) and potential difference (PD) were measured. Conductance (G) was then calculated from PD and I_{sc} according to Ohm's law.

The PD was clamped to zero using a voltage-clamp apparatus (EVC-4000, World Precision Instruments, Sarasota, FL) and I_{sc} responses to various stimuli monitored using digital data acquisition (MP100, Biopac Systems, Santa Barbara, CA) and analysis (AcKnowledge 3.0.3, Biopac) software.

2.6 Reverse Transcriptase-Polymerase Chain Reaction

Colonic iNOS mRNA expression was examined using reverse-transcriptase polymerase chain reaction (RT-PCR). Whole thickness colonic tissues (~150-200 mg) were placed in 2 ml of TRIzol reagent (GIBCO BRL, Gaithersburg, MD), and RNA was isolated according to previously described methods (155). The method used for RT-PCR was modified slightly from that described previously (156). The housekeeping gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Briefly, 1 μg of RNA from each sample was reverse transcribed at 42°C using Superscript RNase H Reverse Transcriptase (GIBCO BRL, Gaithersburg, MD) and appropriate reaction mixture (containing 2 μl of 10X PCR buffer, 2 μl of 10 mmol/L deoxynucleoside triphosphate [dNTP] stock, and 2 μl of N_6 random hexamer stock). The

enzyme was then deactivated by heating the sample to 95°C for 10 minutes. After the reaction, 2 μl of complementary DNA (cDNA) was mixed with 2 μl of 2 mmol/L dNTP stock and 5 μl of 10X PCR buffer. The iNOS upstream primer (2 μl; ~20 pmol) and iNOS downstream primer (2 μl; ~20 pmol) were then added to each tube).

DNA amplification was conducted under the following conditions: denaturation at 94°C for 1 minute, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. To ensure complete denaturation of the DNA with no background polymerase activity, T*aq* DNA polymerase was added to the PCR mixture during the hot start of cycle 1. Optimal coamplification of each primer with GAPDH was determined during preliminary trials. The iNOS/GAPDH genes were coamplified for 26/22 cycles. Hence, the GAPDH upstream and downstream primers were added to the iNOS PCR mixture during the hot start of cycle 5.

After separation of the PCR products on a 1% agarose gel containing 10 μg of ethidium bromide, a Polaroid (Cambridge, MA) picture of the gel was taken under UV light. Using a densitometer and National Institutes of Health software, quantities of each product were normalized to control levels of GAPDH and expressed as densitometry units. The iNOS and GAPDH products were made using primers based on published sequences (Table 2.2).

2.7. Statistical Analysis

All data are presented as the mean ± SEM. Comparisons between two experimental groups were performed using an unpaired Student's t test. Comparisons among three or

more experimental groups were carried out using a one-way analysis of variance followed by the appropriate post-hoc test. Details of the specific post tests used are provided in the individual chapters. With all analyses, an associated probability (*P* value) less than 5% was considered significant.

2.8. Materials

TNBS was purchased from Fluka Chemika (Buchs, Switzerland). PCR buffer, dNTPs, N_6 random hexamers, and RNasin were purchased from Pharmacia Biotech (Baie d'Urfé, QC, Canada). Superscript and TRIzol were obtained from GIBCO BRL. The chemicals utilized in the MPO activity assay and Ussing chamber studies were purchased from Sigma (St. Louis, MO, USA).

Table 2.1. Criteria for the macroscopic scoring of colonic damage.

Feature	Score
Ulceration	
Normal appearance	0
Focal hyperemia, no ulcers	1
Ulceration without hyperemia or bowel wall thickening	2
Ulceration with inflammation at 1 site	3
Ulceration/ inflammation at 2 or more sites	4
Major sites of damage extending >1 cm along the length of the colon	5
When an area of damage extended >2 cm along the length of the colon, the score is increased by 1 for each additional cm of involvement	6-10
	plus
Adhesions	
No adhesions	0
Minor adhesions (colon can be separated from other tissue with little effort)	1
Major adhesions	2
Diarrhea	
No	0
Yes	1
Thickness	
The maximal bowel wall thickness (x), in mm, was added	x
	Total Score

Table 2.2. Primer Sequences for RT-PCR

Gene	Primer Sequences	Amplicon Length (base pairs)	Reference
iNOS	Upstream, 5'-ACA ACA GGA ACC TAC CAG CTC A-3'	651	Lyons *et al.*(157)
	Downstream, 5'-GAT GTT GTA GCG CTG TGT GTC A-3'		
GAPDH	Upstream, 5'-CGG AGT CAAC GGA TTT GGT CT AT-3'	306	Wong *et al.*(158)
	Downstream, 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'		

Chapter 3

THE ROLE OF NITRIC OXIDE IN AN EXPERIMENTAL MODEL OF COLITIS

3.1 Introduction

Ulcerative colitis and Crohn's disease are inflammatory bowel diseases (IBD) characterized by extensive ulceration of the intestine and alterations in smooth muscle, enteric nerve and epithelial function (39). However, the etiological basis of these disorders is not fully understood. In recent years it has been postulated that over-production of nitric oxide (NO) might contribute to the tissue injury observed in IBD. In support of this hypothesis, several investigators have reported a correlation between the extent of tissue injury and nitrate levels in colonic biopsies from patients with IBD (144-146). Similarly, studies using animal models of colitis have suggested a correlation between the extent of colonic injury and the levels of nitric oxide synthase activity (143,147).

Several groups have examined the effects of inhibition of nitric oxide synthase (NOS) on the severity of tissue injury in experimental colitis, but the results have been discrepant. The effects of NOS inhibitors have varied from amelioration of intestinal inflammation (147,159-162), to no effect on colonic injury (163), to exacerbation of tissue injury (164,165). One explanation for these conflicting results is that the NOS inhibitors used were not isoform-selective. Inhibition of the constitutive isoforms of NOS may be harmful to the tissue. Thus, others have examined the effectiveness of selective inhibitors of inducible NOS (iNOS) for the treatment of experimental colitis.

However, the results of these studies have also been inconsistent. Some investigators have reported iNOS-selective inhibitors to be beneficial in reducing macroscopic damage (143,160,166,167), while others have reported these drugs to have unfavourable effects or no effect (163,168). Administration of slow-releasing NO donors has also been shown to reduce, rather than augment, colonic injury in experimental colitis (169,170). Furthermore, studies using iNOS gene knockout mice suggest that iNOS-derived NO might be beneficial, rather than detrimental, during inflammation. These mice exhibited increased susceptibility to acetic acid- and TNBS-induced colonic injury compared to wild type mice (149,171).

The mechanism through which NO might be deleterious to tissue is not well understood. Harmful effects of NO have been attributed primarily to its reactivity with other biological molecules. For example, NO can react with superoxide (O_2^-) to form the highly reactive and unstable peroxynitrite ion ($ONOO^-$) (172). Interestingly, NOS has also been shown to produce superoxide (79,81-83). Peroxynitrite is believed to play an important role in tissue injury through the oxidation and nitration of proteins (172-174). For example, purines and pyrimidines can be oxidized, while antioxidant enzymes such as glutathione transferase and glutathione peroxidase can be inactivated through nitration (87). Peroxynitrite can also be formed through the reaction of NO (or even its end-product nitrite) with the neutrophilic enzyme myeloperoxidase (MPO) (175). Since increased neutrophil infiltration and increased levels of superoxide and NO have been demonstrated in IBD, it is possible that NO mediates tissue injury as a result of enzymatic and/or non-enzymatic peroxynitrite formation. Furthermore, peroxynitrite can become protonated to form peroxynitrous acid (ONOOH) which subsequently could undergo

homolytic cleavage to form the strong oxidizing agents hydroxyl radical (HO•) and nitrogen dioxide (NO_2•) (173). These two oxidants can produce detrimental effects on cells through inactivation of metabolic enzymes and transport proteins (86,90,93).

The aim of this study was to clarify the roles of NOS isoforms in experimental colitis. First, we characterized iNOS and endothelial NOS (eNOS) expression in TNBS-induced colitis. Second, we tested the effects of a series of NOS inhibitors (non-selective, iNOS-selective and nNOS–selective) on colonic damage and granulocyte infiltration in experimental colitis. Finally, given the potential for reactions of NO with antioxidant enzymes and oxidants, it is possible that affecting the production of NO would change the oxidative state of the tissue. Thus, we determined the effects of NOS inhibition on colonic levels of free radicals (superoxide, hydroxyl radical).

3.2 Materials & Methods

Induction of colitis

Colitis was induced in male Wistar rats (175-200 g) by intracolonic adminstration of TNBS according to the method described in chapter 2.

NOS inhibitors

In studies examining the effects of NOS inhibition on colonic damage, seven groups of rats were compared. One group consisted of untreated rats (controls) while in the others the rats received TNBS intracolonically. Of the TNBS-treated rats, one group was treated with vehicle, another group was treated intraperitoneally once-daily with L-nitroarginine methyl ester (L-NAME; 30 mg/kg), while the others were treated intraperitoneally, twice-daily with: aminoguanidine (25 mg/kg), L-N^6-iminoethyllysine (L-NIL; 3 mg/kg), 1400W (3 mg/kg), or 7-nitroindazole (7-NI; 20 mg/kg). These doses of the NOS inhibitors have previously been shown to produce significant NOS inhibition (147,176-179).

Seven days after intracolonic TNBS administration, the rats were killed by cervical dislocation and the extent of colonic injury and inflammation were assessed. Colonic tissue was taken for immunohistochemical analysis of iNOS and eNOS expression, as well as for measurement of iNOS activity and MPO activity. In all experiments, the minimum sample size per group was six.

Assessment of colonic injury and inflammation

The distal colon was removed, opened by longitudinal incision, and the severity of macroscopic damage was scored using previously described criteria (chapter 2).

Samples of macroscopically inflamed tissue, or from the same sites of the colon in rats devoid of damage, were taken for measurement of MPO activity.

iNOS activity

Colonic tissue samples (~100 mg) from untreated or TNBS-treated rats were frozen with liquid nitrogen and stored at –70°C until assayed for iNOS activity. iNOS activity was determined by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline in the presence of the calcium chelator EGTA. The assay was carried out with slight modifications of a previously described procedure (180). Briefly, tissues were homogenized in ice-cold Tris buffer (5 ml/g tissue; pH 7.4 containing 1 mM L-citrulline, 0.1 mM EDTA, 1 mM dithiothreitol, 10 μ/ml leupeptin, 2 μg/ml aprotonin, 10μg/ml trypsin inhibitor, and 1 mM PMSF) using a Polytron homogenizer. Samples were then centrifuged at 12,000 *g* for 10 min and the supernatants collected. The supernatants (50 μl) were then diluted in 50 μl Tris buffer (pH 7.4) and incubated at 37°C for 15 min with 1 mM $CaCl_2$, 1 mM EGTA, 10 μM FAD, 1 mM NADPH, 10 μg/ml calmodulin, 0.1 mM tetrahydrobiopterin, and 10 μM L-arginine containing 60 nM L-[^{14}C] arginine. The reaction was stopped by addition of 1 ml of cold buffer (containing 20 mM HEPES, 1 mM EGTA, and 1 mM EDTA). Each sample was loaded onto columns containing 1 ml of Dowex (50W-X8 100-200) cation exchange resin. The eluent was collected, added to 10 ml of scintillation cocktail (Ecolite, ICN Biomedicals, Irvine, CA) and the radiolabelled products counted using a Wallac scintillation counter (Turku, Finland)

Immunohistochemical detection of iNOS and eNOS and RT-PCR detection of iNOS mRNA

Tissue sections taken for immunohistochemistry were fixed in formalin for 24 h, and then embedded in parrafin. After deparaffinization, the sections were incubated in 0.3% hydrogen peroxide-methanol solution for 15 min to eliminate endogenous peroxidase activity. The sections were blocked with normal horse serum for 1 h at room temperature, then incubated overnight (4°C) with the primary antibodies for iNOS (monoclonal IgG) or for eNOS (rabbit polyclonal IgG) (Santa Cruz) at dilutions of 1:500. Following washes with Tris-buffered saline, the sections were incubated with a biotinylated universal secondary anti-IgG antibody. The sections were washed again, then incubated with Streptavidin for 30 min. Finally, the slides were incubated with the substrate hydrogen peroxide-diaminobenzamide solution for 1 min, and iNOS or eNOS expression examined using a light microscope. To be ceratin that non-specific staining was not due to the secondary antibody used, immunohistochemistry controls were performed. This included negative control sections in which tissues were incubated with secondary antibody without prior incubation with primary antibody.

iNOS mRNA expression was examined in colonic tissues taken from control rats and rats administered intracolonic TNBS 7 days earlier. Examination of iNOS mRNA expression was carried out by RT-PCR according to the procedure outlined in chapter 2.

Free radical detection by chemiluminescence

Free radical production was measured by chemiluminescent detection of N,N'-dimethyl-diacridinium (lucigenin) reactivity as previously described (181). Briefly, colonic tissue samples (~100 mg) were immediately placed in 1 ml of cold Tris buffer

(pH 7.4). Following the collection of tissues from each rat, the Tris buffer was replaced with 1 ml of Tris buffer (37°C) containing N,N'-dimethyl-diacridinium (lucigenin; 250 μM) and glucose (5 mM). Following a 2 min equilibration period, lucigenin-induced chemiluminescence was measured over a 200 sec interval using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Chemiluminescence in the presence of superoxide dismutase (SOD; 300 U/ml) was then measured in the same tissue following the addition of 1 ml of fresh Tris buffer containing lucigenin. Superoxide levels were determined to be the difference between the colonic tissue chemiluminescence in the presence and absence of superoxide dismutase. Similarly, addition of salicylate (5 mM), was used to determine what proportion of the lucigenin-induced chemiluminescence was attributable to hydroxyl radical (182,183). These measurements were performed using fresh tissues that were not previously exposed to SOD. Thus, hydroxyl radical levels were determined to be the difference between chemiluminescence in the presence and absence of salicylate.

In vitro effects of NO donors and NOS inhibitors on lucigenin chemiluminescence were also examined in colonic tissue (~ 100 mg) taken from TNBS-treated rats. As described above, 1 ml of Tris buffer containing lucigenin (250 μM) and glucose (5 mM) was added to each tissue sample. In addition, L-NAME (100 μM), AG (1 mM), sodium nitrite (100 μM), sodium nitroprusside (SNP; 50 μM), S-nitrosoacetylpenacillamine (SNAP; 100 μM) or vehicle was added to each sample. The concentrations selected for the NO donors were based on those previously shown to elicit epithelial secretion and muscle relaxation in rat tissue *in vitro* (122,123,184,185). The concentrations chosen for the NOS inhibitors were based on those previously shown to

have the desired NOS inhibition and selectivity (186-188). Following a 2-min equilibration period, lucigenin-induced chemiluminescence was measured over an interval of 200 sec.

Statistical Analysis

Comparisons among groups of data were made using a one way analysis of variance followed by a Student-Newman-Keuls test. With all analyses, an associated probability (P value) of less than 5% was considered significant.

3.3 Results

Effects of NOS inhibitors on colonic damage and granulocyte infiltration

Intracolonic administration of TNBS caused extensive injury to the colon characterized by severe ulceration, adhesion of the colon to other organs, and diarrhea (Figure 3.1). Administration of L-NAME, a non-selective NOS inhibitor, did not affect the severity of macroscopic damage in the colon compared to vehicle treatment (Figure 3.1). Treatment with the selective iNOS inhibitors aminoguanidine, L-NIL, or 1400W, or with the selective nNOS inhibitor 7-NI, also did not affect the severity of macroscopic damage in the colon. Intracolonic administration of TNBS caused significant granulocyte infiltration into the colonic tissue as indicated by elevated MPO activity (~16 fold higher than controls) and as confirmed by histology. With the exception of L-NIL, the NOS inhibitors did not affect the levels of granulocyte infiltration in the colonic tissue (Figure 3.1).

NOS activity and expression in TNBS-induced colitis

iNOS activity was significantly elevated in rats with colitis (by ~ 110%) compared to controls. Administration of L-NAME, aminoguanidine, L-NIL or 1400W significantly reduced iNOS activity by 60%, 40%, 75% and 50% respectively (Figure 3.2). In contrast, 7-NI did not affect iNOS activity in the colon of TNBS-treated rats.

iNOS expression was low in controls. Intracolonic TNBS administration resulted in increased iNOS expression (Figure 3.3A.) which was mainly localized to areas of ulceration. iNOS was primarily expressed in inflammatory cells within the lamina propria and in epithelial cells. In contrast, eNOS expression was localized to the lining of blood vessels and did not appear to be different in tissues from TNBS-treated versus

control rats. iNOS mRNA expression was not detectable in the colon of control rats, but was induced 7 days after induction of colitis (Figure 3.3B.).

Effects of NOS inhibition on colonic free radical production in vivo

Colonic tissue from untreated control rats exhibited low levels of total free radical production (58.7 ± 3.2 relative light units/mg tissue). Colonic tissue from rats with TNBS-induced colitis displayed significantly increased free radical production (~ 4-fold) compared to tissue from control rats. Treatment with NOS inhibitors did not affect free radical production in the inflamed colon when compared to vehicle. Superoxide production in colonic tissue taken from rats with colitis was significantly increased compared to that in tissue of control rats. Likewise, inflamed colonic tissues from rats treated with NOS inhibitors (L-NAME, AG, 7-NI) displayed significantly elevated superoxide levels compared to tissues from control rats. However, colonic superoxide levels were significantly reduced in these tissues compared to that in vehicle-treated rats (Figure 3.4).

Hydroxyl radical production in colonic tissue from rats with colitis was not significantly different from that in control rats. In contrast, colonic tissues from L-NAME- and AG-treated rats with colitis displayed significantly elevated levels of hydroxyl radical when compared to control and vehicle-treated rats with colitis (Figure 3.4). Colonic tissues from 7-NI-treated rats did not display any change in hydroxyl radical production when compared to tissues taken from control rats.

Effects of NOS inhibitors and NO donors on free radical production in vitro

To examine the effects of NO on free radical production, *in vitro* experiments with NOS inhibitors and NO donors were performed. Unlike the effects of L-NAME and

AG on colonic superoxide and hydroxyl radical production *in vivo*, neither NOS inhibitor had an effect on free radical production *in vitro* (Figure 3.5). In contrast, the NO donors SNP and SNAP reduced free radical production in inflamed colonic tissue by ~40% and ~30%, respectively (Figure 3.5). Addition to the tissue of sodium nitrite, which releases nitrite (an end product of NO), had no effect on colonic free radical production (Figure 3.5).

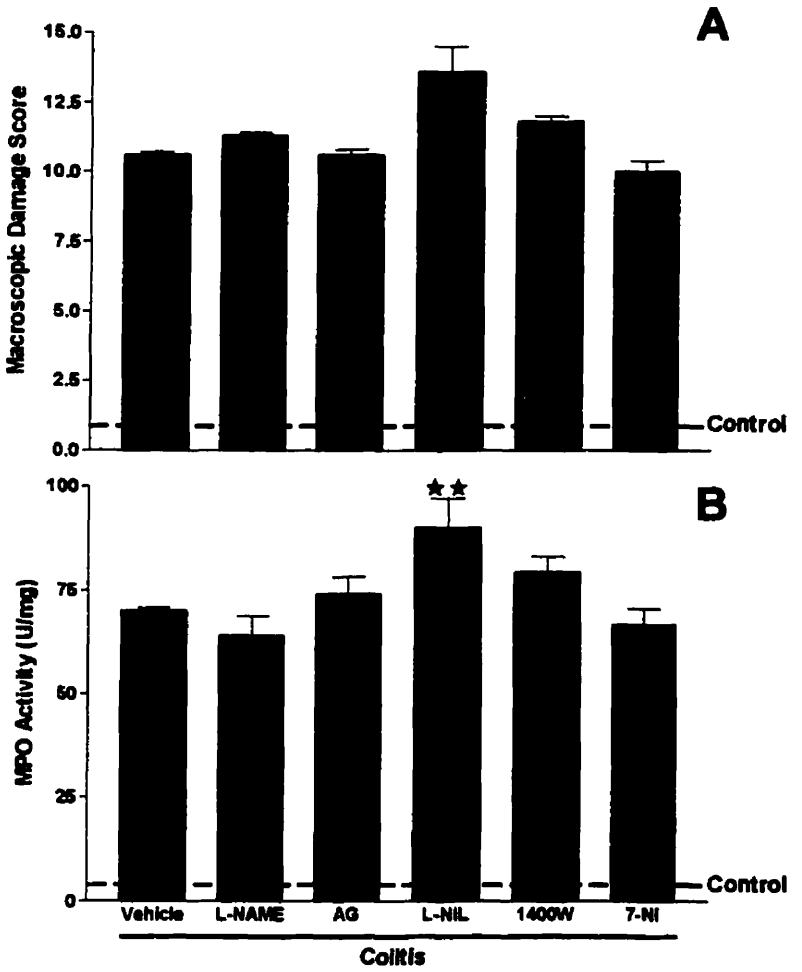

Figure 3.1. Effect of NOS inhibitors on severity of colonic damage (panel A) and myeloperoxidase (MPO) activity (panel B) 7 days after intracolonic administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS). L-nitroarginine methyl ester (L-NAME; 30 mg/kg), aminoguanidine (AG; 25 mg/kg), L-N^6-iminoethyllysine (L-NIL; 3 mg/kg), 1400W (3 mg/kg) and 7-nitroindazole (7-NI; 20 mg/kg) were administered for 7 days. Each bar represents the mean ± SEM, with at least 7 rats per group. **$P < 0.01$ compared with the vehicle-treated colitis group.

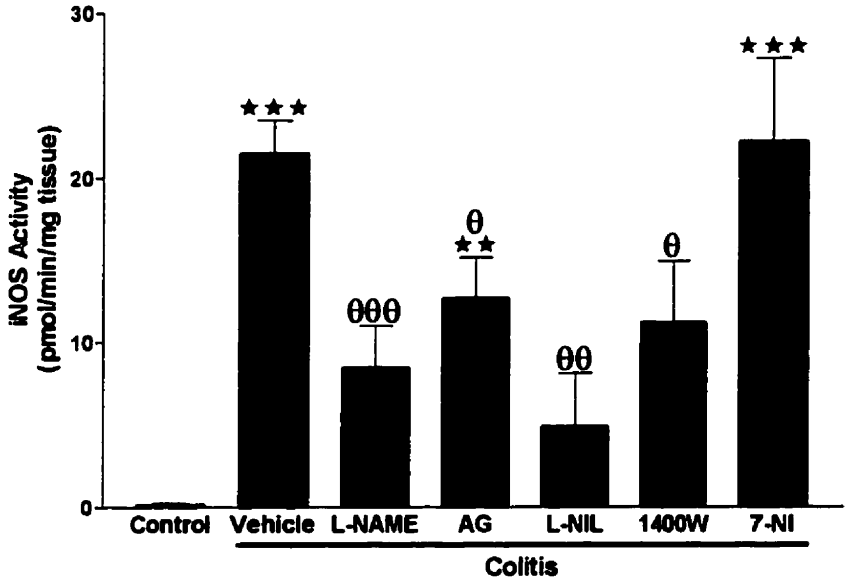

Figure 3.2. Effects of NOS inhibitors (L-nitrourginine methyl ester (L-NAME; 30 mg/kg), aminoguanidine (AG; 25 mg/kg), L-N^6-iminoethyllysine (L-NIL; 3 mg/kg), 1400W (3 mg/kg) and 7-nitroindazole (7-NI; 20 mg/kg) on colonic NOS activity 7 days after intracolonic TNBS. Each bar represents the mean ± SEM, with at least 5 rats per group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the control group. $^{\theta}P<0.05$, $^{\theta\theta}P<0.01$, $^{\theta\theta\theta}P<0.001$ compared with the vehicle-treated group.

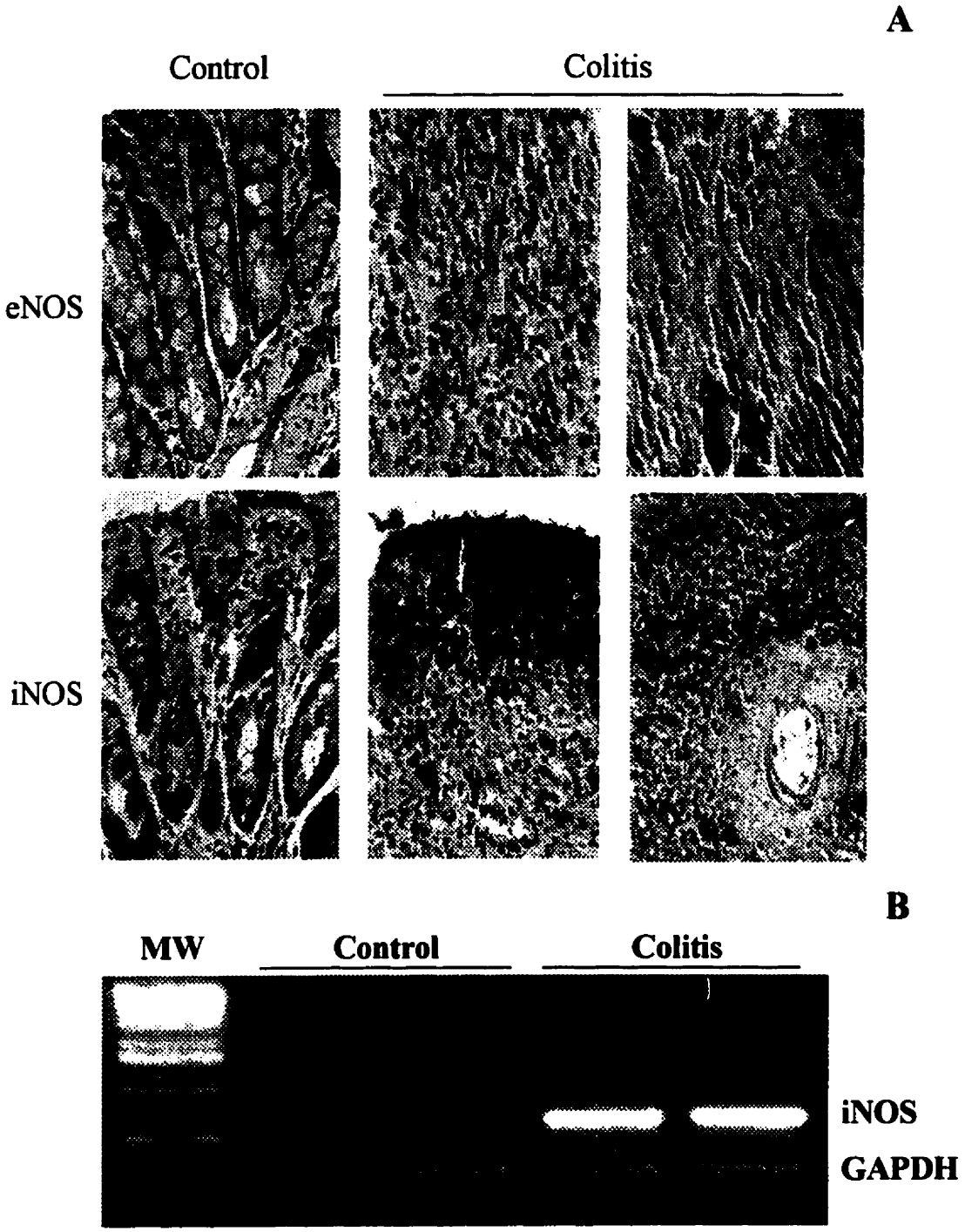

Figure 3.3. (A) iNOS and eNOS immunohistochemistry and (B) iNOS mRNA expression 7 days after intracolonic administration of TNBS. Tissue from control and TNBS-treated rats are shown. The original magnification of the tissue was X 200.

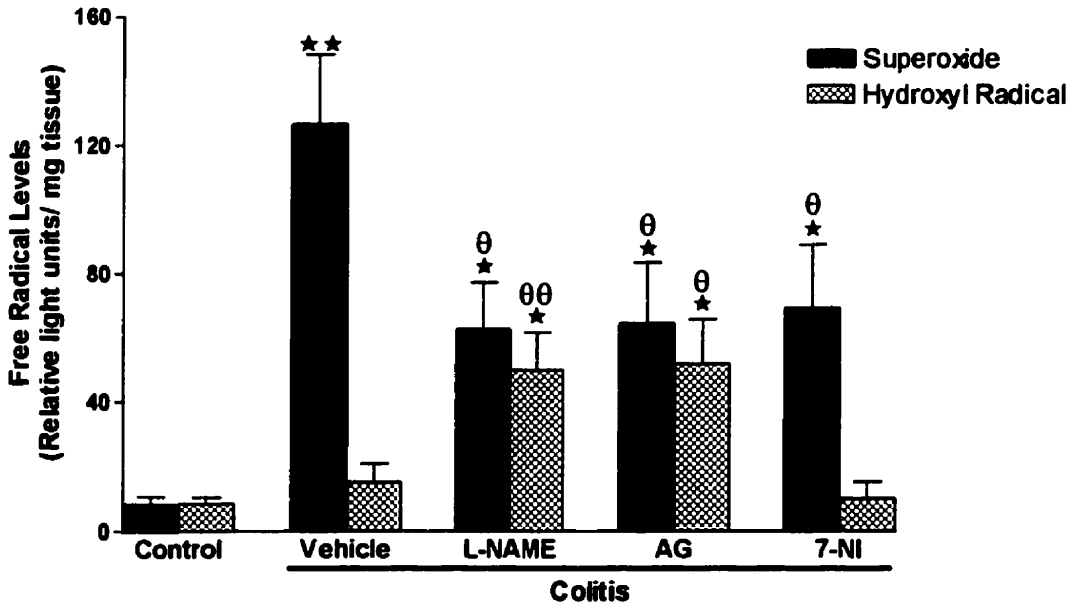

Figure 3.4. Colonic free radical production 7 days after intracolonic administration of TNBS and treatment with either vehicle, L-nitroarginine methyl ester (L-NAME; 30 mg/kg), aminoguanidine (AG; 25 mg/kg), or 7-nitroindazole (7-NI; 20 mg/kg). Each bar represents the mean ± SEM, with at least 5 rats per group. $^{*}P<0.05$, $^{**}P<0.01$ compared to the control group. $^{\theta}P<0.05$, $^{\theta\theta}P<0.01$ compared to the vehicle-treated colitis group.

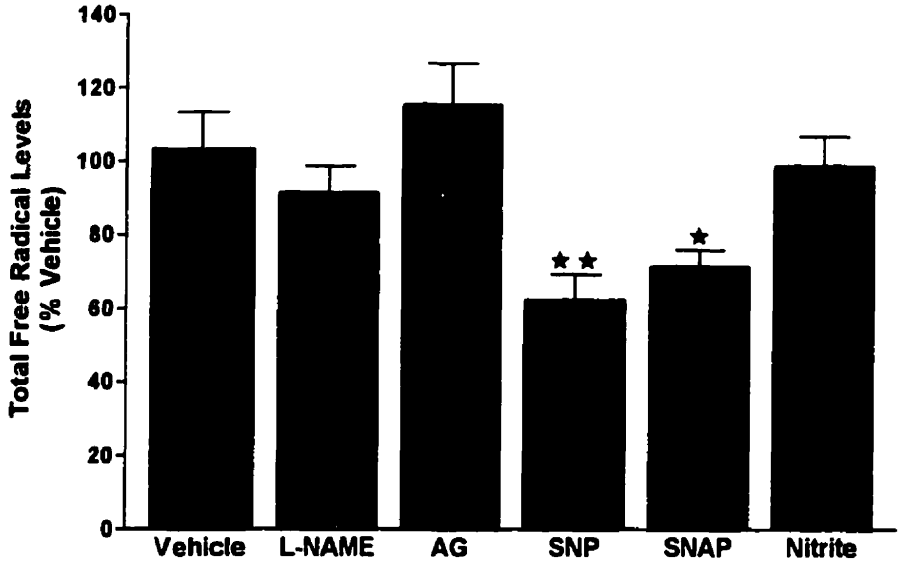

Figure 3.5. In vitro effects of NOS inhibitors (L-nitroarginine methyl ester (L-NAME; 100 μM) and aminoguanidine (AG; 1 mM)) and NO donors (sodium nitroprusside (SNP; 50 μM), S-nitrosoacetylpenacillamine (SNAP; 100 μM), and sodium nitrite (100 μM) on free radical production as determined by lucigenin-induced chemiluminescence in inflamed colonic tissue. Each bar represents the mean ± SEM, with at least 5 rats per group. *P<0.05, **P<0.01 compared to the vehicle group.

3.4. Discussion

Miller *et al.* (86) suggested that the concept that low levels of NO are good and high levels of NO are bad is an oversimplification. What determines the extent of tissue injury during an inflammatory event includes not only which NO-derived species are formed and in what proportion to one another, but also what the levels of antioxidants relative to oxidants are. For example, in experimental colitis it has previously been shown that concomitant with an increase in iNOS expression there is a decrease in tissue SOD levels (189).

Numerous studies have been performed to test the hypothesis that NO is a mediator of intestinal injury, but these have produced conflicting results. In the present study, we have tested the effects of non-selective, iNOS-selective and nNOS-selective inhibitors in the TNBS-induced model of colitis in the rat. Our results showed that despite significant inhibition of NOS activity, the NOS inhibitors tested did not reduce colonic damage or granulocyte infiltration.

Beckman *et al.* (172) proposed the idea that NO is the "bad guy" in inflammation, causing tissue injury subsequent to its conversion to peroxynitrite. However, if this is the case, why did we not observe an attenuation of damage when NOS was inhibited? It is now known that all three isoforms of NOS can produce superoxide (79,81-83). iNOS may be a particularly important source of superoxide in that it is the most prevalent isoform during an inflammatory state. The mechanism of superoxide production by iNOS differs from that of eNOS and nNOS. iNOS superoxide production occurs at the reductase domain, whereas superoxide production by eNOS and nNOS occurs at the

oxygenase domain. Given the difference in the mechanism of superoxide production among the isoforms, it is possible that certain NOS inhibitors inhibit both NO and superoxide production derived from one isoform, but not necessarily that derived from the others (81,84,85).

In this study, we were interested in examining the effects of NOS inhibitors on free radical production in the colon. Our results revealed that neither selective nor non-selective NOS inhibitors had an effect on total free radical production. However, superoxide levels were reduced by the NOS inhibitors, regardless of which isoform of NOS the inhibitors were selective for. It should be noted that from these studies it is not possible to distinguish between superoxide derived from sources such as NADPH oxidase and that derived from the various NOS isoforms. Nevertheless, the reduction in superoxide levels in tissues from rats treated with non-selective and iNOS-selective inhibitors was accompanied by an increase in hydroxyl radical production. Therefore, it is possible that a reduction in NO levels results in a diversion of superoxide to other pathways such as the iron-catalyzed Haber-Weiss reaction, which would result in the production of hydroxyl radical ($H_2O_2 \rightarrow HO\bullet + OH^-$) (190). Given the potentially harmful reactions a strong oxidizing agent such as hydroxyl radical can undergo *in vivo*, its formation is clearly not favourable for the surrounding tissue. In contrast to L-NAME and AG, the selective nNOS inhibitor 7-NI did not increase hydroxyl radical production. This suggests that NO derived from iNOS and/or eNOS, but not from nNOS, might be abrogating the production of other free radicals such as hydroxyl radical. This may be explained by the fact that NO from different NOS isoforms is found in different cells, and therefore, may undergo different reactions depending on the other chemical species

present within the particular cell. Tissues from 7-NI treated rats continued to exhibit the same level of total free radical production as those from other TNBS-treated rats. These findings may explain why there was no attenuation of damage observed in 7-NI treated rats. It is possible that despite inhibiting NO production, colonic injury occurs due to the production of other cytotoxic free radicals. The results of this study point to at least one potential culprit, the hydroxyl radical. Further studies examining the roles of other free radicals such as $NO_2\bullet$ and $O_2^{\cdot}$, as well as their effects on antioxidant enzymes, must be completed in order to determine what effect different nitrogen oxides have on colonic tissue during a bout of inflammation.

Finally, the *in vitro* reduction of chemiluminescence by the NO donors SNP and SNAP is consistent with the hypothesis that NO can scavenge superoxide. Furthermore, the lack of any effect on colonic free radical production by direct addition of sodium nitrite suggests that the form of NO released into the inflammatory mileu is a critical determinant of whether deleterious free radicals are produced. Thus, while SNP and SNAP release forms of nitrogen oxide that affect free radical production, nitrite does not.

In summary, NOS inhibitors failed to reduce colonic injury sustained during an inflammatory insult of the colon. Furthermore, NOS inhibitors did not affect total free radical production in the colon, but did change the composition of the free radicals that were produced. Thus, the positive effects of nitric oxide as a scavenger of free radicals may outweigh its properties as the origin of harmful nitrogen oxide derivatives. Determining which free radical species are present in an inflammatory milieu may be critical to predicting the clinical outcome of disease. Further studies are required in order to determine which nitrogen oxide species are deleterious to the body.

Chapter 4

COLONIC EPITHELIAL HYPO-RESPONSIVENESS FOLLOWING COLITIS: ROLE OF INDUCIBLE NITRIC OXIDE SYNTHASE

4.1 Introduction

The colonic epithelium is continually exposed to bacteria, bacterial products and dietary antigens that are capable of inducing or exacerbating mucosal inflammation. The epithelium performs an important barrier function, preventing the entry of these factors into the lamina propria. The secretion of water by the colonic epithelium is a further defensive mechanism aimed at diminishing the ability of pathogens to adhere to the epithelium, as well as diluting noxious luminal agents (191). Water secretion by intestinal epithelial cells occurs passively, driven by an osmotic force generated through the active transport of chloride ions. The motor activity of the intestine also contributes to this defensive response by assisting in the physical expulsion of the luminal contents. Although normally a defensive response, excessive secretion of water or impaired absorption of water (and electrolytes) can lead to diarrhea, as can impaired motor function. On the other hand, impaired electrolyte (and therefore water) secretion could predispose the colon to infection and inflammation.

There is a great deal of evidence that during an acute bout of colitis, epithelial secretory function is profoundly altered (124,138,192). A number of inflammatory mediators, such as prostaglandins and leukotrienes, are capable of stimulating chloride secretion by colonocytes (54,193). However, large increases in chloride secretion have

not been noted in studies of colonic secretion in inflammatory bowel disease or in animal models of colitis (194-196). It is possible that a bout of colitis produces long-lasting changes in function (i.e., down-regulation of secretory responses to inflammatory mediators) that persist after resolution of the mucosal inflammation. If such changes occurred in epithelial permeability and/or secretory function, they may render the colon more susceptible to subsequent inflammatory events.

In the present study, we have investigated the possibility that inflammation of the colon results in significant long-term alterations in epithelial permeability and secretion. Given recent evidence that nitric oxide can modulate intestinal permeability and secretion (7,118,121), and that NO derived from iNOS may contribute to altered secretory function in acute colitis (124), we have also examined the role of iNOS-derived NO in mediating prolonged epithelial dysfunction after colitis.

4.2 Methods and Materials

Induction of Colitis

Colitis was induced in male, Wistar rats using the method described in chapter 2. A second group of rats was treated intracolonically with 50% ethanol (the vehicle for TNBS). A control group consisted of rats that were left untreated during the course of the study.

Rats were killed by cervical dislocation 6, 9, or 12 weeks following induction of colitis. Controls (age-matched to the other rats) were killed at the same times. Rats treated with 50% ethanol were killed at the 6 wk time-point. Within each group, some rats were used for assessment of colonic injury and inflammation, while others were used for electrolyte transport studies. The minimum sample size per group in all experiments was 5.

Electrolyte Flux Studies

The distal colon was removed from each rat and placed in Ussing-type chambers containing oxygenated Krebs buffer as described in chapter 2. After a 20 min equilibration period, the basal transport parameters of short circuit current (I_{sc}) and potential difference (PD) were measured. Conductance (G) was calculated from PD and I_{sc} according to Ohm's law.

The PD was clamped to zero and the short circuit current (I_{sc}) responses to various stimuli measured. Electrical field stimulation (EFS; 50V, 10 Hz, 5s) was delivered with a Grass S48 stimulator and SIU50 stimulus isolation unit (Grass Instruments, Warwick, RI). In the same colonic preparations, I_{sc} responses to the secretagogues IBMX (300 μM)

and carbachol (1 μM) added to the serosal side of the tissue were also recorded. Isc was allowed to recover to a stable baseline between application of drugs or EFS.

In other Ussing chamber experiments, ^{22}Na (4 μCi) and ^{36}Cl (2 μCi) were added to the bathed tissues and after a 20-min equilibration period, steady-state unidirectional electrolyte fluxes ($\mu Eq \bullet cm^{-2}h^{-1}$) were measured during four 5-min intervals, as previously described (138,197).

Permeability Measurements

In separate Ussing chamber experiments, the permeability marker ^{51}Cr-labeled EDTA (24 μCi) was added either to the mucosal or serosal side of the tissues taken from rats 6 wk after TNBS administration or from age-matched controls. The tissues were allowed to equilibrate in the Ussing chambers for 20 min before unidirectional ^{51}Cr-EDTA fluxes were measured during four 5-min intervals, as previously described (138,197).

Measurement of Nitric Oxide Synthase Activity

Colonic tissue samples (~100 mg) from control and TNBS-treated rats were frozen with liquid nitrogen and stored at –70°C until assayed for NOS activity. NOS activity was determined by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as previously described. Briefly, tissues were homogenized using a Polytron homogenizer in 0.5 ml of ice-cold buffer (pH 7.4; containing 10 nM HEPES, 320 nM sucrose, 1 nM dithiothreitol, 0.1 nM EDTA, 0.01 mg/ml soybean trypsin inhibitor, 0.01 mg/ml leupeptin, and 0.002 mg/ml aprotinin) followed by centrifugation for 10 min at 14 000 *g* at 4°C. Supernatants (20 μl) were then incubated at 37°C for 10 min in 50 μl of NOS assay buffer (containing 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM valine,

0.3 mM NADPH, 3.15 mg/ml L-arginine, and 157 pM L-[^{14}C]arginine). Addition of EGTA (1 mM) was used to determine the activity of Ca^{2+}-independent NOS. The reaction was stopped by addition of 0.5 ml of Dowex (50W-X8 100-200) cation exchange resin and the mixture was then loaded onto columns containing 1 ml of Dowex resin. Eluent was collected and a 1 ml aliquot measured for radiolabelled products using a Wallac scintillation counter (Turku, Finland).

NOS activity was defined as the rate of L-[^{14}C]citrulline formation. NOS activity that was abolished by EGTA was considered as constitutive NOS (cNOS), whereas that not inhibited by EGTA incubation was considered inducible NOS.

Detection of iNOS mRNA Expression

Colonic iNOS mRNA expression was examined using RT-PCR as outlined in chapter 2. Briefly, whole thickness colonic tissues from rats killed 6 weeks after induction of colitis were placed in 2 ml TRIzol reagent (GIBCO BRL, Gaithersburg, MD). Total RNA was isolated from each sample, and RT-PCR performed as previously described (chapter 2). The iNOS and GAPDH products were made using primers based on published sequences (see chapter 2).

Drug Treatments

Dexamethasone was tested for its ability to reverse the hypo-responsiveness of the epithelium following colitis. Five weeks after induction of colitis, dexamethasone (1 mg/kg; i.p.) was administered once-daily for 4 days. Rats were killed 48 h following the final dose of dexamethasone and the colon was removed for electrolyte transport studies or for RT-PCR and MPO analyses.

Experiments examining the role of nitric oxide derived from iNOS were performed *in vitro* using the Ussing chamber technique. After a 20-min equilibration period, a selective iNOS inhibitor (L-N_6-(1-iminoethyl) lysine; L-NIL, 3 μM), was added serosally. Isc responses to EFS, IBMX, and carbachol were determined after a 10-min incubation period. This concentration of L-NIL was selected because it has been reported to significantly decrease iNOS activity without affecting cNOS activity (198).

Materials

L-[^{14}C]arginine was purchased from Dupont NEN Research Products (Mississauga, ON, Canada); 50W-X8 Dowex resin from BioRad (Hercules, CA); dexamethasone, IBMX, carbachol, EGTA, L-valine, and L-arginine from Sigma Chemical Co. (St. Louis, MO). iNOS and GAPDH primers were synthesized by University Core DNA Services (Calgary, AB, Canada).

Statistical Analysis

With the exception of the iNOS activity data, comparisons among groups of data were made using a one way analysis of variance followed by a Student-Newman-Keuls test. The iNOS activity data was analyzed using the Mann-Whitney non-parameteric test.

4.3 Results

Colonic Injury and Inflammation

As shown in Figure 4.2, the severe inflammation that was apparent 3 days after intracolonic administration of TNBS/ethanol or ethanol alone had resolved within 6 wk to a point where the colonic damage score was not significantly different from that of age-matched, control rats. Similarly, the colons of TNBS- and ethanol-treated rats were not histologically different from controls (Figure 4.1). Likewise, colonic MPO activity in rats treated with TNBS-ethanol or ethanol alone had recovered to the levels seen in controls (Figure 4.2). In the rats killed 9 or 12 wk after TNBS administration, the colon was macroscopically (damage scores: 0.5 ± 0.2 in controls vs 1.3 ± 0.5 in rats 9 wk and 1.2 ± 0.6 in rats 12 wk after TNBS administration) indistinguishable from controls.

Epithelial Permeability

Measurement of unidirectional fluxes of ^{51}Cr-EDTA, as summarized in Table 4.1, demonstrated that there were no significant differences in the flux of this molecule in either direction between the rats treated with TNBS and the controls at either time point examined. These data, taken together with the lack of significant differences in conductance of the epithelium in these two groups (Table 4.1), strongly suggest that there was no difference between the groups in terms of epithelial permeability.

Electrolyte Transport

Basal transport parameters (Isc, PD, and conductance) of the distal colon taken from rats killed 6 wk after TNBS administration were not different from controls (conductance data shown in Table 4.1). Tissue from rats killed 6 wk after intracolonic

administration of 50% ethanol also exhibited basal transport parameters not significantly different from those of controls. Furthermore, basal colonic Na^+ and Cl^- ion fluxes or rats receiving TNBS 6 wk earlier were not significantly different from those of control rats (Table 4.2). However, the distal colon from rats killed 6 wk after TNBS administration had significantly depressed I_{sc} responses to EFS, IBMX, and carbachol (Figure 4.3). Moreover, at both 9 and 12 wk post-TNBS administration, the I_{sc} responses to EFS and carbachol remained significantly depressed, while the response to IBMX had recovered to that observed in controls. The hypo-responsiveness observed after induction of colitis with TNBS was not a non-specific response to tissue injury, as tissues taken from rats 6 wk after intracolonic administration of 50% ethanol, which caused extensive colonic injury (Figure 4.2), responded to EFS and carbachol stimulation in a comparable manner to that observed in controls (e.g., response to EFS: 50% ethanol: 21.6 ± 3.6 vs. 22.7 ± 6.1 $\mu A/cm^2$ in controls; response to IBMX: 50% ethanol: 71.7 ± 15.8 vs. 68.8 ± 10.7 $\mu A/cm^2$ in controls; response to carbachol: 50% ethanol: 28.9 ± 5.4 vs. 31.2 ± 7.3 $\mu A/cm^2$ in controls). The hypo-responsiveness observed after induction of colitis was also not likely to be a result of neuronal changes, as tissues from both control and TNBS-treated rats pre-incubated with the sodium channel blocker tetrodotoxin (TTX; 1 μM) did not exhibit altered Isc responses to IBMX and carbachol compared with tissues pre-incubated with vehicle (Table 4.3). The reason there was a TTX-resistant response to EFS is not known. However, it has previously been suggested that there are TTX-insensitive neurons in the myenteric plexus of the gut (199,200). Therefore, it is possible that some of these neurons are activated upon EFS-stimulation and are responsible for the TTX-insensitive response.

NOS Activity and mRNA Expression

In control rats, iNOS activity in the colonic tissue was very low or undetectable. In rats killed 6 wk after induction of colitis, iNOS activity was quite variable, but significantly ($P<0.05$) elevated above that in the controls (Figure 4.4). These rats also exhibited significantly ($P<0.01$) elevated iNOS mRNA expression in the distal colon compared to controls (Figure 4.5). Expression of iNOS mRNA was not observed in rats killed 6 wk after intracolonic 50% ethanol.

Effects of Dexamethasone

Treatment with dexamethasone 5 wk after intracolonic TNBS administration had no effect on basal I_{sc}. The I_{sc} response to IBMX in rats treated with dexamethasone was not significantly different from control rats and significantly elevated compared with TNBS-treated rats receiving saline. Interestingly, the I_{sc} response to EFS after dexamethasone treatment was not significantly different from that in either untreated controls or TNBS-treated rats receiving saline. In contrast, the I_{sc} response to carbachol after dexamethasone treatment was not significantly different from TNBS-treated rats receiving saline and remained significantly depressed compared with control rats (Fig. 4.6). Dexamethasone treatment had no effect on the basal I_{sc} of TNBS-treated rats and had no effect on transport responses in tissues from control rats (e.g., response to EFS: rats treated with vehicle: 25.8 ± 4.8 vs. 31.2 ± 4.9 $\mu A/cm^2$ in rats treated with dexamethasone; response to IBMX: rats treated with vehicle: 80.8 ± 15.8 vs. 83.1 ± 8.8 $\mu A/cm^2$ in rats treated with dexamethasone; response to carbachol: rats treated with vehicle: 41.0 ± 9.1 vs. 47.7 ± 7.9 $\mu A/cm^2$ in rats treated with dexamethasone). Treatment

with dexamethasone abolished the increase in iNOS mRNA expression caused by intracolonic TNBS treatment 6 wk earlier (Figure 4.5).

Effects of L-NIL

In vitro exposure to L-NIL resulted in a normalization of the I_{sc} responses to EFS and IBMX, but not to carbachol, in tissues from rats killed 6 wk after TNBS administration (Figure 4.7). L-NIL had no effect on the responses to any of the three stimuli in tissues from controls.

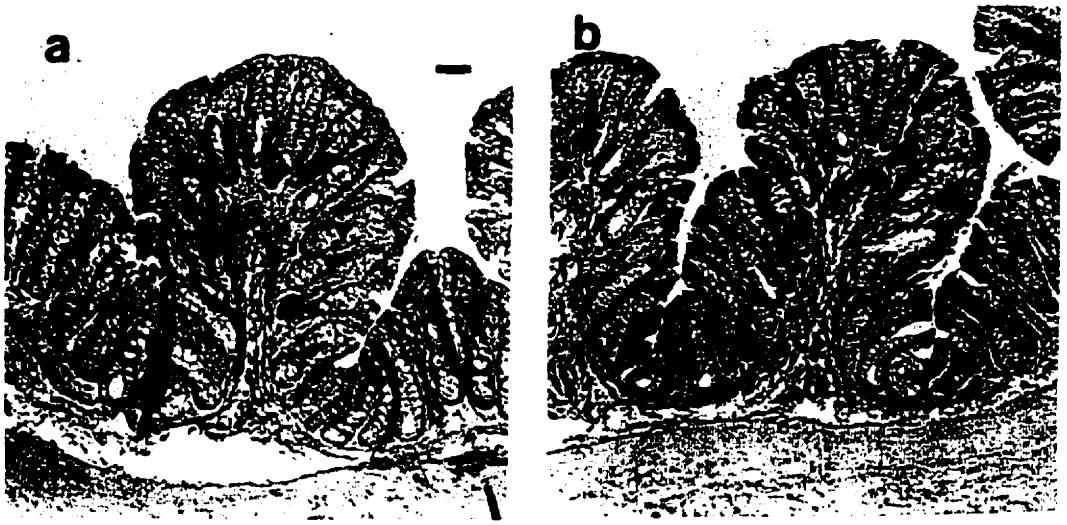

Figure 4.1. Microscopic appearance of the distal colon 6 wk after intracolonic TNBS administration. Distal colonic tissues from control (A) and TNBS-treated (B) rats are shown. The latter were killed 6 wk after intracolonic administration of TNBS. Tissue architecture is normal and the level of immunocytes within the lamina propria are comparable to those seen in age-matched control rats. The bar at top of panel A represents 100 μm.

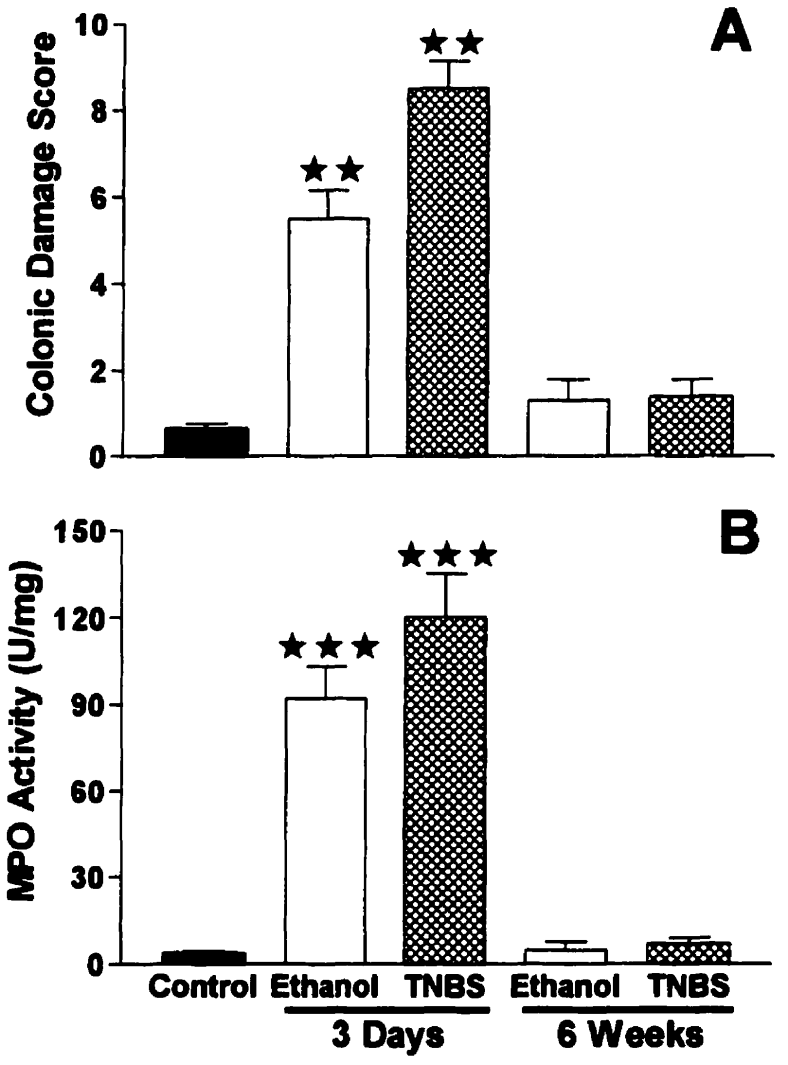

Figure 4.2. Colonic macroscopic damage scores (panel A) and myeloperoxidase (MPO) activity (panel B) 3 days and 6 weeks after intracolonic administration of ethanol or TNBS/ethanol. Each bar represents the mean ± S.E.M., with 5-16 rats per group. **P<0.01, ***P<0.001 compared to the control group.

Table 4.1. Colonic epithelial permeability 6 and 9 weeks after induction of colitis.

	Control	TNBS
6 Weeks Post-TNBS		
mucosal to serosal EDTA flux[a]	1.69 ± 0.22	2.68 ± 0.51
serosal to mucosal EDTA flux[a]	2.29 ± 0.42	3.57 ± 1.45
conductance[b]	32 ± 4	33 ± 6
9 Weeks Post-TNBS		
mucosal to serosal EDTA flux[a]	2.72 ± 0.53	3.12 ± 0.19
serosal to mucosal EDTA flux[a]	1.65 ± 0.15	2.47 ± 0.53
conductance[b]	36 ± 4	29 ± 4

[a]Results for flux are expressed as the amount of ^{51}Cr-EDTA that moved across the epithelium as a percentage ($x10^{-3}$) of the amount added to one side of the chamber (mean ± SEM). [b]Results for conductance are expressed as mSiemens/cm^2 (mean ± SEM). Each group consisted 5 or 6 rats. There were no significant differences among the groups. Control rats were untreated, but were age-matched to the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-treated rats.

Table 4.2. Colonic epithelial sodium and chloride fluxes 6 weeks after induction of colitis.

Group	J_{Na^+}			J_{Cl^-}		
	m→s	s→m	Net	m→s	s→m	Net
Control	5.39 ± 0.42	4.35 ± 0.33	0.72 ± 0.59	7.25 ± 0.44	6.30 ± 0.32	0.95 ± 0.51
TNBS	4.51 ± 0.48	3.89 ± 0.53	0.61 ± 0.75	6.48 ± 0.44	5.63 ± 0.38	0.86 ± 0.64

Na^+ and Cl^- flux values are expressed in $\mu Eq \bullet cm^{-2} \bullet h^{-1}$ (mean ± SEM; n≥ 5). m → s denotes mucosal-to-serosal flux, whereas s → m denotes serosal-to-mucosal flux. Control rats were untreated, but were age-matched to the rats treated intracolonically with 2,4,6-trinitrobenzenesulfonic acid (TNBS) 6 wk earlier.

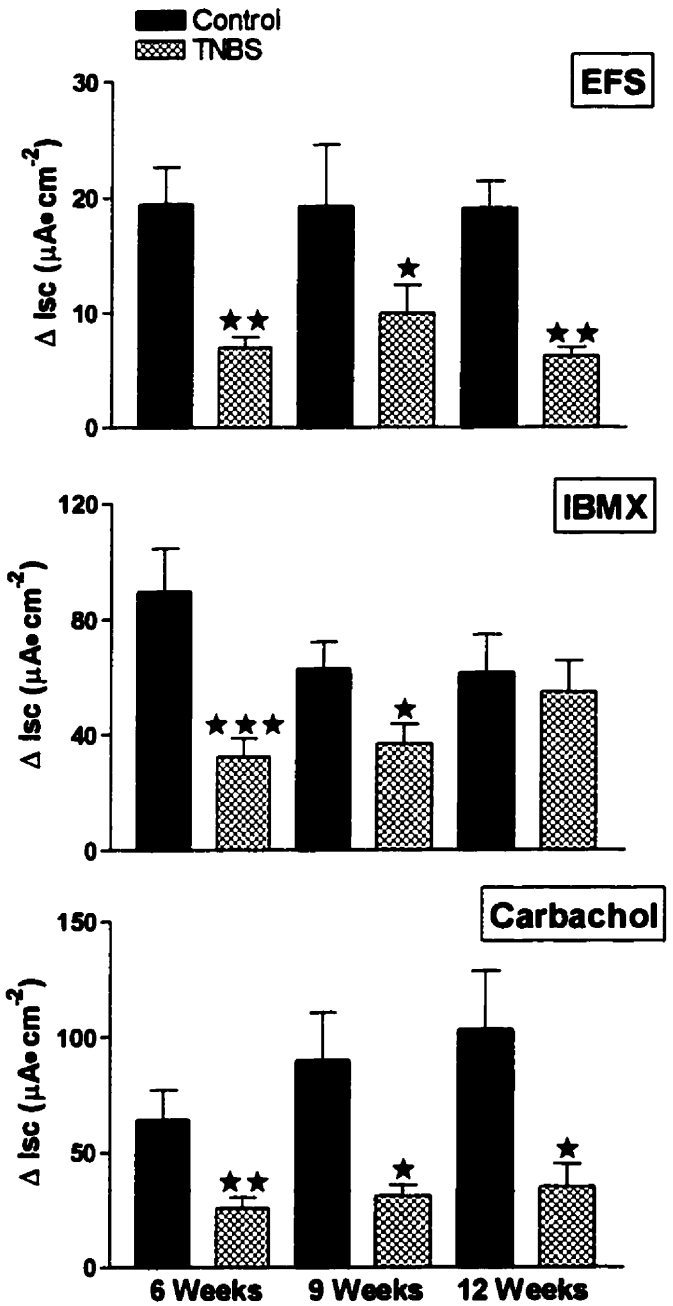

Figure 4.3. Epithelial short circuit current (I_{sc}) responses to electrical field stimulation (EFS), IBMX, and carbachol 6, 9, or 12 weeks after induction of colitis. Each bar represents the mean ± S.E.M., with 5 to 7 rats per group. *P<0.05, **P<0.01 ***P<0.001 compared to the control group at the same time point.

Table 4.3. Effects of Tetrodotoxin on colonic epithelial I_{sc} responses.

Group	EFS	IBMX	Carbachol
Control-Vehicle	100.0 ± 10.8	99.9 ± 32.5	100.0 ± 26.3
Control-TTX	25.8 ± 7.3***	104.2 ± 24.7	124.4 ± 32.7
TNBS-Vehicle	57.9 ± 12.3**	32.7 ± 12.8*	33.3 ± 13.7*
TNBS-TTX	9.5 ± 4.6***	25.4 ± 9.1*	32.8 ± 9.7*

Results are expressed as % control-vehicle short circuit current (I_{sc}) response to electrical field stimulation (EFS), IBMX, and carbachol (mean ± SEM; n≥5). *P<0.05, **P<0.01, ***P<0.001 compared with control-vehicle response to each stimulant. Control rats were untreated, but were age-matched to the rats treated intracolonically with 2,4,6-trinitrobenzenesulfonic acid (TNBS) 6 weeks earlier.

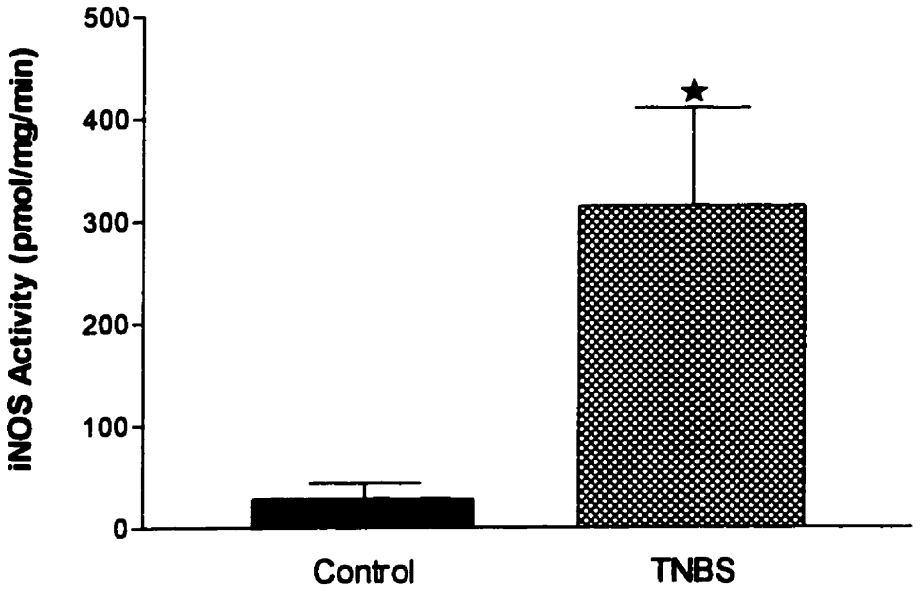

Figure 4.4. iNOS enzyme activity in rats 6 weeks after induction of colitis. Each bar represents the mean ± S.E.M., with 6 rats in the control group and 8 rats in the TNBS group. $^{*}P<0.05$ compared to the control group.

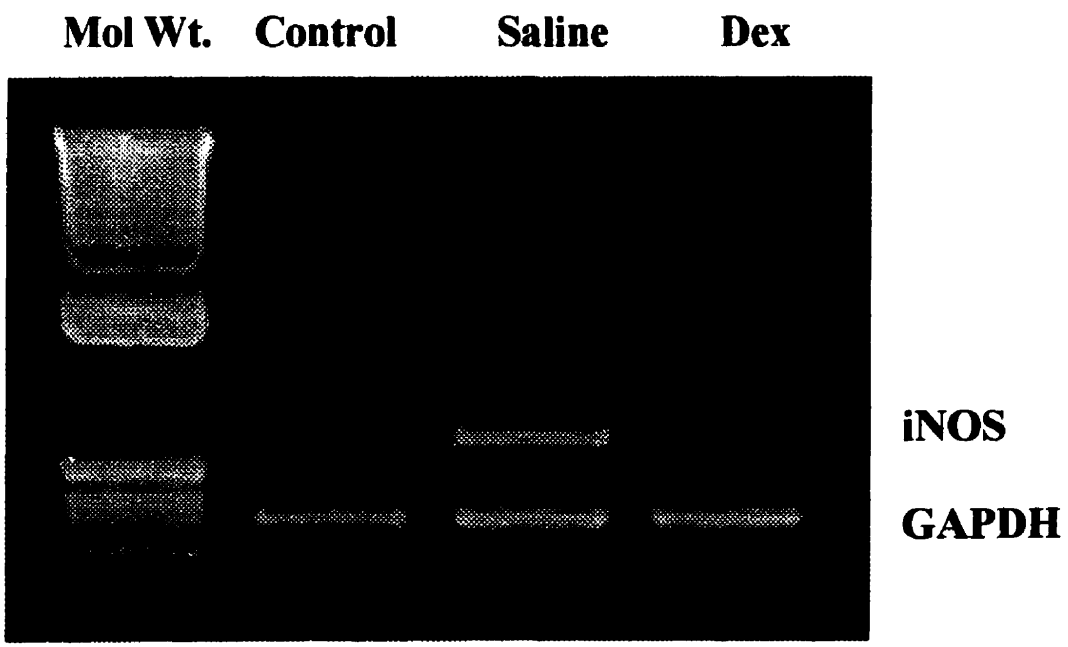

Figure 4.5. iNOS mRNA expression in the distal colon: effects of dexamethasone (1 mg/kg; once-daily for 4 days) five weeks after intracolonic TNBS administration. Representative gel of iNOS expression as determined by RT-PCR of tissue from control rats, TNBS-treated rats receiving saline, and TNBS-treated rats receiving dexamethasone. MW, molecular weight markers. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

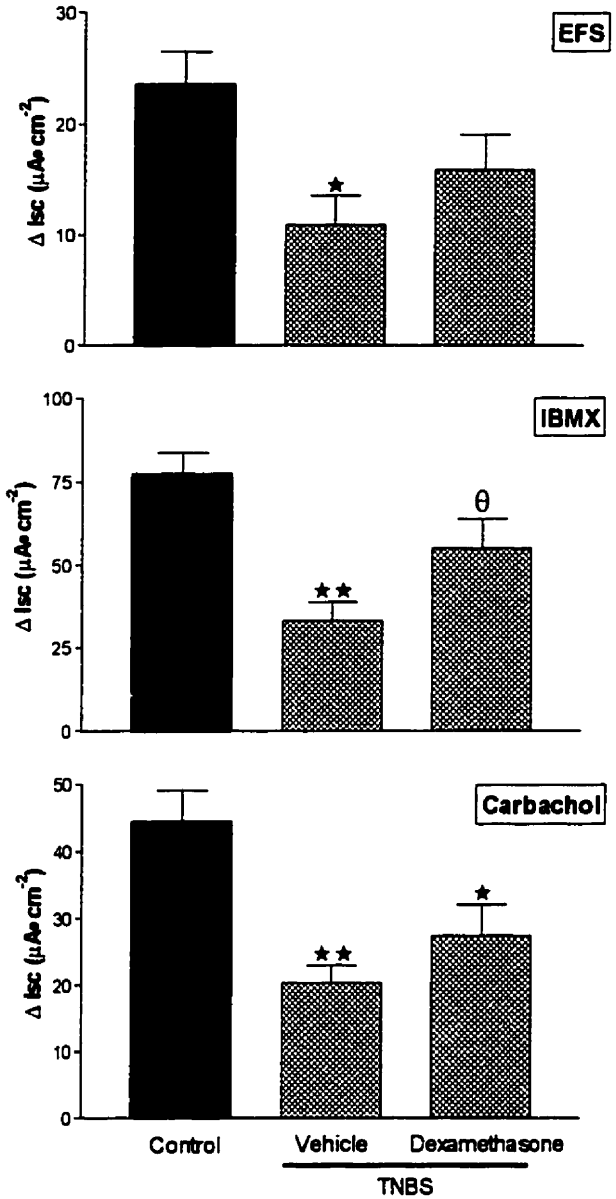

Figure 4.6. Effects of dexamethasone (1 mg/kg; once-daily for 4 days) on epithelial short circuit current (I_{sc}) responses to EFS, IBMX and carbachol, five weeks after intracolonic TNBS administration. Each bar represents the mean ± S.E.M., with 6 to 10 rats per group. *P<0.05, **P<0.01 compared to the control group response to the same secretagogue. $^{\theta}$P<0.05 compared to control rats that received TNBS and five weeks later received vehicle (saline) in place of dexamethasone.

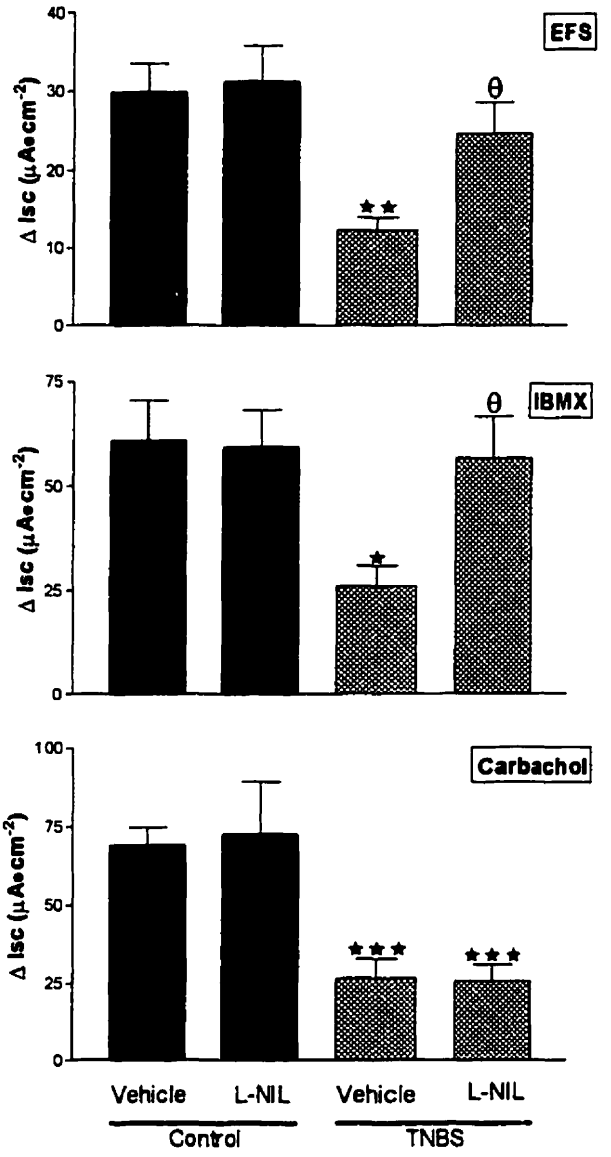

Figure 4.7. Colonic epithelial short circuit current (I_{sc}) responses to EFS, IBMX, and carbachol: effects of a selective iNOS inhibitor (L-NIL; 3 μM). Each bar represents the mean ± S.E.M., with 5 to 10 rats per group. *P<0.05, **P<0.01, and ***P<0.001 compared to the control group response to the same secretagogue. $^{\theta}$P <0.05 compared to rats that received TNBS intracolonically and five weeks later received vehicle (distilled water) in place of L-NIL.

4.4 Discussion

An acute bout of colitis is associated with substantial alterations in epithelial function, including a profound hypo-responsiveness to secretagogues (138). Few studies have investigated intestinal function during the post-inflammatory period. In the present study we demonstrated that epithelial hypo-responsiveness to a variety of secretagogues extended for up to 12 wk after induction of the inflammatory response.

Exposure of the rat colon to TNBS in 50% ethanol caused an acute inflammatory response characterized by mucosal ulceration, bowel wall thickening, and adhesion formation, as previously described (201). Furthermore, there was an acute granulocyte influx as shown histologically and by the significant increase in colonic MPO activity. By 6 wk after the initial administration of TNBS the colonic damage score and MPO had returned to the levels of control rats. Tissue conductance and the serosal-to-mucosal flux of ^{51}Cr-EDTA were similar to those observed in control rats, indicating that there was no persistent permeability defect after resolution of the acute phase of inflammation. Despite the fact that, on the basis of these parameters, the colon was not inflamed at 6 wk post-TNBS, segments of this tissue mounted in Ussing chambers exhibited significant hypo-responsiveness to EFS or to serosal application of IBMX and carbachol. Furthermore, compared with control rats, iNOS expression and iNOS activity were increased 6 wk post-TNBS. The effects on responsiveness to secretagogues and on iNOS expression and activity were specific to the inflammatory response and were not simply a function of generalized tissue injury. Intracolonic administration of 50% ethanol alone

caused an acute colonic damage and granulocyte infiltration, but did not cause epithelial hyporesponsiveness after 6 wk. Furthermore, iNOS mRNA expression was not elevated 6 wk after intracolonic 50% ethanol.

Prolonged epithelial dysfunction after acute inflammation may be due to structural changes that persist after resolution of the acute phase of the inflammatory response or to ongoing mediator release. Although we have not investigated the former possibility in the present study, the fact that L-NIL reversed the hypo-responsiveness suggests that a "structural change" did not account for the hypo-responsiveness, at least in the case of EFS- and IBMX-evoked secretion. The role of NO in epithelial function is controversial. Several studies have shown that *in vitro* application of NO-donating compounds to intestinal tissue in Ussing chambers stimulated chloride secretion (118,119,122). Furthermore, endogenous NO has been implicated in the diarrhea caused by administration of castor oil (202) or magnesium sulfate (203), although whether these were secretory or malabsorptive forms of diarrhea was not determined. In contrast to these studies, it has been shown that endogenous NO may be antisecretory. Inhibition of NOS activity exacerbated cholera toxin- and prostaglandin-induced secretion in rats (120,121), perhaps through effects on enteric neurons. Our data are in keeping with these latter observations in that chronic elevation of iNOS activity may exert a potent antisecretory tone on the colonic epithelium. iNOS is expressed during periods of active inflammation in animal models of colitis (163) and in inflammatory bowel disease (204). The evidence for iNOS expression after resolution of acute inflammation is less clear. We have shown that elevation of iNOS mRNA expression and iNOS activity persist for at least 6 wk post-TNBS. Inhibition of iNOS mRNA expression by dexamethasone and

of iNOS activity using the selective iNOS inhibitor L-NIL resulted in responses to EFS and IBMX that were similar to those observed in control rats. Dexamethasone inhibits iNOS mRNA expression by increasing I-κB expression and decreasing nuclear factor-κB (205). In addition, dexamethasone has been shown to decrease mast cell reactivity and number (206,207). Mast cells can affect epithelial transport through their release of histamine and 5-HT which stimulate secretion. Therefore, it is forseeable that inhibition of a mast cell response by dexamethasone might explain why there was only a partial reversal of epithelial hypo-responsiveness by dexamethasone, whereas a more dramatic effect by L-NIL. L-NIL is a selective inhibitor of iNOS activity in mouse colon at the concentration used in this study (124). Interestingly, inhibition of NOS expression and activity by L-NIL did not reverse the hypo-responsiveness to carbachol. This suggests that iNOS-derived NO selectively inhibits cAMP-mediated secretion. NO has been shown in other systems to reduce intracellular cAMP (208). Neurally evoked chloride secretion is a composite response to neurotransmitter release from cholinergic and vasoactive intestinal peptidergic (VIPergic) submucosal secretomotor neurons, implying that both Ca^{2+}-dependent (acetylcholine) and cAMP-dependent (VIP) mechanisms are involved in the response to EFS (209). Furthermore, these data indicate that the hypo-responsiveness to carbachol is mediated by an undetermined, NO-independent mechanism.

The prolonged hypo-responsiveness to EFS, IBMX and carbachol may have important implications for host defense against invading pathogens and antigens. Secretion of chloride ions and water is characteristic of enterocytes near the base of the crypt region and is thought to "flush" bacteria, bacterial products, and antigens away

from the epithelium (210). The intestinal neuroimmune system links this secretory function to motility to effectively remove harmful microorganisms and antigens from the intestinal lumen (191). An inability to respond to these luminal stimuli would compromise this defense mechanism, potentially leading to translocation of bacteria, toxins, or antigens across the epithelium, with subsequent inflammation or endotoxemia. Our data are consistent with the hypothesis that chronic synthesis of NO through iNOS may also predispose the intestine to recurrent bouts of inflammation and injury.

Chapter 5

IN VIVO COLONIC EPITHELIAL DYSFUNCTION AND BACTERIAL TRANSLOCATION FOLLOWING A BOUT OF INFLAMMATION

5.1. Introduction

Inflammatory bowel disease (IBD) is characterized by periods of remission and relapse. Little is known regarding the factors that trigger relapses and there are few therapeutic options for prolonging remission. One of the most accepted theories for the pathogenesis of IBD is that an infectious agent triggers the initial inflammatory event in genetically susceptible individuals (211). An impaired immune response then results in a failure of the normal down-regulation of the inflammatory response, leading to excessive tissue injury. Thus far, however, a specific infectious agent responsible for triggering IBD has not been identified. Another possibility is that a genetically susceptible individual who suffers a bout of inflammation, perhaps due to an infection, becomes more susceptible to recurrent inflammation induced by the commensal flora. For example, it is possible that a bout of colonic inflammation causes long-term changes in the ability of the tissue to resist infection, thereby predisposing the individual to recurrent bouts of infection and inflammation. If that individual were also genetically predisposed in some way, such as an impaired mucosal immune response, these recurrent bouts of inflammation would lead to extensive tissue destruction and the generation of the symptoms that typify IBD (pain, bleeding, diarrhea, etc.).

The intestinal epithelium plays an essential role in host defence by preventing the entry of bacteria and other potentially harmful substances into the lamina propria. The secretion of water and mucus by the epithelium assists in preventing the adherence and invasion of microbes, as well as diluting luminal toxins. Impairment of epithelial barrier function and/or secretion could therefore result in an increased susceptibility to infection and inflammation. Inflammation of the gastrointestinal mucosa in various species is associated with the release of a number of soluble mediators that have the capacity to influence intestinal epithelial function (57,212,213). For example, interleukin (IL)-4 and prostaglandin D_2 have been shown to decrease epithelial chloride secretion, while IL-4, IL-13, and interferon-γ have been shown to diminish epithelial barrier function (9,43,47,,53). Nitric oxide (NO) has also been shown to modulate epithelial barrier function and secretion, but its role is somewhat controversial. Our own findings have been in agreement with an anti-secretory role for NO (chapter 4)(214). It has previously been demonstrated, in various species, that inflammation of the colon is accompanied by an acute reduction in epithelial responsiveness to secretagogues (124,138,192,196). We have further shown that in the rat such changes are long-lasting, at least when assessed *in vitro* (chapter 4). The epithelial dysfunction observed was attributed, at least in part, to the release of NO from the inducible isoform of nitric oxide synthase (iNOS) (chapter 4). While epithelial secretion was impaired for a prolonged period of time after a bout of colitis, permeability of the colonic epithelium (*in vitro*) to ^{51}Cr-EDTA was normal (chapter 4).

In the present study, the possibility that colonic epithelial secretory dysfunction occurs *in vivo* in response to clinically relevant stimuli (bacterial toxin, antigen), and that

it persists for many weeks after resolution of a bout of colonic inflammation have been examined. Moreover, the hypothesis that small intestinal inflammation could produce prolonged alterations in colonic epithelial secretion has been tested. Finally, whether or not the functional consequences of altered secretory function following a bout of colitis or enteritis might include an increase in bacterial colonization of the colon, and bacterial and/or neutrophil translocation across the epithelium has been examined.

5.2. Methods & Materials

Induction of colonic inflammation

Colitis was induced in male Wistar rats by intracolonic administration of TNBS dissolved in 0.5 ml of 50% ethanol (vol/vol), as described in chapter 2 (151). A group of age-matched rats, which were left untreated during the course of the study, served as controls. In all experiments the minimum sample size per group was 5.

Colonic fluid secretion was measured 6 wk after TNBS administration and in controls. In separate groups of rats, permeability of the gastrointestinal tract to ^{51}-Cr-EDTA was assessed (72 h and 6 weeks post-TNBS). The measurement of permeability to ^{51}Cr-EDTA involved placing rats in individual metabolic cages and collecting urine over a period of 12 hours. The rats (n=6 per group) were given 100 μCi of ^{51}Cr-EDTA (33 Ci/mmol) orally, after which the collection of urine was initiated. The urine samples were then counted in a gamma spectrometer and the fractional excretion of the ^{51}Cr-EDTA was calculated.

Induction of small intestinal inflammation

Sprague-Dawley rats were infected with ~3000 stage-III *Nippostrongylus brasiliensis* larvae, as previously described (215). A group of age-matched rats served as controls. In this model, infection of the small intestine (primarily jejunum) occurs 60-120 h after the administration of the larvae. The infection and the associated small intestinal inflammation is typically cleared by 18 to 21 days after infection (216,217,218). Tissue for histological analysis was taken from the small intestine and

colon 52 days after administration of the larvae. A separate group of rats was used for measurement of colonic fluid secretion (see below).

Samples of the small intestine and colon of control rats, and of rats infected with *N. brasiliensis* 52 days earlier, were fixed in Carnoy's solution and processed using routine histological techniques. Sections (7 μm) were mounted on glass slides and stained with hematoxylin and eosin. The sections were then assessed for degree of inflammation by an observer who was unaware of the treatments the rats had received.

Carrageenan-airpouch model

In order to determine if extraintestinal inflammation would affect colonic fluid secretion, the carrageenan airpouch model was used. An airpouch was induced as previously described (219). Briefly, 20 ml of air was injected subcutaneously on the back of the rat on the first day. Two and five days later, another 10 ml of air was injected at the same site. Twenty-four hours later, carrageenan (2 ml of a 1 % w/v solution in sterile saline) was injected into the air pouch. Six hours following carrageenan injection, colonic fluid secretion was measured. A separate group of rats in which airpouches were not induced by carageenan served as controls.

In vivo colonic fluid secretion

Rats were fasted for 24 h, anesthetized with 20% urethane (6 ml/kg) and a laparotomy was performed. Colonic loops (~5 cm) were formed using suture to tie off each end. Care was taken not to disrupt the blood and nerve supplies to the gut. A cannula was inserted into one of the ends of each loop. Clostridium difficile-derived toxin A (10 μg) dissolved in 2 ml of Tyrode's buffer (133 mM NaCl, 4.7 mM KCl, 1.84 mM $MgSO_4$, 20 mM $NaHCO_3$; pH 7.4) was injected into the colonic loops. In control rats, loops were

injected with Tyrode's buffer alone. In other experiments, 2 ml of Tyrode's buffer containing the phosphodiesterase inhibitor IBMX (300 μM) or vehicle were injected instead of toxin A. IBMX was used as another agonist because we have previously found that the epithelium of the previously inflamed colon is hypo-responsive to this secretagogue *in vitro* (220). After 2 h (in the case of IBMX) and after 3 h (in the case of toxin A), the loops were carefully excised and the volume of fluid recovered from the loop was recorded. The time allowed for fluid accumulation in the colon was based on preliminary experiments showing that peak fluid accumulation occurred 2 h after IBMX stimulation and 3 h after toxin A stimulation (Figure 5.1). Any changes in the volume of fluid during this period would reflect the difference between secretion and absorption. However, it has been well established that accumulation of fluid in the colon following administration of C. difficile toxin A or IBMX is due to active secretion, with little effect of these agents on absorption (221,221-224). Thus, in the present study we have used the volume of fluid recovered from the colonic loops as an index of secretion. The dry weight of the tissue was determined after desiccation at 60°C for 24 h. Changes in fluid accumulation after administration of C. difficile toxin A could also be attributable to epithelial damage caused by this agent. To test this hypothesis, we used leakage of the lysosomal enzyme lactate dehydrogenase into the lumen as an index of cellular disruption. A spectrophotometric assay for lactate dehydrogenase was performed on samples of the luminal fluid from vehicle- and toxin A-treated rats according to a previously described protocol (225).

Role of enteric nerves

In order to examine the role of nerves in the epithelial hypo-responsiveness observed after colitis, the effects of the sodium channel blocker tetrodotoxin (TTX), were examined. *C. difficile* toxin A has previously been shown to elicit its fluid secretory effect in the intestine via a neurally-mediated mechanism (226). Therefore, TTX (10 μM), at a concentration that significantly reduced colonic fluid secretion stimulated by toxin A (7.3 ± 1.0 μl/ mg tissue in TTX-treated rats vs 13.2 ± 2.0 μl/ mg tissue in vehicle-treated rats), or vehicle was added to Tyrode's buffer containing IBMX. The IBMX-stimulated fluid secretory responses in untreated control rats and rats 6 wk after induction of colitis were then compared following TTX or vehicle treatment.

Role of iNOS

In order to determine the contribution of iNOS to any changes in colonic fluid secretion induced by a prior bout of colitis, rats were treated with L-N^G-iminoethyl-lysine (L-NIL; 3 mg/kg; i.p.) or vehicle (saline) 10 min prior to measurement of colonic fluid secretion. This dose of L-NIL was chosen because it has previously been shown to inhibit iNOS selectively; that is, it reversed an endotoxin-induced decrease in blood pressure, but had no effect on the blood pressure of normal rats (227).

Mucus secretion

Rats (n=5-9 per group) that had been fasted for 24 h were given ^{3}H-glucosamine (20 μCi) intraperitoneally in order to quantify mucus secretion (31). To examine basal mucus secretion, the rats were killed 3 h after injection of the labeled glucosamine. The colon was excised and the fecal contents removed prior to gentle scraping of the luminal surface with a glass slide. These samples were subsequently processed for quantification of the labeled glycoproteins (see below). To examine stimulated mucus secretion, rats

were anesthetized with sodium pentobarbital (65 mg/kg i.p.) 3 h after injection of the labeled glucosamine. Colonic loops (~2 cm long) were formed using suture to tie off each end and a cannula was inserted into one end of the loop. Carbachol (300 μl of a 10 mM solution) was instilled into the colonic loop and left in place for 30 min. At the end of this period, the luminal surface of the loop was gently scraped with a glass slide, as above. The samples collected in both sets of experiments (basal and carbachol-stimulated) were placed in 5 ml of PBS and were then vortex mixed. After centrifugation at 1000 *g* for 10 min, the supernatant was mixed with an equal volume of 10% trichloroacetic acid/1% phosphotungstic acid for 30 min at 4°C in order to precipitate the glycoproteins. Following centrifugation (2000 *g*; 10 min), each pellet was mixed with 4 ml of scintillation cocktail (Ecolite, ICN Biomedicals, Irvine, CA) and counted in a liquid scintillation counter. The amount of radiolabeled glycoprotein secreted was then calculated as cpm/mg of protein/cm of colon.

Antigen-induced colonic secretion in vitro

In experiments examining colonic epithelial responses to antigen, Hooded-Listar rats were sensitized by intraperitoneal injection of a 1.0 ml solution containing 10 μg of ovalbumin and 1.0 mg of aluminum hydroxide adjuvant, prepared as previously described (228). Adjuvant alone was injected in control animals. Two days later, sensitized and non-sensitized control rats were divided into two groups. Colitis was induced in one group of sensitized and one group of control rats by intracolonic administration of TNBS, as described above, while the remaining rats were left without any further treatment. Following a six-week time period to allow for the colonic inflammation to resolve, blood was taken from each rat for measurement of ovalbumin-

specific reaginic antibody titres by the passive cutaneous anaphylaxis (PCA) reaction (229). Rats were considered sensitized if the serum diluted to ≤1:32 caused a positive PCA reaction.

The distal colon from sensitized and control rats was excised and gently washed of fecal contents. The colon was stripped of its external muscle by blunt dissection and a segment of mucosa from each rat was mounted in Ussing-type diffusion chambers (Navicyte, Sparks, NV). Experiments were carried out as described in detail previously (chapter 2). Briefly, after a 20 min equilibration period, the short-circuit current (I_{sc}) response to serosally applied ovalbumin (100 μg/ml) was measured. This concentration of ovalbumin was chosen because it has previously been shown to cause a maximal Isc response in the rat (59). Following the completion of these experiments, luminal fluid from the Ussing chambers was taken for measurement of rat mast cell protease (RMCP)-II concentrations. The release of this protease is a sensitive index of mucosal mast cell degranulation (230). Briefly, this entailed protein precipitation by addition of an equal volume of 10% TCA to the luminal fluid, followed by centrifugation at 12,000 *g* for 30 minutes at 4°C. Protein samples from each tissue were then run on a 12.5% sodium dodecyl-sulfate polyacrylamide gel and RMCP-II levels measured by Western blot detection using a rabbit anti-RMCP-II antibody.

BrdU staining of proliferating cells

The detection of proliferating cells in the colonic epithelium was compared among three groups of rats. One group of rats was treated intracolonically with TNBS, another was treated intracolonically with 50% ethanol, and the third group was left untreated. Six wk later all the rats were dosed with 5'-bromo-2'-deoxyuridine (BrdU;

150 mg/kg; i.p.). One hour later the rats were anesthetized with pentobarbitol (6.5 mg/100 g) and perfused with 10% formalin. The colon was removed and cryostat sections (10 μm) were mounted on chromium phosphate-gelatin coated slides. Immunohistochemistry was then performed using anti-BrdU and goat anti-donkey CY3 secondary antibody (Jackson Laboratories). Briefly, tissues were washed in Tris buffer containing 0.1% Triton X-100 followed by a 30 min incubation in 1 M HCl. After two washes in Tris buffer, the slides were incubated with primary antibody (1:500 dilution of anti-BrdU) overnight at 4°C. Following another series of washes, the slides were incubated with secondary antibody (1:1000 dilution of donkey anti-rat) at room temperature for 4 hours. The slides were examined using a fluorescence microscope and a CY3 filter by an observer who was unaware of the group that each slide was from.

Bacterial translocation and colonic bacterial levels

Rats were killed by cervical dislocation six weeks after TNBS administration or 52 days after infection with *N. brasiliensis*. Controls were killed at the same time. The mesenteric lymph node complex and spleen were removed from each rat using sterile technique, as previously described (231). Each tissue was then weighed and homogenized in 5 ml of sterile PBS. Two serial 10-fold dilutions were made of each sample and 0.1 ml aliquots of each undiluted and diluted sample were plated onto MacConkey and blood agar plates. Following incubation at 37°C for 48 h, bacterial counts were performed as previously described (231). Any mesenteric lymph node or splenic tissue having more than 20 colony forming units (CFU) per gram of tissue was classified as positive for translocation of bacteria.

To determine total colonic bacterial levels (luminal plus adherent), a segment of colon including luminal contents was placed in 5 ml of sterile PBS. To determine the number of bacteria adherent to the colonic tissue, a second tissue sample (excluding the luminal contents) was placed in 5 ml of PBS and processed as previously described by Deitch *et al.* (232). The samples were then vortexed vigorously for 5 seconds, then transferred to tubes containing sterile PBS and vortexed again. This was repeated a total of four times. Finally, both tissue samples (for total and for adherent bacteria) were weighed, homogenized, and diluted prior to plating 0.1 ml of each dilution on MacConkey and blood agar plates. Following incubation at 37°C for 48 h, bacterial counts were performed.

To examine the contribution of iNOS-derived NO to changes in bacterial colonization and translocation after a bout of colitis, rats treated 6 weeks earlier with TNBS (n=6) were treated twice-daily with L-NIL (3 mg/kg; i.p.) for 7 days, beginning 5 weeks after intracolonic administration of TNBS. A second group of rats (n=6) was treated with vehicle (saline) instead of L-NIL.

Measurement of fecal excretion of granulocyte marker protein (GMP)

In separate rats, feces were collected over a 24-hour period and fecal excretion of granulocyte marker protein (GMP) was measured by ELISA as previously described (233). Briefly, fecal samples were homogenized for 20-30 sec on ice in 4 ml of buffer (containing 0.25 mM Thimerosal and 10 mM $CaCl_2$; pH 8.4) per gram of feces. The samples were then centrifuged at 4°C for 20 min at 10,000 *g* and the supernatants collected and stored at –70°C until analysis was performed. GMP was quantified by enzyme-lined immunosorbent assay as previously described (234). GMP is an iron-

binding protein found in neutrophils (235). Therefore, this was used as an index of transepithelial neutrophil migration.

Statistical analysis

All data are expressed as the mean ± SEM. Comparisons among groups of data were made using a one-way ANOVA followed by the Student-Neuman-Keuls Test, with the exception of the bacterial translocation incidences, where the Fisher's Exact Test was used. With all analyses, an associated probability (P value) of less than 5% was considered significant.

Materials

Alcian blue 8GX, IBMX, ovalbumin and L-NIL were obtained from Sigma Chemical Co. (St. Louis, MO). *Clostridium difficile* toxin A was purchased from Techlab Inc. (Blacksburg, VA). Safranine was obtained from Fisher Scientific (Nepean, ON, Canada). The bacterial culturing materials were obtained from Becton-Dickinson (Cockeysville, MD). RMCP-II and the antibody to this protease were obtained from Moredun Animal Health (Glasgow, UK). ^{3}H-glucosamine was obtained from ICN Biomedicals Inc. (Costa Mesa, CA). All other materials were obtained from VWR (Edmonton, AB, Canada) or Sigma Chemical Co. (St. Louis, MO).

5.3 Results

The results described in chapter 4 established that by six weeks after intracolonic administration of TNBS, macroscopic damage scores and myeloperoxidase activity (MPO) in the colon were not significantly different from those in control rats. Furthermore, six weeks after TNBS administration, the histological appearance of the colon and the permeability of the colon to ^{51}Cr-EDTA (*in vitro*) were indistinguishable from those of naïve controls. In the present study, we have confirmed that permeability to ^{51}Cr-EDTA *in vivo* is not significantly different from that in age-matched controls (Figure 5.2). While the acute colitis at 72 h after TNBS administration was associated with a 6-fold increase in permeability to ^{51}Cr-EDTA, the permeability had returned to normal by 6 weeks after TNBS administration.

Effects of a prior bout of colitis on colonic secretion

In control rats, toxin A from *C. difficile* caused approximately a 5–fold increase in fluid secretion into the colonic loops over that observed in vehicle-treated controls (Figure 5.3, panel A). In rats in which colitis had been induced 6 weeks earlier, the fluid accumulation in colonic loops after exposure to toxin A was significantly lower than that in toxin A-treated controls (reduced by 53%; Figure 5.3, panel A). More striking was the observation that the secretory response to IBMX was completely abolished in the rats that had previously had colitis (Figure 5.3, panel B).

Any change in fluid accumulation after exposure to *C. difficile* toxin A could be due, at least in part, to exudation of intracellular and extracellular fluid subsequent to injury. Thus, differences among the treatment groups could be attributable to differences

in the severity of damage, rather than to differences in secretion. To test this hypothesis, we measured levels of lysosomal enzyme lactate dehydrogenase in the luminal fluid samples as an index of damage. We found that there were no significant differences in luminal levels of lactate dehydrogenase among the four groups (as a percent of the control group treated with vehicle: control/toxin A: 98 ± 9%; TNBS/vehicle: 104 ± 35%; TNBS/toxin A: 74 ± 11%).

While a prior bout of colitis resulted in markedly diminished fluid secretion, it did not significantly affect basal or carbachol-stimulated colonic mucus secretion (Figure 5.4). Exposure to carbachol for 30 min caused a ~20-fold increase in ^{3}H-glucosamine-labeled glycoprotein release in both control and post-colitis rats.

The effects of antigen (ovalbumin) on chloride secretion by the colon was examined *in vitro* using tissue from in post-colitis rats and from controls. Addition of the antigen to the serosal side of the colonic tissue from control, sensitized rats resulted in a 10-fold increase in short-circuit current, which is generated primarily by active Cl^- secretion into the lumen (Figure 5.5). When the same experiments were performed in control rats that had not been sensitized to the antigen, no secretory response was observed. Antigen challenge of colonic tissue from sensitized, post-colitis rats did not elicit a secretory response (Figure 5.5). Indeed, the response to antigen was no different from that in post-colitis rats than had not been sensitized to the antigen. The absence of a secretory response to antigen in the sensitized post-colitis rats was not due to a failure of the antigen to cause mucosal mast cell degranulation, as we observed a significant increase (3-fold) in the luminal levels of the mucosal mast cell protease, RMCP-II, in these rats. A doubling of RMCP-II concentrations in the luminal side of the Ussing

chamber was noted in control, sensitized rats following challenge with the antigen. Moreover, all rats used in this study as "sensitized" were confirmed to be sensitized by a positive PCA test.

Effects of a prior bout of enteritis or extraintestinal inflammation on colonic secretion

Colonic tissue that was fixed 52 days after *N. brasiliensis* infection did not exhibit any histological evidence of inflammation. Toxin A caused a significant increase (2.8-fold) in fluid accumulation in the colon of control rats (Figure 5.6). In colonic loops from rats previously infected with *N. brasiliensis*, toxin A caused significantly less fluid accumulation than in controls (33% reduction). There was no significant difference in the amount of fluid recovered from toxin A- versus vehicle-treated loops in rats previously infected with *N. brasiliensis*.

IBMX caused a significant increase (~ 1.7-fold) in fluid accumulation in control rats (Figure 5.7). A similar level of fluid secretion was observed in rats in which a carrageenan-airpouch was induced.

Effects of TTX

The colonic fluid secretion hypo-responsiveness to IBMX after a bout of colitis was not a result of neural input to the epithelium. Rats treated with intracolonic TNBS 6 wk earlier continued to exhibit reduced fluid secretion responses to IBMX in the presence of the neuronal blocker TTX when compared to the secretory responses in control rats (Figure 5.8). TTX did not have an effect on IBMX-stimulated colonic fluid secretion responses in control rats when compared to vehicle (Figure 5.8).

Effects of iNOS inhibition

Treatment with L-NIL 10 min prior to toxin A administration did not have any effect on colonic fluid accumulation in control rats (Figure 5.9). Similarly, L-NIL pretreatment did not affect colonic secretion in rats treated with TNBS 6 weeks earlier; that is, the volume of fluid recovered from toxin A-treated colonic loops in post-colitis rats remained significantly reduced compared to the volume recovered from controls. In contrast, in IBMX-stimulated colonic loops, the volume of fluid recovered in post-colitis rats pretreated with L-NIL was not different from the volume of fluid recovered from control rats (i.e., L-NIL partially reversed the hyposecretion; Figure 5.9).

Colonic bacterial levels and bacterial and neutrophil translocation: effects of a bout of colitis

Despite the normal macroscopic and histological appearance of the colon, there were marked changes in bacterial colonization of the colon 6 weeks after induction of colitis. We observed a 16-fold increase in the number of aerobic bacteria in the colon (tissue + lumen) of post-colitis rats compared to controls (Table 5.1). However, there was no difference in the number of adherent aerobic bacteria between control and post-colitis rats. Total gram-negative bacterial levels in the colon were also not different between control and post-colitis rats. Aerobic bacterial translocation to either the mesenteric lymph nodes or spleen was observed in 17 of the 22 rats (77%) examined 6 weeks after administration of TNBS, significantly greater ($p<0.0005$) than the 23% incidence (5 of 22) in control rats. In terms of the types of bacterial colonization in the post-colitis rats versus controls, there were few major differences. *Eschericia coli* and *Enterococci* spp. were the predominant bacteria in both control and post-colitis rats. Neutrophil

translocation across the colonic mucosa, as detected by fecal granulocyte marker protein excretion, was also significantly elevated 6 wk after the induction of colitis (Figure 5.7.).

The increase in bacterial colonization and translocation across the colonic mucosa was not simply due to tissue damage caused by TNBS. This was demonstrated by the fact that rats treated intracolonically with ethanol 6 wk earlier did not exhibit changes in bacterial colonization (gram negative bacteria: 4.6 ± 0.1 log CFU/ g tissue in the ethanol treated group vs 4.0 ± 0.6 log CFU/ g tissue in the control group; total aerobes: 5.3 ± 0.1 log CFU/ g tissue in the 50% ethanol treated group vs 4.4 ± 0.6 log CFU/ g tissue in the control group). Similarly, the rats treated with ethanol 6 wk earlier did not have a significantly different incidence of bacterial translocation compared to control rats (25% translocation in ethanol-treated rats vs 23% translocation in control rats).

In post-colitis rats treated with L-NIL for 7 days, the incidence of bacterial translocation was 50% (3 of 6 rats), not significantly different from the rate observed in naïve rats, nor from the rate observed in post-colitis rats treated with the vehicle (83%; 5 of 6 rats). Similarly, the total numbers of bacteria in the colon of rats treated with L-NIL (7.07 ± 1.00 log CFU/mg) were not significantly different from those in the rats treated with vehicle (7.00 ± 0.20 log CFU/mg).

Colonic bacterial levels and bacterial translocation: effects of a bout of enteritis

In rats that had been infected with N. brasiliensis 52 days earlier, the colonic aerobic bacterial numbers were 10-fold higher ($p<0.05$) than in controls (7.8 ± 0.6 log CFU/g versus 6.8 ± 0.4 log CFU/g, respectively). Unlike the case in the post-colitis rats, there was also a significant increase (25-fold) in the numbers of Gram-negative bacteria in the colon of the post-enteritis rats compared to controls (6.1 ± 0.6 log CFU/g versus 4.7 ± 0.6

log CFU/g, respectively; $p<0.01$). The incidence of bacterial translocation in rats that previously had enteritis was 54% (7 of 13 rats), significantly greater ($p<0.05$) than the 23% incidence in controls.

Epithelial cell proliferation: effects of a bout of colitis

Induction of colitis 6 wk earlier resulted in significantly increased (~ 1.8-fold) epithelial cell proliferation in the colon when compared to untreated controls (Figure 5.10). This increase in proliferation rate was not associated with a non-specific response to tissue injury, as colonic tissues taken from rats 6 wk after intracolonic administration of 50% ethanol, which caused extensive damage, did not display increased proliferation rates compared to controls.

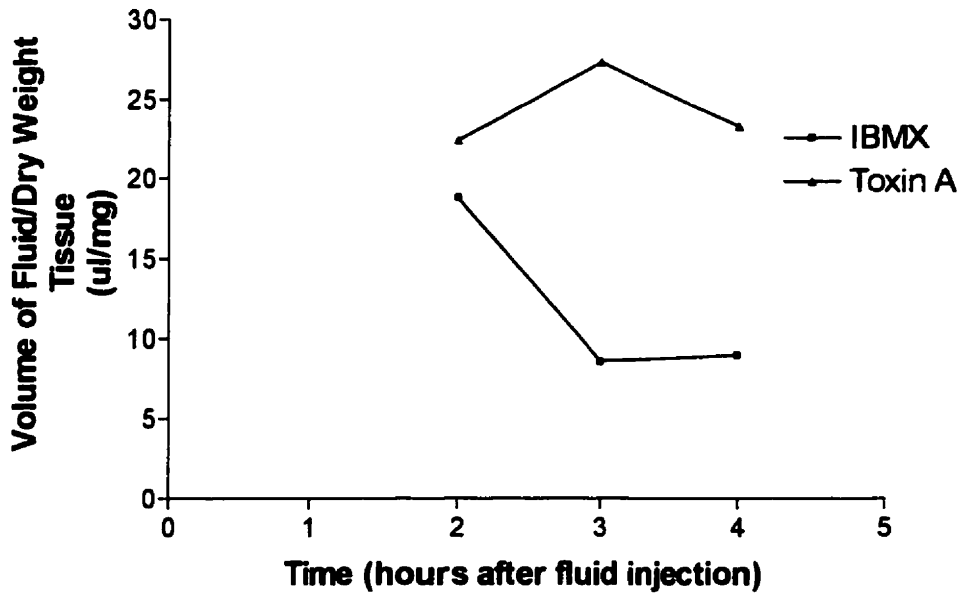

Figure 5.1. Preliminary study illustrating peak fluid accumulation in the colon of control rats after IBMX or C. difficile toxin A stimulation.

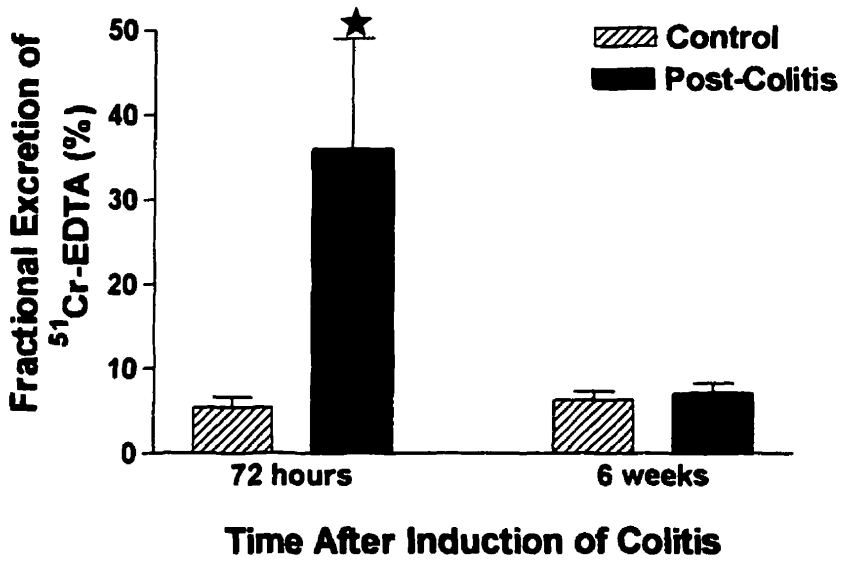

Figure 5.2. Permeability of the gastrointestinal tract to the small molecular weight marker (^{51}Cr-EDTA) in rats 72 h or 6 wk after induction of colitis and in age-matched, control rats. Results are expressed as the mean ± SEM fractional excretion of the labeled permeability probe (measured in urine over a 12 h period after its oral administration). Each group consisted of at least 6 rats. *P<0.05 versus the corresponding control group.

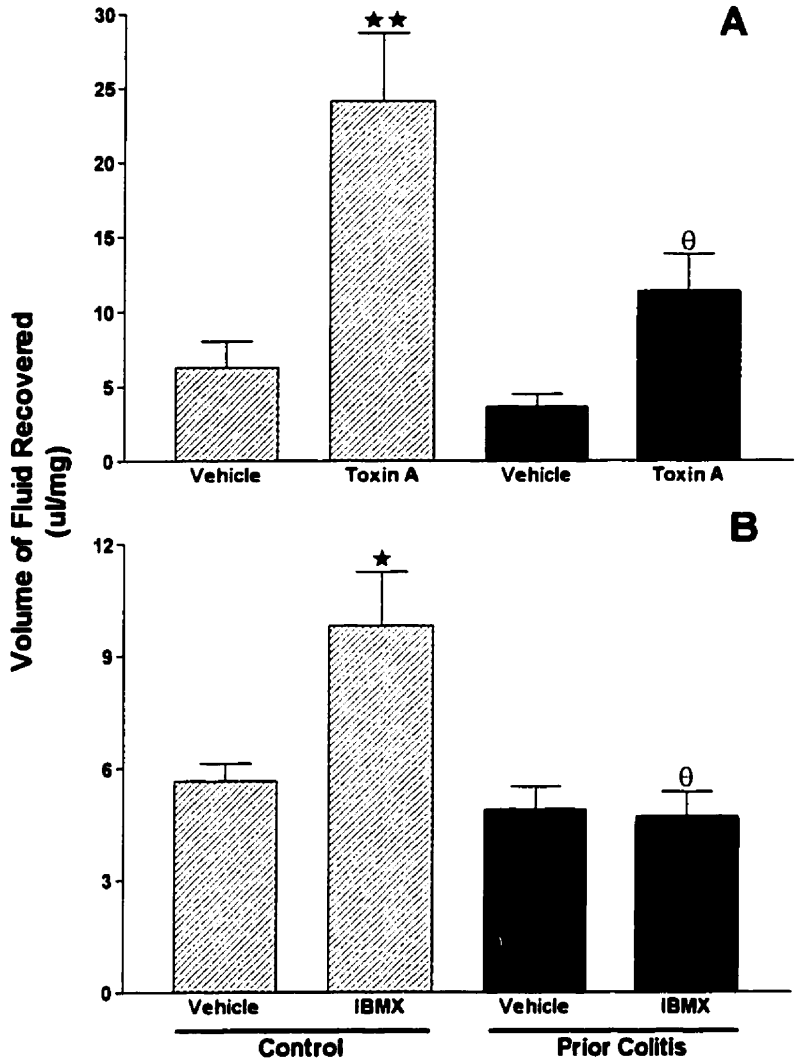

Figure 5.3. (A) C. difficile toxin A- (5 μg/ml) or (B) IBMX- (300 μM) stimulated colonic fluid secretion after a bout of colitis. Colonic fluid levels were measured 3 h after administration of toxin A, IBMX or vehicle. Each bar represents the mean ± SEM, with 5-8 rats per group. *P<0.05, **P<0.01 compared to the corresponding vehicle-treated group. $^{\theta}$P<0.05 compared to the corresponding control group.

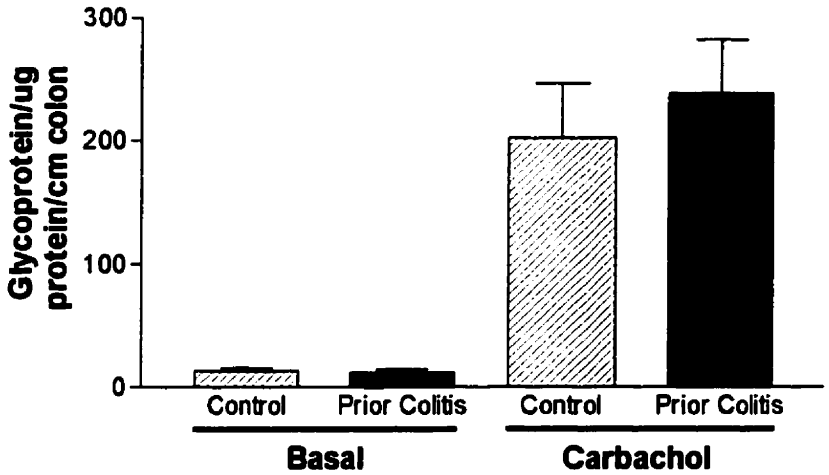

Figure 5.4. Basal and carbachol-stimulated mucus secretion after a bout of colitis. ^{3}H-glucosamine-labeled glycoprotein levels were measured as an index of mucus secretion under basal conditions and after stimulation with carbachol (10 μM). Each bar represents the mean ± SEM, with 5-9 rats per group. There were no significant differences between the control and post-colitis groups.

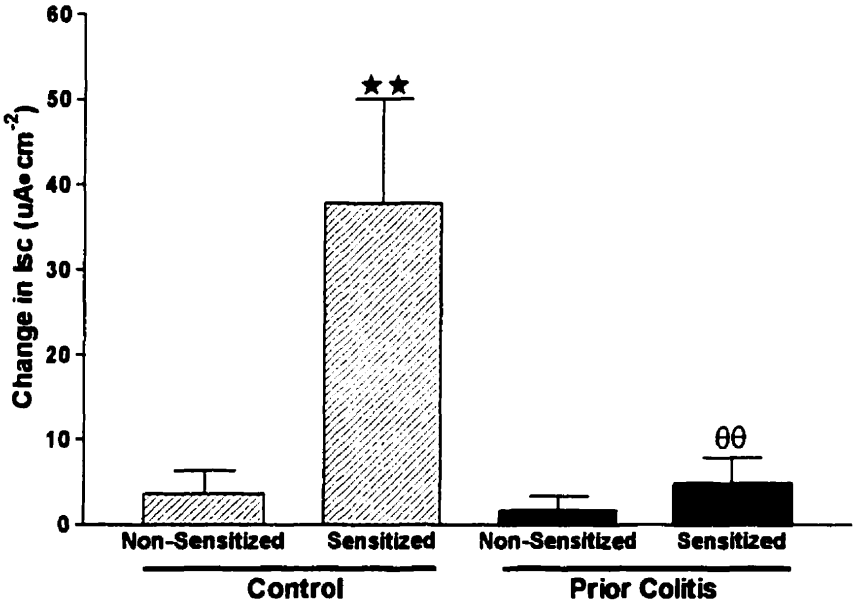

Figure 5.5. Colonic epithelial short circuit current (I_{sc}) responses to ovalbumin challenge after a bout of colitis. Tissues from rats killed 6 weeks after induction of colitis and age-matched controls were mounted in Ussing chambers. In each case, subgroups of rats were sensitized to ovalbumin. $^{**}P<0.01$ versus to the corresponding non-sensitized group. $^{\theta\theta}P<0.01$ versus the corresponding control group.

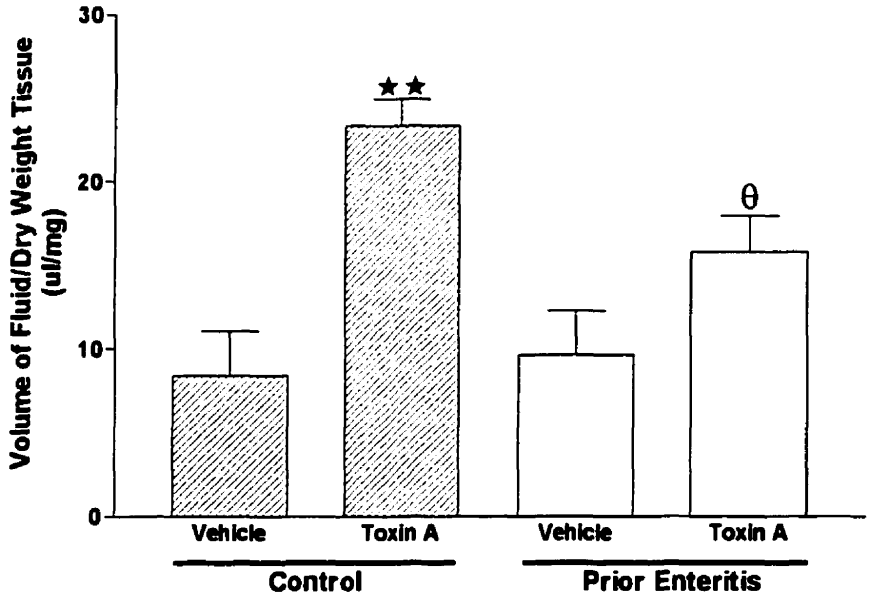

Figure 5.6. Effects of a prior bout of enteritis on C. difficle toxin A-stimulated colonic fluid secretion. Colonic fluid levels were measured 3 h after stimulation with toxin A (5 μg/ml) or vehicle. Each bar represents mean ± SEM, with 6-9 rats per group. $^{**}P<0.01$ compared to the corresponding vehicle-treated group. $^{\theta}P<0.05$ compared to the corresponding control group.

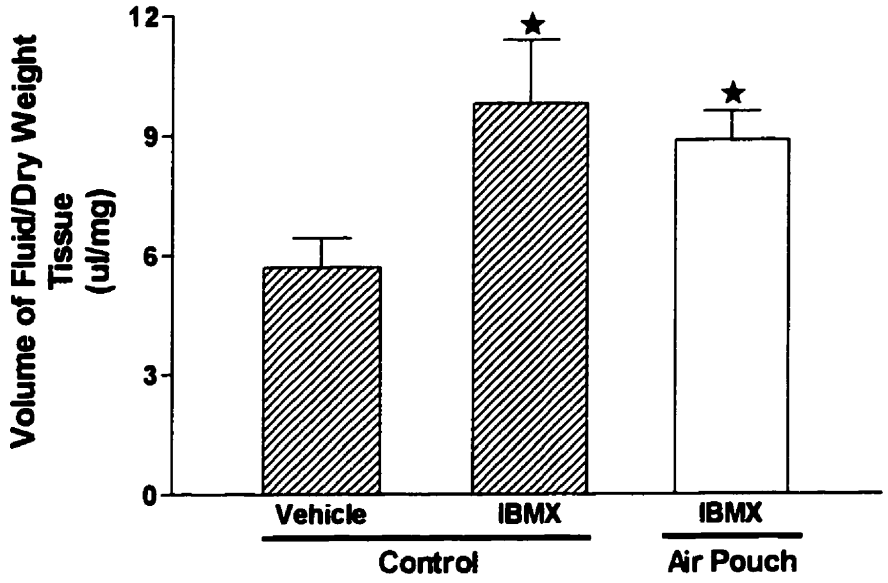

Figure 5.7. Effects of extraintestinal inflammation on C. difficle toxin A-stimulated colonic fluid secretion. Colonic fluid secretion was measured 6 h after administration of a 1% carrageenan solution in to the airpouch. Untreated rats served as controls. Each bar represents the mean ± SEM, with 8-9 rats per group. $^{*}P<0.05$ compared to the vehicle-treated control group. There were no significant differences between the IBMX-treated control and airpouch groups.

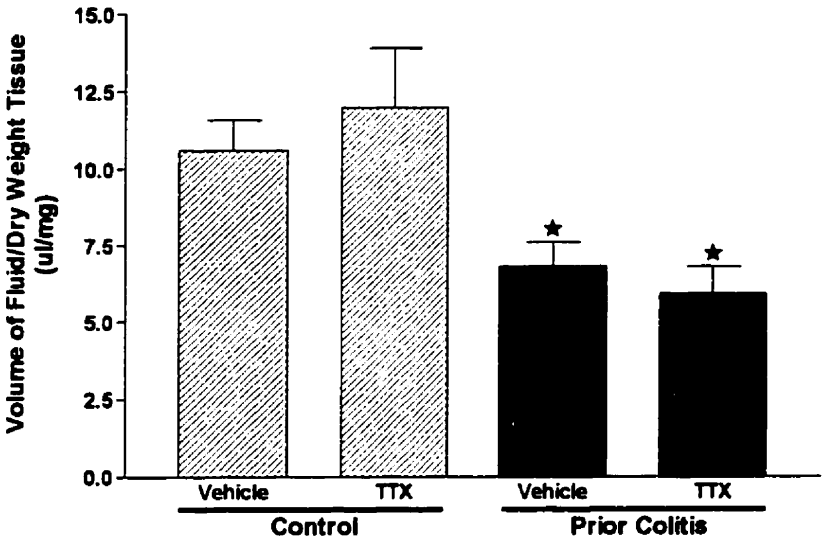

Figure 5.8. Effects of the sodium channel blocker tetrodotoxin (10 μM) on IBMX-stimulated colonic fluid secretion. Each bar represents the mean ± SEM, with 5-10 rats per group. *P<0.05 compared to the corresponding control group.

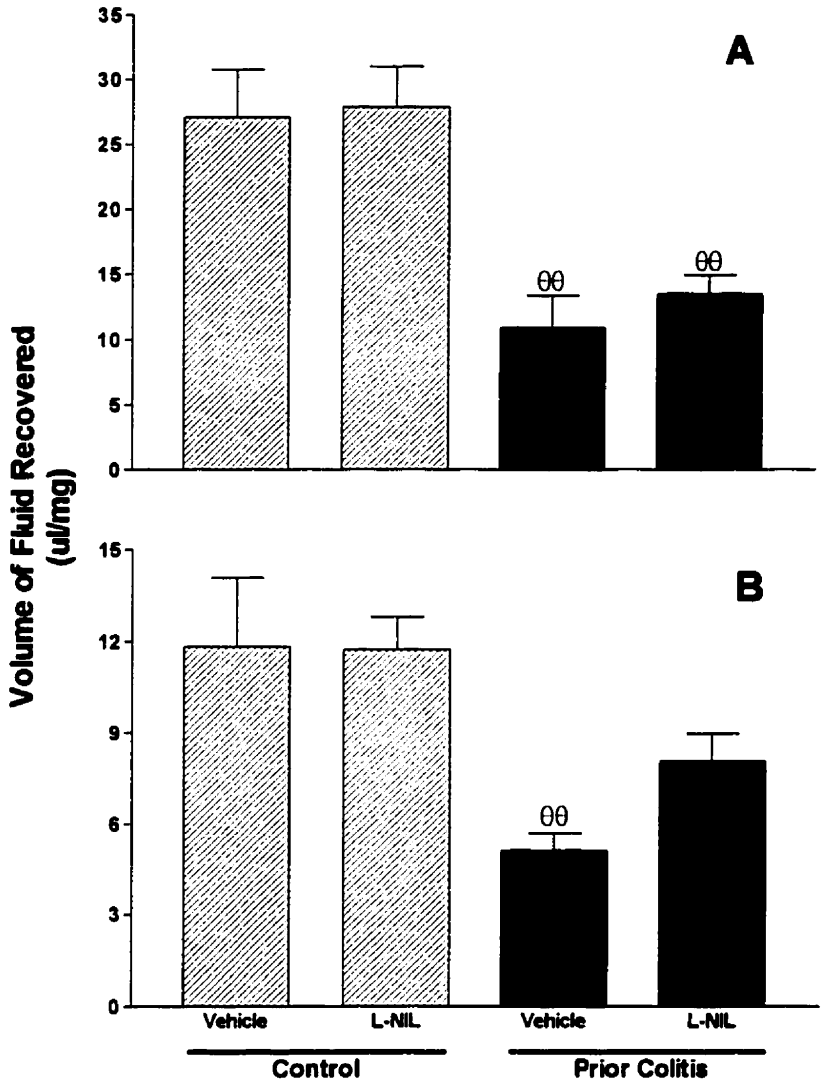

Figure 5.9. Effects of a selective iNOS inhibitor, L-NIL (3 mg/kg), on in vivo colonic fluid responses to (A) C. difficile toxin A (5 μg/ml) and (B) IBMX (300 μM). Each bar represents the mean ± SEM, with 7-9 rats per group. $^{\theta\theta}P<0.01$ versus to the corresponding control group.

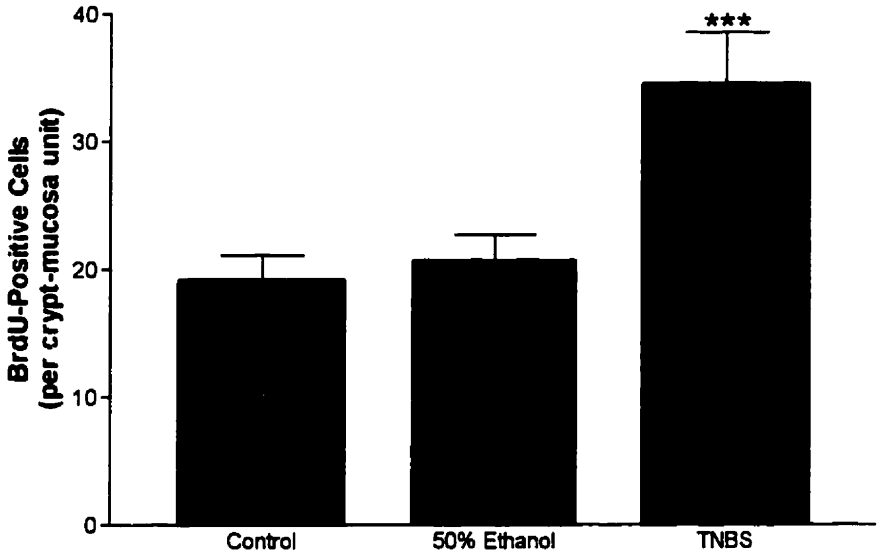

Figure 5.10. Number of proliferating cells in the colonic epithelium 6 wk after intracolonic administration of TNBS or ethanol. Staining of colonic epithelial cells with 5'-bromo-2'-deoxyuridine was assessed six weeks after intracolonic administration of TNBS/50% ethanol, or ethanol alone. Each bar represents the mean ± SEM, with 5-13 rats per group. ***$P<0.001$ versus the control group.

Table 5.1. Colonic Bacterial Levels and Bacterial Translocation in Control and Post-Colitis Rats

	n	Colonic Bacteria Levels (log_{10} CFU/g)				Incidence of Bacterial Translocation (%)
		Tissue + Lumen		Adherent		
		Total Aerobes	Gram-Negative	Total Aerobes	Gram-Negative	
Control	22	5.3 ± 0.5	4.4 ± 0.9	4.4 ± 0.6	4.0 ± 0.6	23
Prior Colitis	22	6.5 ± 0.3 ($p<0.05$)	4.2 ± 0.9	5.0 ± 0.5	3.7 ± 0.6	77 ($p<0.0005$)

These studies were performed in rats killed 6 weeks after induction of colitis with trinitrobenzene sulfonic acid and in age-matched, controls. Bacterial colonization was measured in two ways: from samples of the colonic tissue plus the luminal contents (Tissue + Lumen) and from samples of tissue that were repeatedly washed to remove all but the adherent bacteria (Adherent).

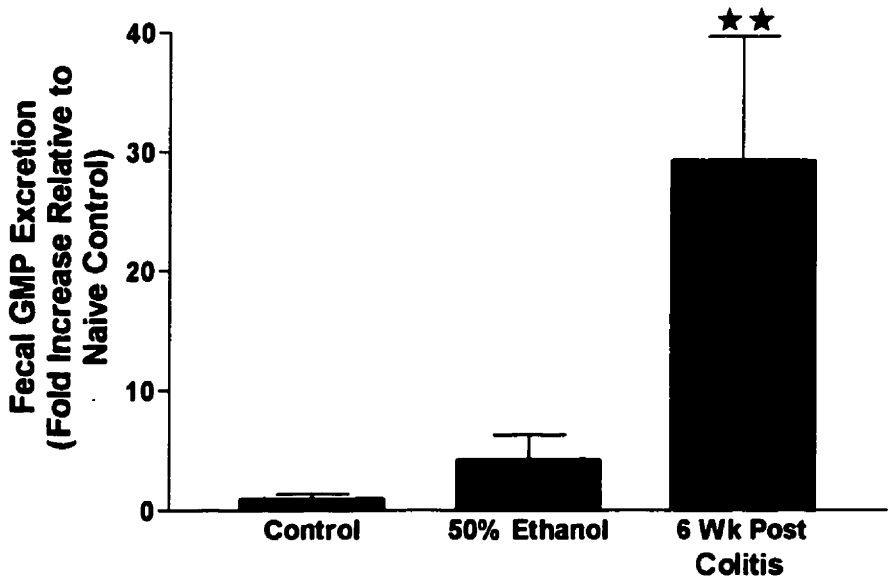

Figure 5.11. Fecal granulocyte marker protein (GMP) excretion following a bout of colitis. Each bar represents mean ± SEM, with 5-6 rats per group. $^{**}P < 0.01$ versus the control group.

5.4 Discussion

Epithelial secretion of electrolytes, water and mucus is an important mechanism of host defence against microbes and toxins in the intestinal lumen. During inflammatory reactions, this response may be altered as a result of the release of soluble mediators such as prostaglandins, nitric oxide and various cytokines. However, following the resolution of inflammation, epithelial dysfunction can persist for prolonged periods of time. We previously demonstrated that epithelial dysfunction *in vitro* persists for at least 12 weeks after the episode of inflammation, but this dysfunction was not seen in rats only given the ethanol vehicle (chapter 4). In the present study, we have extended this observation by demonstrating that colonic epithelial hypo-responsiveness can also be observed *in vivo* for at least 6 weeks after induction of colitis, and after resolution of small intestinal inflammation. The secretory dysfunction was a generalized effect, rather than being specific to a single stimulus. Thus, the hypo-responsiveness was observed *in vivo* with two secretagogues that have distinct mechanisms of action (*C. difficile* toxin A and IBMX), while secretory dysfunction *in vitro* was demonstrated in a model of antigen-induced epithelial chloride secretion. Moreover, the rats that had previously been subjected to colitis or enteritis exhibited significant increases in bacterial colonization of the colon, consistent with the notion that colonic epithelial secretion normally limits bacterial colonization. The impairment of colonic secretion may also have accounted for the significant increase in bacterial translocation in the rats that previously had experienced a bout of colitis or enteritis. Interestingly, the tripling of the amount of bacterial translocation in post-colitis rats occurred despite the epithelial permeability to a

small molecular weight probe (EDTA) not being significantly different from that in normal rats.

The mechanism for epithelial hypo-responsiveness following a bout of colitis is not yet fully understood. In previous *in vitro* studies in which epithelial hypo-responsiveness was found to persist for more than 12 weeks after a bout of colitis, inhibition of iNOS was found to normalize the responsiveness of the colonic epithelium to electrical field stimulation and IBMX, but not to carbachol (chapter 4). Based on these data, we proposed that NO might contribute to the hypo-responsiveness by affecting cAMP-mediated (such as that activated by IBMX), but not Ca^{2+}-mediated (such as that activated by carbachol) pathways of secretion. Similarly, our *in vivo* findings in this study demonstrate that L-NIL treatment partially reversed the epithelial hypo-responsiveness to IBMX, but had no effect on *C. difficile* toxin A-stimulated secretion. *C. difficile* toxin A is believed to elicit its effects through Ca^{2+}-dependent pathways (228,228,236). *C. difficile* toxin A has also been shown to elicit epithelial toxicity. Consequently, it was possible that the difference in fluid accumulation levels observed between control and post-colitis rats could have been due to increased susceptibility to toxin A-induced damage in the latter group. However, cellular injury (as determined by lactate dehydrogenase release) in toxin A-treated versus vehicle-treated colonic loops from control and post-colitis rats were not significantly different.

It is important to note that acute administration of L-NIL (iNOS inhibitor) resulted in a partial reversal of the hypo-responsiveness to IBMX. This indicates that the epithelial dysfunction was not attributable to a loss of the cellular apparatus for active Cl^- section (e.g., loss of expression of the chloride transporter). The results of the present

study are consistent with the hypothesis that the defect lies at the level of the enterocyte, rather than in cells in the lamina propria that can influence epithelial chloride secretion (e.g., nerves, mast cells). For example, in both the present study and in our previous *in vitro* study, we observed that the hypo-responsiveness was evident with a number of different secretagogues that act through different pathways. Moreover, in the present study we observed a complete absence of an epithelial secretory response to antigen in sensitized, post-colitis rats despite the fact that the antigen still caused mucosal mast cell degranulation.

The mechanism of NO-mediated hypo-responsiveness is not clear, but may be due to the effect of NO on adenylate cyclase activity, an effect on the Na^+-K^+-ATPase, or both. Cyclic AMP-dependent pathways regulate chloride secretion in intestinal epithelial cells, and NO has been shown to inhibit the generation of cAMP in other systems (208). NO can decrease cAMP generation in an intestinal epithelial cell line (Dr. W. MacNaughton, personal communication). NO has also been shown to reduce expression of the α subunit of the Na^+-K^+-ATPase in a kidney cell line (237). Na^+-K^+-ATPase provides the electrochemical gradient that promotes chloride entry into the enterocyte, with subsequent secretion through the apical CFTR chloride channel. However, as this effect of NO is against a transcriptionally regulated event, it is unlikely to account for suppression of activity in a system such as epithelial chloride secretion, which can be rapidly reversed by inhibition of the generation of NO.

As mentioned above, in addition to secretory dysfunction, we observed that following resolution of colitis or enteritis there was an increase in the number of bacteria in the colon and an increase in bacterial translocation to lymphatic organs such as the

mesenteric lymph nodes and spleen. These data point to a functional consequence of epithelial hyposecretion following a bout of intestinal inflammation. The inability to "flush away" bacteria could contribute to the elevated colonization in the mucus layer, which in turn could cause an increase in bacterial translocation. The fact that we have observed a marked increase in bacterial and neutrophil translocation in the absence of any detectable change in permeability suggests that caution should be exercised in drawing conclusions from studies that use permeability probes as a sole index of "barrier function".

Another important finding was that following a bout of colitis, the colonic epithelium displayed increased cell proliferation when compared to tissue from control rats. This suggests that the epithelium may be functionally different after a bout of inflammation, as increased proliferation could result in a more immature (crypt-like) epithelium. That is, epithelial cells may migrate from the crypt to the surface epithelia without allowing for sufficient time for their maturation to become surface absorptive cells. This may have implications for epithelial function. For example, the tight junctions of crypt cells are more permeable than those of surface epithelial cells. Therefore, it is possible that following a bout of inflammation, an increased proliferation rate resulting in a phenotypically crypt-like epithelium may predispose the colon to increased translocation (i.e., by neutrophils, bacteria) across the mucosa. On the other hand, epithelial cell proliferation does not result in detectable changes in permeability to the small molecular weight probe EDTA (figure 4.1). The reason for this is not known, however, it is possible that the procedure used to measure epithelial permeability was simply not sensitive enough to detect the changes in permeability resulting in increased

bacterial translocation. Another possibility is that the increased bacterial translocation was due to transcellular passage of bacteria across the epithelium. If this were the case, a paracellular epithelial marker such as EDTA would not reveal any changes in permeability.

The findings of this study may have important implications for understanding the pathogenesis of IBD and irritable bowel syndrome. The possibility that an intestinal infection could be an early event in the etiopathogenesis of IBD has already been discussed. Several studies have reported that in a significant percentage of patients with irritable bowel syndrome, the symptoms can be traced to a bout of intestinal infection (2, 4,5,238). The present study has demonstrated that a prior bout of intestinal inflammation can cause long-lasting changes in epithelial function that could contribute to an increased propensity for infection and/or inflammation. There is also evidence of long-term changes in nerve and muscle function after a bout of colitis in humans and in animal models (37,39,40,42). In addition, myenteric nerve function has been shown to be altered not only at the site of the inflammatory reaction, but also at more distant, non-inflamed sites (9,36,41). These changes, along with the hypo-responsiveness of the epithelium, could contribute not only to reduced mucosal defense, but also to the generation of symptoms (diarrhea, pain) in patients with IBD or irritable bowel syndrome.

Chapter 6

GENERAL DISCUSSION

Inflammation causes alterations in smooth muscle contractility and enteric nerve function (39,41,42). These changes have been proposed to contribute to symptoms of diarrhea and pain associated with inflammatory disorders such as IBD (e.g., diarrhea, pain). Likewise, in post-infectious IBS, it is believed that a bout of inflammation results in alterations in intestinal function that may be associated with the chronic symptoms of abdominal discomfort, fecal urgency and bloating. Given the evidence that alterations in smooth muscle and nerve function can occur in areas of the gut not directly exposed to the inflammatory insult, it is possible that inflammation has other long-term consequences for intestinal function. The epithelium serves as an important part of the intestinal barrier against bacteria and other noxious substances in the lumen of the intestine. This may be through the secretion of electrolytes, water and mucus, or simply the nature of the tight junctions that prevent the translocation of luminal contents across the mucosa. As a result, if a bout of inflammation were to impair normal epithelial function, it is forseeable that one would be predisposed to a subsequent bout of inflammation or infection. Thus, the goal of this project was to examine whether a bout of inflammation can affect epithelial function, particularly during the post-inflammatory period.

This dissertation provides a description of two studies examining the effects of inflammation on colonic epithelial function. In addition, the first study examines the role

of NO in acute intestinal inflammation. Details of each study are provided in the corresponding chapters. In the following discussion, a brief overview of the main findings, the implications of these results, and some limitations of the research are provided. Future studies that may be pursued as follow-up experiments are also discussed.

6.1. Role of Nitric Oxide in Colitis

In the study described in chapter 3, the interaction of NO, superoxide and hydroxyl radical in TNBS-induced colitis was examined. In this study, the role of NO derived from the different NOS isoforms in acute inflammation was examined using a variety of NOS inhibitors. Macroscopic damage and granulocyte infiltration were not reduced by non-selective, iNOS-selective or nNOS-selective inhibitors. A review of the literature encompassing the effects of NOS inhibition in experimental colitis demonstrates that the results have been conflicting. Some investigators have attributed the discrepant results to the fact that many of the NOS inhibitors used were not isoform-selective. As a result, inhibition of the constitutively expressed eNOS and nNOS isoforms may have detrimental effects on the intestine with respect to damage. However, studies examining the effects of iNOS-selective isoforms have also been inconsistent. Another explanation proposed to explain this controversy is that the chemically reactive form of nitrogen oxide present is a critical determinant of whether NO is beneficial or detrimental to the tissue. That is, it is important to determine which nitrogen oxide species (nitroxyl ion, nitrosonium, nitrogen dioxide or nitric oxide) are present in the

tissue, as each species will undergo different reactions *in vivo*. The discrepant results of NOS inhibitors in colitis may also be explained by the fact that the various forms of nitrogen oxide described above interact with other potentially harmful free radicals such as superoxide. The subsequent formation of reactive species such as peroxynitrite and hydroxyl radical also precludes the destructive manner in which nitric oxide may act. The interaction of these reactive nitrogen and oxygen intermediates with antioxidant enzymes such as glutathione peroxidase and glutathione transferase also adds complexity to the regulation of oxidants within the intracellular milieu. Grisham *et al.* (87) demonstrated that these enzymes can be inactivated through nitration by nitrogen oxide species (i.e., nitroxyl ion, nitronium ion, peroxynitrite). Therefore, when a NOS inhibitor is administered *in vivo*, it is important to consider not only which reactive nitrogen and oxygen species are formed, but also the oxidative state of the tissue. For example, the study conducted in chapter 5 revealed that although nitric oxide production was inhibited by NOS inhibitors, superoxide production was also suppressed. Furthermore, as superoxide production was reduced in tissues from NOS inhibitor treated rats, there was an elevation of hydroxyl radical levels. Thus, the lack of a beneficial effect of non-selective and iNOS-selective inhibitors in colitis may have been attributable to a change in the tissue from superoxide production to hydroxyl radical production. On the other hand, the nNOS-selective inhibitor 7-NI did not attenuate macroscopic damage or granulocyte infiltration, despite a reduction in superoxide production and a lack of stimulation of hydroxyl radical production. Interestingly, total free radical production remained elevated in all NOS inhibitor-treated groups regardless of the isoform specificity of the drugs. This was also true of colonic tissue from 7-NI-treated rats which

suggests that free radicals other than superoxide and hydroxyl radical must be present at the site of inflammation. Therefore, although NOS inhibition may eliminate or reduce the levels of NO-derived free radicals, other potentially detrimental reactive species such as oxygen-derived intermediates may be produced. In theory, the inhibition of NO production, particularly that derived from iNOS, should reduce the level of free radicals produced at the site of inflammation and this should attenuate the damage caused by the inflammatory insult. However, the data from this study demonstrated that inhibition of NOS does not reduce the production of potentially harmful free radicals. Rather, the production of free radicals such as superoxide is changed to the production of other free radicals such as hydroxyl radical. Finally, the *in vitro* findings of this study point to the importance of the different nitrogen oxide derived species at the site of inflammation. NO is believed to interact with superoxide to form peroxynitrite (172). Therefore, it is not surprising that the addition of NO donors such as SNP and SNAP reduced free radical production within the inflamed colon. However, the fact that sodium nitrite, which releases nitrite, did not elicit the same effects as SNP and SNAP suggests that the nitrogen oxide-species released is very important to consider in the realm of the inflammatory milieu.

In summary, this study disproved our initial hypothesis that iNOS-selective inhibitors are beneficial in acute inflammation of the colon, as well as provided evidence that NO might be beneficial in inflammation by affecting the production of other free radicals.

6.2. Effects of Inflammation *in vitro*

Chapter 4 focused on epithelial function with respect to electrolyte secretion and permeability following an inflammatory insult. This study examined whether inflammation of the colon due to intracolonic TNBS administration caused long-term changes in epithelial function *in vitro*. At a time-point 6 wk following the induction of colitis, the colonic epithelium appeared histologically and macroscopically normal. Furthermore, tissue conductance and serosal-to-mucosal flux of the permeability marker ^{51}Cr-EDTA were normal, indicating the lack of any persistent permeability defect. However, consistent with the initial hypothesis, epithelial function was altered following a bout of colitis. The epithelium was hypo-responsive to EFS, IBMX and carbachol stimulation with respect to electrolyte secretion. This phenomenon was not attributed to neuronal irregularities as TTX did not affect epithelial transport responses to IBMX and carbachol compared to tissues pre-incubated with vehicle. It is also unlikely that structural changes in the epithelium such as alterations in the expression of the CFTR chloride channel caused the observed hypo-responsiveness. Evidence to support this included the fact that L-NIL treatment reversed the hypo-responsiveness to IBMX and EFS. Therefore, the hypo-responsiveness was at least partially attributed to elevated levels of NO derived from the inducible isoform of NOS. These data provide evidence that a bout of inflammation can have long-term effects on colonic epithelial electrolyte secretion. This may have consequences for mucosal defense as impaired epithelial secretion may predispose the intestine to frequent adherence and translocation of pathogens and antigens. Given these findings, one question that remains unanswered is

the mechanism through which epithelial function is altered long after the inflammation has resolved. From these data, although NO is implicated in the observed hypo-responsiveness, the molecular mechanisms through which NO alters epithelial electrolyte transport are not known. Increased cAMP levels have been shown to increase CFTR translocation to the cell membrane. Thus, it is still possible that a decrease in cAMP levels by NO could cause acute changes in surface expression of CFTR (239,240). That is, decreased CFTR expression could cause reduced secretory responsiveness that may be reversed by inhibition of NO synthesis by L-NIL. Furthermore, the hypo-responsiveness to carbachol stimulation was not reversed by L-NIL. This suggests that a bout of inflammation results in other changes that affect electrolyte transport. Other inflammatory mediators that affect electrolyte transport may persist in the intestinal mucosa despite macroscopic resolution of inflammation. These could include cytokines or eicosanoids that have previously been shown to alter electrolyte transport. For example, prostaglandin D_2 which has been found to be elevated up to 6 wk after induction of colitis (Dr. M. Ajeubor, personal communication), may exhibit an anti-secretory effect on the epithelium. Other experiments examining the mechanisms through which the epithelium was hypo-responsive were conducted in the study described in chapter 5 and are discussed below.

6.3. Effects of Inflammation *in vivo*

In the subsequent study described in chapter 5, we set out to determine whether the changes in epithelial function observed *in vitro* (chapter 4) were also characteristic of

the colon *in vivo*. We also examined whether the epithelium was hyporesponsive to the more "clinically relevant agonists," *C. difficile*-derived toxin A and ovalbumin. Once again, our hypothesis was confirmed by the findings that with respect to fluid secretion, rats exhibited hypo-responsiveness to toxin A and IBMX stimulation 6 wk following the induction of colitis. The colonic epithelium was also hyporesponsive to ovalbumin stimulation. Furthermore, small intestinal, but not extra-intestinal, inflammation was found to induce changes in colonic epithelial function. These findings are consistent with the notion that an inflammatory insult in one part of the intestine can induce changes in other, non-inflamed parts of the gut. This has been previously demonstrated with respect to smooth muscle contractility and enteric nerve function (39,41,42). Moreover, the findings of the present study suggest that this is not a generalized phenomenon. That is, although inflammation in the small intestine can affect epithelial function in the colon, peripheral inflammation did not exert similar effects on the colonic epithelium. These findings are consistent with the hypothesis that inflammation in one part of the intestine might affect epithelial function in another as a result of neurally-mediated effects. For instance, Nocerino *et al.* (207) demonstrated that exposure of the jejunum to cholera toxin resulted in a secretory response not only in the exposed intestinal segment, but also in the colon. This phenomenon was attributed to the enteric nervous system as transection of the intestine from the colon eliminated the secretory response in the colon (207). Thus, as observed in this study, extra-intestinal inflammation that does not affect enteric nerve function would not be expected to affect colonic epithelial function. The hypo-responsiveness of the epithelium observed *in vivo* was also not attributed to a neural defect, in that TTX did not change the epithelial secretory responses to IBMX

stimulation. However, the epithelial changes were at least partially attributed to iNOS-derived NO. L-NIL partially reversed the hypo-responsiveness to IBMX but had no effect on toxin A-stimulated fluid secretion. Thus, other inflammatory-induced changes must be present in the tissue for epithelial fluid secretion to be altered. *C. difficile* toxin A causes epithelial secretion as a result of changes in cytoplasmic calcium levels, induction of cytoskeletal changes in epithelial cells, activation of mucosal mast cells, recruitment of neutrophils and release of substance P from sensory afferent nerves (18). Therefore, it is possible that changes in any of these factors as a result of an inflammatory episode might contribute to the secretory hypo-responsiveness. For instance, altered nerve function has previously been shown to occur following a bout of inflammation (40,42), and it possible that the hypo-responsiveness was due to hypo-responsive enteric nerves or altered nerve-mast cell interactions. Further experiments must be conducted in order to test such a hypothesis.

Another possible mechanism underlying the prolonged hypo-responsiveness of the epithelium after colitis is that changes in epithelial cell proliferation result in an altered epithelial phenotype. The results of this study indicated that there is increased cell proliferation in the colon 6 wk after induction of colitis. This raises the possibility that the colonic epithelium might contain increased numbers of immature crypt-like cells during the post-inflammatory period. That is, epithelial cells may migrate along the crypt-surface epithelium axis at a faster rate than normal due to the increased proliferation rate. This may not allow for the complete maturation of cells from secretory crypt to absorptive surface epithelial cells as the migration progresses. As a result, it is possible that crypt cells have different capacities to secrete fluid and electrolytes in

response to secretagogues when compared to surface epithelial cells (241,242). Intuitively, this would be expected to result in a hyper-responsive epithelium as crypt cells are secretory, whereas surface epithelial cells are absorptive. However, it is possible that a prior bout of colitis affects the maturation of epithelial cells in a manner that reduces their ability to secrete, although the mechanism by which this would occur is unknown.

Normally the epithelium secretes fluid and electrolytes in order to "flush away" bacteria, toxins and other noxious substances (210). Therefore, the findings of a hypo-responsive epithelium following a bout of inflammation *in vivo* suggest that the intestine may be more susceptible to infection or inflammation. Examination of bacterial translocation following a bout of inflammation revealed that the epithelium was in fact more conducive to the translocation of bacteria. Furthermore, 6 wk after the induction of colitis, increased levels of bacteria were present in the colon. These data indicate that alterations in epithelial function following a bout of inflammation are associated with increased bacterial colonization and translocation across the mucosa. This was observed at the same time that elevated iNOS activity was detected in the colon (chapter 4). These results are consistent with the findings that increased bacterial translocation in septicemia is attributed to induction of iNOS expression (140,141). This suggests that the altered epithelial secretory and barrier function were at least partially due to iNOS-derived NO. However, the increased bacterial translocation occurred despite the lack of detection of permeability changes in the intestine. Moreover, translocation of neutrophils across the epithelium was persistent in the intestine during this post-inflammatory period. Examination of epithelial permeability and mucus secretion *in vivo* did not reveal any

significant alterations 6 wk after the induction of colitis. These data suggest that changes in the "epithelial barrier function" are not necessarily detected as changes in permeability of a small molecular weight probe.

This study provided strong evidence that epithelial changes occur following a bout of inflammation. However, a limitation of this study is the fact that the method used to examine fluid secretion did not directly differentiate between changes in fluid absorption versus fluid secretion. That is, theoretically, an increase in the volume of fluid recovered from the colon could have been due to increased fluid secretion, decreased fluid absorption, or both. As previously described in chapter 4, we assumed that the increased fluid recovered following stimulation with agents such as toxin A and IBMX was likely due to increased fluid secretion. The reason for this is that *C. difficile* toxin A and IBMX have both been shown to cause fluid accumulation in the colon as a result of active chloride secretion (221,224,243). Another limitation of the method used to measure fluid secretion was that it was not possible to know what was being secreted when detecting changes in the volume of fluid recovered. The increased fluid secretion could have been due to increased water and electrolyte secretion and/or mucus secretion. However, it was shown that mucus secretory responses were not different between control rats and rats with colitis 6 wk earlier. This suggested that changes in the volume of fluid recovered due to mucus secretion would be similar in control and treated rats. Thus, the limitations described above are important to consider when interpreting the epithelial responses in this study.

Another limitation of this study was with respect to the link between bacterial translocation and epithelial dysfunction. The findings of this study include the fact that a

bout of inflammation resulted in epithelial dysfunction and increased bacterial colonization and translocation across the mucosa. However, the link between these two findings was correlative. That is, we simply showed an association between these factors following a bout of inflammation and did not directly provide evidence that epithelial dysfunction caused increased bacterial translocation. This is not to say that epithelial dysfunction did not result in increased bacterial translocation, but rather the fact that when interpreting these results it is important to realize that we have demonstrated correlation and not causation.

6.4. Future Considerations & Directions

In the study examining the role of nitric oxide in colitis, a series of NOS inhibitors (non-selective, iNOS-selective and nNOS-selective) did not have an effect on the extent of macroscopic damage or MPO activity. Interestingly, despite NOS inhibition there was no change in total free radical production when compared to vehicle treatment in rats with colitis. Furthermore, superoxide production was reduced in rats treated with all NOS inhibitors, whereas hydroxyl radical production was elevated with non-selective and iNOS-selective inhibitor treatment. These results illustrate the importance of interactions among free radicals. Thus, one question that comes to mind based on these findings is which free radical species, whether it be oxygen- or nitrogen-derived, are present in the inflamed colon. Secondly, how does altering the levels of NO produced in the inflamed colon affect which free radicals are produced? Answering these questions is difficult due to the limitations of what can be detected experimentally. As technological

advancements are made, our ability to detect and distinguish between the various free radical species should some light as to which species are important determinants of tissue destruction versus tissue protection.

The findings of this project also shed some light on possible mechanisms of intestinal dysfunction following a bout of inflammation. Nevertheless, many questions remain in our understanding of inflammation-induced effects on intestinal function. In this project, we have demonstrated that epithelial dysfunction persists despite the lack of macroscopic evidence of inflammation-induced damage to the mucosa, and that this could lead to increased susceptibility to subsequent infection/inflammation. Although, we have provided some speculation (i.e., iNOS-derived NO and increased cell proliferation) as to why the epithelial dysfunction occurred, further experiments examining the mechanism for the epithelial hypo-responsiveness are necessary. For example, determining the role of other inflammatory mediators such as PGD_2 in electrolyte transport during the post-inflammatory period may be beneficial to understanding the mechanism of epithelial hypo-responsiveness. Furthermore, experiments examining whether intracellular changes (i.e., in signal transduction pathways) occur within the epithelial cell following a bout of inflammation might be helpful in determining whether changes in the epithelial cell itself make the epithelium hypo-responsive. Given the proposed mechanism of toxin A-stimulated secretion, it would also be particularly interesting to examine the long-term effects of inflammation on the cytoskeletal structure of epithelial cells, as well as the effects on mast cells and enteric nerves.

Finally, with regards to the long-term effects of inflammation, it is also important to know whether changes in mast cell-nerve interaction and/or pain perception exist in the colon. The relevance of this is that knowing what other aspects of intestinal function are altered by a bout of inflammation may help us understand what factors influence the underlying susceptibility to relapse in IBD, and/or symptoms of abdominal pain and diarrhea in IBS. Moreover, determining the mechanisms of change in intestinal function are essential to understanding whether changes in smooth muscle, nerve, and epithelial function are unrelated to each other or rather a direct result of one another. This study revealed that in association with altered epithelial secretion, increased bacterial translocation was characteristic of the colon following a bout of colitis. Although it was previously described that iNOS-derived NO might be responsible for this phenomenon, it is also possible that other aspects of the mucosal defence system are altered as a result of prior inflammation. For example, altered production and secretion of IgA could forseeably result in increased antigen and bacterial translocation across the mucosa. As a result, it would not be unreasonable to examine the expression and function of luminal IgA following a bout of inflammation in future studies.

In summary, the data of this project suggest that inflammation has long-term effects on epithelial function that may predispose the intestine to subsequent exposure to luminal antigens and microbial pathogens. With respect to human disease, in particular post-infectious IBS, the findings of this study have clinical significance. Despite the macroscopic appearance of an intact epithelium, previous inflammatory episodes might predispose the intestinal tract to continual symptoms of gut dysfunction. For example, IBS patients experience nausea, bloating and diarrhea (34) despite the lack of any

clinically evident signs of inflammmation or intestinal injury. In this study mucus secretion was not altered following a bout of inflammation. However, unlike the case with ulcerative colitis, IBS patients have been found to have diarrhea in association with increased mucus secretion (34). As a result, the relevance of these findings to IBD and IBS must be considered bearing these differences in mind.

References

1. Stenson WF. Yamada T, editors.Textbook of Gastroenterology. 2 ed. Philadelphia: JB Lippincott Company; 1995;Inflammatory Bowel Disease. p. 1748-806.

2. McKendrick MW, Read NW. Irritable bowel syndrome--post salmonella infection [see comments]. J Infect 1994;29(1):1-3.

3. Neal KR, Hebden J, Spiller R. Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients [see comments]. BMJ 1997;314(7083):779-82.

4. Rodriguez LA, Ruigomez A. Increased risk of irritable bowel syndrome after bacterial gastroenteritis: cohort study. BMJ 1999;318(7183):565-6.

5. LaMont JT, Trnka YM. Therapeutic implications of Clostridium difficile toxin during relapse of chronic inflammatory bowel disease. Lancet 1980;1(8165):381-3.

6. Kubes P. Nitric oxide modulates epithelial permeability in the feline small intestine. Am J Physiol 1992;262(6 Pt 1):G1138-G1142

7. Kanwar S, Wallace JL, Befus D, Kubes P. Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. Am J Physiol 1994;266(2 Pt 1):G222-G229

8. McKay DM, Baird AW. Cytokine regulation of epithelial permeability and ion transport [In Process Citation]. Gut 1999;44(2):283-9.

9. Madara JL, Stafford J. Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. J Clin Invest 1989;83(2):724-7.

10. Madsen KL, Lewis SA, Tavernini MM, Hibbard J, Fedorak RN. Interleukin 10 prevents cytokine-induced disruption of T84 monolayer barrier integrity and limits chloride secretion. Gastroenterology 1997;113(1):151-9.

11. Nash S, Stafford J, Madara JL. Effects of polymorphonuclear leukocyte transmigration on the barrier function of cultured intestinal epithelial monolayers. J Clin Invest 1987;80(4):1104-13.

12. Von Ritter C, Grisham MB, Hollwarth M, Inauen W, Granger DN. Neutrophil-derived oxidants mediate formyl-methionyl-leucyl- phenylalanine-induced increases in mucosal permeability in rats. Gastroenterology 1989;97(3):778-80.

13. Wallace JL, Higa A, McKnight GW, MacIntyre DE. Prevention and reversal of experimental colitis by a monoclonal antibody which inhibits leukocyte adherence. Inflammation 1992;16(4):343-54.

14. Kraehenbuhl JP, Pringault E, Neutra MR. Review article: Intestinal epithelia and barrier functions. Aliment Pharmacol Ther 1997;11 Suppl 3:3-8.

15. Mestecky J, Russell MW, Elson CO. Intestinal IgA: novel views on its function in the defence of the largest mucosal surface. Gut 1999;44(1):2-5.

16. Russell MW, Sibley DA, Nikolova EB, Tomana M, Mestecky J. IgA antibody as a non-inflammatory regulator of immunity. Biochem Soc Trans 1997;25(2):466-70.

17. Tagliabue A, Nencioni L, Villa L, Keren DF, Lowell GH, Boraschi D. Antibody-dependent cell-mediated antibacterial activity of intestinal lymphocytes with secretory IgA. Nature 1983;306(5939):184-6.

18. Barrett KE, Keely SJ. Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. Annu Rev Physiol 2000;62:535-72.

19. Marcial MA, Carlson SL, Madara JL. Partitioning of paracellular conductance along the ileal crypt-villus axis: a hypothesis based on structural analysis with detailed consideration of tight junction structure-function relationships. J Membr Biol 1984;80(1):59-70.

20. Madara JL, Trier JS, Neutra MR. Structural changes in the plasma membrane accompanying differentiation of epithelial cells in human and monkey small intestine. Gastroenterology 1980;78(5 Pt 1):963-75.

21. Tapper EJ. Local modulation of intestinal ion transport by enteric neurons. Am J Physiol 1983;244(5):G457-G468

22. Gershon MD. Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. Aliment Pharmacol Ther 1999;13 Suppl 2:15-30.

23. Mynott TL, Guandalini S, Raimondi F, Fasano A. Bromelain prevents secretion caused by Vibrio cholerae and Escherichia coli enterotoxins in rabbit ileum in vitro [published erratum appears in Gastroenterology 1997 Oct;113(4):1425]. Gastroenterology 1997;113(1):175-84.

24. Rolfe V, Levin RJ. Enterotoxin Escherichia coli STa activates a nitric oxide-dependent myenteric plexus secretory reflex in the rat ileum. J Physiol (Lond) 1994;475(3):531-7.

25. Beubler E, Kollar G, Saria A, Bukhave K, Rask-Madsen J. Involvement of 5-hydroxytryptamine, prostaglandin E2, and cyclic adenosine monophosphate in cholera toxin-induced fluid secretion in the small intestine of the rat in vivo. Gastroenterology 1989;96(2 Pt 1):368-76.

26. Holtug K, Hansen MB, Skadhauge E. Experimental studies of intestinal ion and water transport. Scand J Gastroenterol Suppl 1996;216:95-110.

27. Phillips TE, Phillips TH, Neutra MR. Regulation of intestinal goblet cell secretion. III. Isolated intestinal epithelium. Am J Physiol 1984;247(6 Pt 1):G674-G681

28. Perdue MH, Chung M, Gall DG. Effect of intestinal anaphylaxis on gut function in the rat. Gastroenterology 1984;86(3):391-7.

29. Neutra MR, O'Malley LJ, Specian RD. Regulation of intestinal goblet cell secretion. II. A survey of potential secretagogues. Am J Physiol 1982;242(4):G380-G387

30. Moore BA, Sharkey KA, Mantle M. Neural mediation of cholera toxin-induced mucin secretion in the rat small intestine. Am J Physiol 1993;265(6 Pt 1):G1050-G1056

31. Belley A, Chadee K. Prostaglandin E(2) stimulates rat and human colonic mucin exocytosis via the EP(4) receptor. Gastroenterology 1999;117(6):1352-62.

32. Halm DR, Halm ST. Secretagogue response of goblet cells and columnar cells in human colonic crypts [corrected and republished in Am J Physiol 2000 Jan;278(1):212-33]. Am J Physiol 1999;277(3 Pt 1):C501-C522

33. Plaisancie P, Barcelo A, Moro F, Claustre J, Chayvialle JA, Cuber JC. Effects of neurotransmitters, gut hormones, and inflammatory mediators on mucus discharge in rat colon. Am J Physiol 1998;275(5 Pt 1):G1073-G1084

34. Hasler WL, Owyyang C. Yamada T, editors.Textbook of Gastroenterology. 2 ed. Philadelphia: JB Lippincott Company; 1995; 81, Irritable bowel syndrome. p. 1833-55.

35. Vermillion DL, Collins SM. Increased responsiveness of jejunal longitudinal muscle in Trichinella-infected rats. Am J Physiol 1988;254(1 Pt 1):G124-G129

36. Marzio L, Blennerhassett P, Chiverton S, Vermillion DL, Langer J, Collins SM. Altered smooth muscle function in worm-free gut regions of Trichinella-infected rats. Am J Physiol 1990;259(2 Pt 1):G306-G313

37. Grossi L, McHugh K, Collins SM. On the specificity of altered muscle function in experimental colitis in rats. Gastroenterology 1993;104(4):1049-56.

38. Wallace JL, Byles NI, Scott RB. Snape WJJr, Collins SM, editors.Effects of Immune Cells and Inflammation on Smooth Muscle and Enteric Nerves. Boca Raton: CRC Press Inc; 1991; 22, Inflammatory Mediators and Gastrointestinal Motility. p. 271-9.

39. Vermillion DL, Huizinga JD, Riddell RH, Collins SM. Altered small intestinal smooth muscle function in Crohn's disease. Gastroenterology 1993;104(6):1692-9.

40. Swain MG, Blennerhassett PA, Collins SM. Impaired sympathetic nerve function in the inflamed rat intestine. Gastroenterology 1991;100(3):675-82.

41. Jacobson K, McHugh K, Collins SM. Experimental colitis alters myenteric nerve function at inflamed and noninflamed sites in the rat. Gastroenterology 1995;109(3):718-22.

42. Jacobson K, McHugh K, Collins SM. The mechanism of altered neural function in a rat model of acute colitis. Gastroenterology 1997;112(1):156-62.

43. Chang EB, Musch MW, Mayer L. Interleukins 1 and 3 stimulate anion secretion in chicken intestine. Gastroenterology 1990;98(6):1518-24.

44. Chiossone DC, Simon PL, Smith PL. Interleukin-1: effects on rabbit ileal mucosal ion transport in vitro. Eur J Pharmacol 1990;180(2-3):217-28.

45. Schmitz H, Fromm M, Bode H, Scholz P, Riecken EO, Schulzke JD. Tumor necrosis factor-alpha induces Cl- and K+ secretion in human distal colon driven by prostaglandin E2. Am J Physiol 1996;271(4 Pt 1):G669-G674

46. Bode H, Schmitz H, Fromm M, Scholz P, Riecken EO, Schulzke JD. IL-1beta and TNF-alpha, but not IFN-alpha, IFN-gamma, IL-6 or IL-8, are secretory mediators in human distal colon. Cytokine 1998;10(6):457-65.

47. Zund G, Madara JL, Dzus AL, Awtrey CS, Colgan SP. Interleukin-4 and interleukin-13 differentially regulate epithelial chloride secretion. J Biol Chem 1996;271(13):7460-4.

48. Colgan SP, Resnick MB, Parkos CA, Delp-Archer C, McGuirk D, Bacarra AE, Weller PF, Madara JL. IL-4 directly modulates function of a model human intestinal epithelium. J Immunol 1994;153(5):2122-9.

49. Holmgren J, Fryklund J, Larsson H. Gamma-interferon-mediated down-regulation of electrolyte secretion by intestinal epithelial cells: a local immune mechanism? Scand J Immunol 1989;30(4):499-503.

50. Keenan CM, Rangachari PK. Eicosanoid interactions in the canine proximal colon. Am J Physiol 1989;256(4 Pt 1):G673-G679

51. Sobhani I, Vidon N, Huchet B, Rambaud JC. Human jejunal secretion induced by prostaglandin E1: a dose-response study. Br J Clin Pharmacol 1991;31(4):433-7.

52. Blume ED, Taylor CT, Lennon PF, Stahl GL, Colgan SP. Activated endothelial cells elicit paracrine induction of epithelial chloride secretion. 6-Keto-PGF1alpha is an epithelial secretagogue. J Clin Invest 1998;102(6):1161-72.

53. Keenan CM, Rangachari PK. Contrasting effects of PGE2 and PGD2: ion transport in the canine proximal colon. Am J Physiol 1991;260(3 Pt 1):G481-G488

54. Jett MF, Marshall P, Fondacaro JD, Smith PL. Action of peptidoleukotrienes on ion transport in rabbit distal colon in vitro. J Pharmacol Exp Ther 1991;257(2):698-705.

55. Smith PL, Chiossone DC, McCafferty GP. Characterization of LTC4 effects on rabbit ileal mucosa in vitro. Naunyn Schmiedebergs Arch Pharmacol 1990;341(1-2):94-100.

56. Traynor TR, Brown DR, O'Grady SM. Neuromodulation of ion transport in porcine distal colon: NPY reduces secretory actions of leukotrienes. Am J Physiol 1995;269(2 Pt 2):R426-R431

57. Wang YZ, Cooke HJ, Su HC, Fertel R. Histamine augments colonic secretion in guinea pig distal colon. Am J Physiol 1990;258(3 Pt 1):G432-G439

58. Crowe SE, Sestini P, Perdue MH. Allergic reactions of rat jejunal mucosa. Ion transport responses to luminal antigen and inflammatory mediators. Gastroenterology 1990;99(1):74-82.

59. Perdue MH, Masson S, Wershil BK, Galli SJ. Role of mast cells in ion transport abnormalities associated with intestinal anaphylaxis. Correction of the diminished secretory response in genetically mast cell-deficient W/Wv mice by bone marrow transplantation. J Clin Invest 1991;87(2):687-93.

60. Browning JG, Hardcastle J, Hardcastle PT, Sanford PA. The role of acetylcholine in the regulation of ion transport by rat colon mucosa. J Physiol (Lond) 1977;272(3):737-54.

61. Traynor TR, Brown DR, O'Grady SM. Regulation of ion transport in porcine distal colon: effects of putative neurotransmitters. Gastroenterology 1991;100(3):703-10.

62. Dharmsathaphorn K, Pandol SJ. Mechanism of chloride secretion induced by carbachol in a colonic epithelial cell line. J Clin Invest 1986;77(2):348-54.

63. Dharmsathaphorn K, Mandel KG, Masui H, McRoberts JA. Vasoactive intestinal polypeptide-induced chloride secretion by a colonic epithelial cell line.

Direct participation of a basolaterally localized Na+,K+,Cl- cotransport system. J Clin Invest 1985;75(2):462-71.

64. Friel DD, Miller RJ, Walker MW. Neuropeptide Y: a powerful modulator of epithelial ion transport. Br J Pharmacol 1986;88(2):425-31.

65. Hardcastle J, Hardcastle PT, Redfern JS. Action of 5-hydroxytryptamine on intestinal ion transport in the rat. J Physiol (Lond) 1981;320:41-55.

66. Kuwahara A, Kuramoto H, Kadowaki M. 5-HT activates nitric oxide-generating neurons to stimulate chloride secretion in guinea pig distal colon. Am J Physiol 1998;275(4 Pt 1):G829-G834

67. Katsuki S, Arnold W, Mittal C, Murad F. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. J Cyclic Nucleotide Res 1977;3(1):23-35.

68. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288(5789):373-6.

69. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A 1987;84(24):9265-9.

70. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327(6122):524-6.

71. Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 1991;351(6329):714-8.

72. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. Cell 1994;78(6):915-8.

73. Dinerman JL, Dawson TM, Schell MJ, Snowman A, Snyder SH. Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. Proc Natl Acad Sci U S A 1994;91(10):4214-8.

74. Lundberg JO, Farkas-Szallasi T, Weitzberg E, Rinder J, Lidholm J, Anggaard A, Hokfelt T, Lundberg JM, Alving K. High nitric oxide production in human paranasal sinuses. Nat Med 1995;1(4):370-3.

75. Kobzik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle [see comments]. Nature 1994;372(6506):546-8.

76. Bonner FT, Stedman G. Feelisch M, Stamler JS, editors.Methods in Nitric Oxide Research. West Sussex: John Wiley & Sons Ltd; 1996; 1, Chemistry, biochemistry, kinetics and metabolism of nitrogen oxides. p. 3-18.

77. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. Cardiovasc Res 1999;43(3):521-31.

78. List BM, Klosch B, Volker C, Gorren AC, Sessa WC, Werner ER, Kukovetz WR, Schmidt K, Mayer B. Characterization of bovine endothelial nitric oxide synthase as a homodimer with down-regulated uncoupled NADPH oxidase activity: tetrahydrobiopterin binding kinetics and role of haem in dimerization. Biochem J 1997;323 (Pt 1):159-65.

79. Xia Y, Zweier JL. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc Natl Acad Sci U S A 1997;94(13):6954-8.

80. Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci U S A 1996;93(13):6770-4.

81. Pou S, Keaton L, Surichamorn W, Rosen GM. Mechanism of superoxide generation by neuronal nitric-oxide synthase. J Biol Chem 1999;274(14):9573-80.

82. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KAJ. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci U S A 1998;95(16):9220-5.

83. Xia Y, Roman LJ, Masters BS, Zweier JL. Inducible nitric-oxide synthase generates superoxide from the reductase domain. J Biol Chem 1998;273(35):22635-9.

84. Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL. Nitric oxide synthase generates superoxide and nitric oxide in arginine- depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci U S A 1996;93(13):6770-4.

85. Wang W, Wang S, Yan L, Madara P, Del Pilar C, Wesley RA, Danner RL. Superoxide production and reactive oxygen species signaling by endothelial nitric-oxide synthase [In Process Citation]. J Biol Chem 2000;275(22):16899-903.

86. Miller MJ, Sandoval M. Nitric Oxide. III. A molecular prelude to intestinal inflammation. Am J Physiol 1999;276(4 Pt 1):G795-G799

87. Grisham MB, Jourd'Heuil D, Wink DA. Review article: chronic inflammation and reactive oxygen and nitrogen metabolism--implications in DNA damage and mutagenesis [In Process Citation]. Aliment Pharmacol Ther 2000;14 Suppl 1:3-9.

88. Hughes MN. Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. Biochim Biophys Acta 1999;1411(2-3):263-72.

89. Wink DA, Feelisch M, Fukuto J, Chistodoulou D, Jourd'Heuil D, Grisham MB, Vodovotz Y, Cook JA, Krishna M, DeGraff WG, et al. The cytotoxicity

of nitroxyl: possible implications for the pathophysiological role of NO. Arch Biochem Biophys 1998;351(1):66-74.

90. Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide [see comments]. Am J Physiol 1995;268(5 Pt 1):L699-L722

91. Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. Chem Res Toxicol 1996;9(5):836-44.

92. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 1990;87(4):1620-4.

93. Grisham MB, Granger DN. Neutrophil-mediated mucosal injury. Role of reactive oxygen metabolites. Dig Dis Sci 1988;33(3 Suppl):6S-15S.

94. Beckman JS, Ischiropoulos H, Zhu L, van der Woerd M, Smith C, Chen J, Harrison J, Martin JC, Tsai M. Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite. Arch Biochem Biophys 1992;298(2):438-45.

95. van d, V, Eiserich JP, O'Neill CA, Halliwell B, Cross CE. Tyrosine modification by reactive nitrogen species: a closer look. Arch Biochem Biophys 1995;319(2):341-9.

96. McDonald LJ, Murad F. Nitric oxide and cyclic GMP signaling. Proc Soc Exp Biol Med 1996;211(1):1-6.

97. Rajfer J, Aronson WJ, Bush PA, Dorey FJ, Ignarro LJ. Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. N Engl J Med 1992;326(2):90-4.

98. Bhagat K, Vallance P. Nitric oxide 9 years on. J R Soc Med 1996;89(12):667-73.

99. Vallance P, Moncada S. Role of endogenous nitric oxide in septic shock. New Horiz 1993;1(1):77-86.

100. Radomski MW, Palmer RM, Moncada S. The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. Biochem Biophys Res Commun 1987;148(3):1482-9.

101. O'Dell TJ, Hawkins RD, Kandel ER, Arancio O. Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. Proc Natl Acad Sci U S A 1991;88(24):11285-9.

102. Nogawa S, Forster C, Zhang F, Nagayama M, Ross ME, Iadecola C. Interaction between inducible nitric oxide synthase and cyclooxygenase-2 after cerebral ischemia. Proc Natl Acad Sci U S A 1998;95(18):10966-71.

103. Wagner C, Jensen BL, Kramer BK, Kurtz A. Control of the renal renin system by local factors. Kidney Int Suppl 1998;67:S78-S83

104. Wilcox CS, Welch WJ, Murad F, Gross SS, Taylor G, Levi R, Schmidt HH. Nitric oxide synthase in macula densa regulates glomerular capillary pressure. Proc Natl Acad Sci U S A 1992;89(24):11993-7.

105. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci U S A 1991;88(11):4651-5.

106. Holzer P, Lippe IT, Jocic M, Wachter C, Erb R, Heinemann A. Nitric oxide-dependent and -independent hyperaemia due to calcitonin gene-related peptide in the rat stomach. Br J Pharmacol 1993;110(1):404-10.

107. Holzer P, Wachter C, Heinemann A, Jocic M, Lippe IT, Herbert MK. Diverse interactions of calcitonin gene related peptide and nitric oxide in the gastric and cutaneous microcirculation. Can J Physiol Pharmacol 1995;73(7):991-4.

108. Whittle BJ, Lopez-Belmonte J, Moncada S. Nitric oxide mediates rat mucosal vasodilatation induced by intragastric capsaicin. Eur J Pharmacol 1992;218(2-3):339-41.

109. Peskar BM, Respondek M, Muller KM, Peskar BA. A role for nitric oxide in capsaicin-induced gastroprotection. Eur J Pharmacol 1991;198(1):113-4.

110. Walder CE, Thiemermann C, Vane JR. Endothelium-derived relaxing factor participates in the increased blood flow in response to pentagastrin in the rat stomach mucosa. Proc R Soc Lond B Biol Sci 1990;241(1302):195-200.

111. Kato S, Kitamura M, Korolkiewicz RP, Takeuchi K. Role of nitric oxide in regulation of gastric acid secretion in rats: effects of NO donors and NO synthase inhibitor. Br J Pharmacol 1998;123(5):839-46.

112. Takeuchi K, Takehara K, Kaneko T, Okabe S. Nitric oxide and prostaglandins in regulation of acid secretory response in rat stomach following injury. J Pharmacol Exp Ther 1995;272(1):357-63.

113. Brown JF, Hanson PJ, Whittle BJ. Nitric oxide donors increase mucus gel thickness in rat stomach. Eur J Pharmacol 1992;223(1):103-4.

114. Price KJ, Hanson PJ, Whittle BJ. Stimulation by carbachol of mucus gel thickness in rat stomach involves nitric oxide. Eur J Pharmacol 1994;263(1-2):199-202.

115. MacNaughton WK, Cirino G, Wallace JL. Endothelium-derived relaxing factor (nitric oxide) has protective actions in the stomach. Life Sci 1989;45(20):1869-76.

116. Diamant NE. Neuromuscular mechanisms of primary peristalsis. Am J Med 1997;103(5A):40S-3S.

117. Kubes P. Ischemia-reperfusion in feline small intestine: a role for nitric oxide. Am J Physiol 1993;264(1 Pt 1):G143-G149

118. MacNaughton WK. Nitric oxide-donating compounds stimulate electrolyte transport in the guinea pig intestine in vitro. Life Sci 1993;53(7):585-93.

119. Tamai H, Gaginella TS. Direct evidence for nitric oxide stimulation of electrolyte secretion in the rat colon. Free Radic Res Commun 1993;19(4):229-39.

120. Schirgi-Degen A, Beubler E. Significance of nitric oxide in the stimulation of intestinal fluid absorption in the rat jejunum in vivo. Br J Pharmacol 1995;114(1):13-8.

121. Beubler E, Schirgi-Degen A. Nitric oxide counteracts 5-hydroxytryptamine- and cholera toxin-induced fluid secretion and enhances the effect of oral rehydration solution. Eur J Pharmacol 1997;326(2-3):223-8.

122. Wilson KT, Xie Y, Musch MW, Chang EB. Sodium nitroprusside stimulates anion secretion and inhibits sodium chloride absorption in rat colon. J Pharmacol Exp Ther 1993;266(1):224-30.

123. Stack WA, Filipowicz B, Hawkey CJ. Nitric oxide donating compounds stimulate human colonic ion transport in vitro. Gut 1996;39(1):93-9.

124. MacNaughton WK, Lowe SS, Cushing K. Role of nitric oxide in inflammation-induced suppression of secretion in a mouse model of acute colitis. Am J Physiol 1998;275(6 Pt 1):G1353-G1360

125. Freeman SL, MacNaughton WK. Ionizing radiation induces iNOS-mediated epithelial dysfunction in the absence of an inflammatory response. Am J Physiol Gastrointest Liver Physiol 2000;278(2):G243-G250

126. Izzo AA, Mascolo N, Capasso F. Nitric oxide as a modulator of intestinal water and electrolyte transport. Dig Dis Sci 1998;43(8):1605-20.

127. Rao RK, Riviere PJ, Pascaud X, Junien JL, Porreca F. Tonic regulation of mouse ileal ion transport by nitric oxide. J Pharmacol Exp Ther 1994;269(2):626-31.

128. Schirgi-Degen A, Beubler E. Involvement of K+ channel modulation in the proabsorptive effect of nitric oxide in the rat jejunum in vivo. Eur J Pharmacol 1996;316(2-3):257-62.

129. Barry MK, Aloisi JD, Pickering SP, Yeo CJ. Nitric oxide modulates water and electrolyte transport in the ileum. Ann Surg 1994;219(4):382-8.

130. Maher MM, Gontarek JD, Jimenez RE, Cahill PA, Yeo CJ. Endogenous nitric oxide promotes ileal absorption. J Surg Res 1995;58(6):687-92.

131. Mourad FH, O'Donnell LJ, Andre EA, Bearcroft CP, Owen RA, Clark ML, Farthing MJ. L-Arginine, nitric oxide, and intestinal secretion: studies in rat jejunum in vivo. Gut 1996;39(4):539-44.

132. Brand HS, Maas MA, Bosma A, Van Ketel RJ, Speelman P, Chamuleau RA. Experimental colitis in rats induces low-grade endotoxinemia without hepatobiliary abnormalities. Dig Dis Sci 1994;39(6):1210-5.

133. Mascolo N, Izzo AA, Barbato F, Capasso F. Inhibitors of nitric oxide synthetase prevent castor-oil-induced diarrhoea in the rat. Br J Pharmacol 1993;108(4):861-4.

134. Wapnir RA, Wingertzahn MA, Teichberg S. L-arginine in low concentration improves rat intestinal water and sodium absorption from oral rehydration solutions [see comments]. Gut 1997;40(5):602-7.

135. Collins SM. Is the irritable gut an inflamed gut? Scand J Gastroenterol Suppl 1992;192:102-5.

136. Collins SM, Barbara G, Vallance B. Stress, inflammation and the irritable bowel syndrome. Can J Gastroenterol 1999;13 Suppl A:47A-9A.

137. Chaudhary NA, Truelove SC. Human colonic motility. A comparative study of normal subjects, patients with ulcerative colitis, and patients with the irritable colon syndrome. Gastroenterology 1968;54(4):Suppl-8

138. Bell CJ, Gall DG, Wallace JL. Disruption of colonic electrolyte transport in experimental colitis. Am J Physiol 1995;268(4 Pt 1):G622-G630

139. McKay DM, Croitoru K, Perdue MH. T cell-monocyte interactions regulate epithelial physiology in a coculture model of inflammation. Am J Physiol 1996;270(2 Pt 1):C418-C428

140. Mishima S, Xu D, Lu Q, Deitch EA. Bacterial translocation is inhibited in inducible nitric oxide synthase knockout mice after endotoxin challenge but not in a model of bacterial overgrowth. Arch Surg 1997;132(11):1190-5.

141. Sorrells DL, Friend C, Koltuksuz U, Courcoulas A, Boyle P, Garrett M, Watkins S, Rowe MI, Ford HR. Inhibition of nitric oxide with aminoguanidine reduces bacterial translocation after endotoxin challenge in vivo. Arch Surg 1996;131(11):1155-63.

142. Grisham MB, Jourd'Heuil D, Wink DA. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. Am J Physiol 1999;276(2 Pt 1):G315-G321

143. Grisham MB, Specian RD, Zimmerman TE. Effects of nitric oxide synthase inhibition on the pathophysiology observed in a model of chronic granulomatous colitis. J Pharmacol Exp Ther 1994;271(2):1114-21.

144. Boughton-Smith NK, Evans SM, Hawkey CJ, Cole AT, Balsitis M, Whittle BJ, Moncada S. Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. Lancet 1993;342(8867):338-40.

145. Kimura H, Hokari R, Miura S, Shigematsu T, Hirokawa M, Akiba Y, Kurose I, Higuchi H, Fujimori H, Tsuzuki Y, et al. Increased expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in colonic mucosa of patients with active ulcerative colitis [see comments]. Gut 1998;42(2):180-7.

146. Rachmilewitz D, Stamler JS, Bachwich D, Karmeli F, Ackerman Z, Podolsky DK. Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn's disease. Gut 1995;36(5):718-23.

147. Hogaboam CM, Jacobson K, Collins SM, Blennerhassett MG. The selective beneficial effects of nitric oxide inhibition in experimental colitis. Am J Physiol 1995;268(4 Pt 1):G673-G684

148. Singer II, Kawka DW, Scott S, Weidner JR, Mumford RA, Riehl TE, Stenson WF. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. Gastroenterology 1996;111(4):871-85.

149. McCafferty DM, Miampamba M, Sihota E, Sharkey KA, Kubes P. Role of inducible nitric oxide synthase in trinitrobenzene sulphonic acid induced colitis in mice. Gut 1999;45(6):864-73.

150. Dijkstra G, Moshage H, van Dullemen HM, de Jager-Krikken A, Tiebosch AT, Kleibeuker JH, Jansen PL, van Goor H. Expression of nitric oxide

synthases and formation of nitrotyrosine and reactive oxygen species in inflammatory bowel disease. J Pathol 1998;186(4):416-21.

151. Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 1989;96(3):795-803.

152. Reuter BK, Asfaha S, Buret A, Sharkey KA, Wallace JL. Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. J Clin Invest 1996;98(9):2076-85.

153. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol 1982;78(3):206-9.

154. Schultz J, Corlin R, Oddi F, Kaminker K, Jones W. Myeloperoxidase of the leucocyte of normal human blood. 3. Isolation of the peroxidase granule. Arch Biochem Biophys 1965;111(1):73-9.

155. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. Anal Biochem 1987;162(1):156-9.

156. Foley KP, Leonard MW, Engel JD. Quantitation of RNA using the polymerase chain reaction. Trends Genet 1993;9(11):380-5.

157. Lyons CR, Orloff GJ, Cunningham JM. Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. J Biol Chem 1992;267(9):6370-4.

158. Wong H, Anderson WD, Cheng T, Riabowol KT. Monitoring mRNA expression by polymerase chain reaction: the "primer- dropping" method. Anal Biochem 1994;223(2):251-8.

159. Miller MJ, Sadowska-Krowicka H, Chotinaruemol S, Kakkis JL, Clark DA. Amelioration of chronic ileitis by nitric oxide synthase inhibition. J Pharmacol Exp Ther 1993;264(1):11-6.

160. Aiko S, Fuseler J, Grisham MB. Effects of nitric oxide synthase inhibition or sulfasalazine on the spontaneous colitis observed in HLA-B27 transgenic rats. J Pharmacol Exp Ther 1998;284(2):722-7.

161. Conner EM, Brand S, Davis JM, Laroux FS, Palombella VJ, Fuseler JW, Kang DY, Wolf RE, Grisham MB. Proteasome inhibition attenuates nitric oxide synthase expression, VCAM- 1 transcription and the development of chronic colitis. J Pharmacol Exp Ther 1997;282(3):1615-22.

162. Rachmilewitz D, Karmeli F, Okon E, Bursztyn M. Experimental colitis is ameliorated by inhibition of nitric oxide synthase activity [see comments]. Gut 1995;37(2):247-55.

163. Ribbons KA, Currie MG, Connor JR, Manning PT, Allen PC, Didier P, Ratterree MS, Clark DA, Miller MJ. The effect of inhibitors of inducible nitric

oxide synthase on chronic colitis in the rhesus monkey. J Pharmacol Exp Ther 1997;280(2):1008-15.

164. Dobosz M, Mionskowska L, Dobrowolski S, Dymecki D, Makarewicz W, Hrabowska M, Wajda Z. Is nitric oxide and heparin treatment justified in inflammatory bowel disease? An experimental study. Scand J Clin Lab Invest 1996;56(7):657-63.

165. Pfeiffer CJ, Qiu BS. Effects of chronic nitric oxide synthase inhibition on TNB-induced colitis in rats. J Pharm Pharmacol 1995;47(10):827-32.

166. Southey A, Tanaka S, Murakami T, Miyoshi H, Ishizuka T, Sugiura M, Kawashima K, Sugita T. Pathophysiological role of nitric oxide in rat experimental colitis. Int J Immunopharmacol 1997;19(11-12):669-76.

167. Zingarelli B, Cuzzocrea S, Szabo C, Salzman AL. Mercaptoethylguanidine, a combined inhibitor of nitric oxide synthase and peroxynitrite scavenger, reduces trinitrobenzene sulfonic acid- induced colonic damage in rats. J Pharmacol Exp Ther 1998;287(3):1048-55.

168. Varilek GW, Yang F, Ramanujam KS, et al. Inducible nitric oxide synthase activity is protective in the enterocoliits of IL-10 knockout mice. [Abstract] Gastroenterology 1998;114:(4)A1107

169. Panes J, Salas A, Gironella M, et al. NO supplementation ameliorates DSS-induced colitis in mice. [Abstract] FASEB J 2000;14:(4)A402

170. Wallace JL, Vergnolle N, Muscara MN, Asfaha S, Chapman K, McKnight W, Del Soldato P, Morelli A, Fiorucci S. Enhanced anti-inflammatory effects of a nitric oxide-releasing derivative of mesalamine in rats. Gastroenterology 1999;117(3):557-66.

171. McCafferty DM, Mudgett JS, Swain MG, Kubes P. Inducible nitric oxide synthase plays a critical role in resolving intestinal inflammation. Gastroenterology 1997;112(3):1022-7.

172. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 1996;271(5 Pt 1):C1424-C1437

173. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 1990;87(4):1620-4.

174. Miles AM, Bohle DS, Glassbrenner PA, Hansert B, Wink DA, Grisham MB. Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. J Biol Chem 1996;271(1):40-7.

175. Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van d, V. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 1998;391(6665):393-7.

176. Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, Knowles RG, Stack WA, Filipowicz B, Hawkey CJ. 1400W is a slow, tight binding, and

highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo

Nitric oxide donating compounds stimulate human colonic ion transport in vitro. J Biol Chem 1997;272(8):4959-63.

177. Laszlo F, Whittle BJ. Actions of isoform-selective and non-selective nitric oxide synthase inhibitors on endotoxin-induced vascular leakage in rat colon. Eur J Pharmacol 1997;334(1):99-102.

178. Ferraz JG, Sharkey KA, Reuter BK, Asfaha S, Tigley AW, Brown ML, McKnight W, Wallace JL. Induction of cyclooxygenase 1 and 2 in the rat stomach during endotoxemia: role in resistance to damage. Gastroenterology 1997;113(1):195-204.

179. Handy RL, Moore PK. A comparison of the effects of L-NAME, 7-NI and L-NIL on carrageenan- induced hindpaw oedema and NOS activity. Br J Pharmacol 1998;123(6):1119-26.

180. Davies NM, Sharkey KA, Asfaha S, MacNaughton WK, Wallace JL. Aspirin causes rapid up-regulation of cyclo-oxygenase-2 expression in the stomach of rats. Aliment Pharmacol Ther 1997;11(6):1101-8.

181. Simmonds NJ, Allen RE, Stevens TR, Van Someren RN, Blake DR, Rampton DS. Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. Gastroenterology 1992;103(1):186-96.

182. Hagar H, Ueda N, Shah SV. Role of reactive oxygen metabolites in DNA damage and cell death in chemical hypoxic injury to LLC-PK1 cells. Am J Physiol 1996;271(1 Pt 2):F209-F215

183. Rehman A, Whiteman M, Halliwell B. Scavenging of hydroxyl radicals but not of peroxynitrite by inhibitors and substrates of nitric oxide synthases. Br J Pharmacol 1997;122(8):1702-6.

184. Cocks TM, Angus JA. Comparison of relaxation responses of vascular and non-vascular smooth muscle to endothelium-derived relaxing factor (EDRF), acidified sodium nitrite (NO) and sodium nitroprusside. Naunyn Schmiedebergs Arch Pharmacol 1990;341(4):364-72.

185. Ekblad E, Sundler F. Motor responses in rat ileum evoked by nitric oxide donors vs. field stimulation: modulation by pituitary adenylate cyclase-activating peptide, forskolin and guanylate cyclase inhibitors. J Pharmacol Exp Ther 1997;283(1):23-8.

186. Mourelle M, Vilaseca J, Guarner F, Salas A, Malagelada JR. Toxic dilatation of colon in a rat model of colitis is linked to an inducible form of nitric oxide synthase. Am J Physiol 1996;270(3 Pt 1):G425-G430

187. Griffiths MJ, Messent M, MacAllister RJ, Evans TW. Aminoguanidine selectively inhibits inducible nitric oxide synthase. Br J Pharmacol 1993;110(3):963-8.

188. Yildiz G, Demiryurek AT, Sahin-Erdemli I, Kanzik I. Comparison of antioxidant activities of aminoguanidine, methylguanidine and guanidine by luminol-enhanced chemiluminescence. Br J Pharmacol 1998;124(5):905-10.

189. Seo HG, Takata I, Nakamura M, Tatsumi H, Suzuki K, Fujii J, Taniguchi N. Induction of nitric oxide synthase and concomitant suppression of superoxide dismutases in experimental colitis in rats. Arch Biochem Biophys 1995;324(1):41-7.

190. Grisham MB. A radical approach to treating inflammation. Trends Pharmacol Sci 2000;21(4):119-20.

191. Wood JD. Neuro-immunophysiology of colon function. Pharmacology 1993;47 Suppl 1:7-13.

192. Kachur JF, Keshavarzian A, Sundaresan R, Doria M, Walsh R, de las A, Gaginella TS. Colitis reduces short-circuit current response to inflammatory mediators in rat colonic mucosa. Inflammation 1995;19(2):245-59.

193. Field M, Rao MC, Chang EB. Intestinal electrolyte transport and diarrheal disease (2) [see comments]. N Engl J Med 1989;321(13):879-83.

194. Rask-Madsen J, Jensen PB. Electrolyte transport capacity and electrical potentials of the normal and the inflamed human rectum in vivo. Scand J Gastroenterol 1973;8(2):169-75.

195. Hawker PC, McKay JS, Turnberg LA. Electrolyte transport across colonic mucosa from patients with inflammatory bowel disease. Gastroenterology 1980;79(3):508-11.

196. Goldhill JM, Burakoff R, Donovan V, Rose K, Percy WH. Defective modulation of colonic secretomotor neurons in a rabbit model of colitis. Am J Physiol 1993;264(4 Pt 1):G671-G677

197. McKay DM, Ramage JR, Rangachari PK, Perdue MH. Effect of region, temperature and neuronal blockade on sodium and 51Cr- EDTA transport across canine gastrointestinal mucosae in vitro. Comp Biochem Physiol Comp Physiol 1994;107(4):711-7.

198. Moore WM, Webber RK, Jerome GM, Tjoeng FS, Misko TP, Currie MG. L-N6-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. J Med Chem 1994;37(23):3886-8.

199. Cheng JT, Hsu SY, Shen CL. 4-Aminopyridine induces the release of neuropeptide Y (NPY) to produce an atropine- and tetrodotoxin-resistant contraction in rabbit isolated jejunum. Eur J Pharmacol 1989;174(1):1-7.

200. Gonzalez DR, Morales-Aguilera A. Atropine- and tetrodotoxin-resistant motor activity, responsive to 4-aminopyridine, present in isolated rabbit jejunum. Eur J Pharmacol 1985;114(3):389-92.

201. Wallace JL, Keenan CM. Leukotriene B4 potentiates colonic ulceration in the rat. Dig Dis Sci 1990;35(5):622-9.

202. Mascolo N, Izzo AA, Autore G, Barbato F, Capasso F. Nitric oxide and castor oil-induced diarrhea. J Pharmacol Exp Ther 1994;268(1):291-5.

203. Izzo AA, Gaginella TS, Mascolo N, Capasso F. Nitric oxide as a mediator of the laxative action of magnesium sulphate. Br J Pharmacol 1994;113(1):228-32.

204. Kimura H, Miura S, Shigematsu T, Ohkubo N, Tsuzuki Y, Kurose I, Higuchi H, Akiba Y, Hokari R, Hirokawa M, et al. Increased nitric oxide production and inducible nitric oxide synthase activity in colonic mucosa of patients with active ulcerative colitis and Crohn's disease. Dig Dis Sci 1997;42(5):1047-54.

205. De Vera ME, Taylor BS, Wang Q, Shapiro RA, Billiar TR, Geller DA. Dexamethasone suppresses iNOS gene expression by upregulating I-kappa B alpha and inhibiting NF-kappa B. Am J Physiol 1997;273(6 Pt 1):G1290-G1296

206. Finotto S, Mekori YA, Metcalfe DD. Glucocorticoids decrease tissue mast cell number by reducing the production of the c-kit ligand, stem cell factor, by resident cells: in vitro and in vivo evidence in murine systems. J Clin Invest 1997;99(7):1721-8.

207. Nocerino A, Iafusco M, Guandalini S. Cholera toxin-induced small intestinal secretion has a secretory effect on the colon of the rat [see comments]. Gastroenterology 1995;108(1):34-9.

208. Wang S, Yan L, Wesley RA, Danner RL. Nitric oxide increases tumor necrosis factor production in differentiated U937 cells by decreasing cyclic AMP. J Biol Chem 1997;272(9):5959-65.

209. Brown DR, Miller RJ. Handbook of Physiology - The Gastrointestinal System IV. 1990; 24, Neurohormonal control of fluid and electrolyte transport in intestinal mucosa. p. 527-89.

210. Chang EB, Rao MC. Johnson LR, editors.Physiology of the Gastrointestinal Tract. 3 ed. New York: Raven Press; 1994; 62, Intestinal Water and Electrolyte Transport: Mechanisms of Physiological and Adaptive Responses. p. 2027-82.

211. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 1998;115(1):182-205.

212. Traynor TR, Brown DR, O'Grady SM. Effects of inflammatory mediators on electrolyte transport across the porcine distal colon epithelium. J Pharmacol Exp Ther 1993;264(1):61-6.

213. Wardle TD, Hall L, Turnberg LA. Platelet activating factor: release from colonic mucosa in patients with ulcerative colitis and its effect on colonic secretion. Gut 1996;38(3):355-61.

214. Asfaha S, Bell CJ, Wallace JL, MacNaughton WK. Prolonged colonic epithelial hyporesponsiveness after colitis: role of inducible nitric oxide synthase. Am J Physiol 1999;276(3 Pt 1):G703-G710

215. Befus AD, Bienenstock J. Immunologically mediated intestinal mastocytosis in Nippostrongylus brasiliensis-infected rats. Immunology 1979;38(1):95-101.

216. Hogaboam CM, Befus AD, Wallace JL. Intestinal platelet-activating factor synthesis during Nippostrongylus brasiliensis infection in the rat J Lipid Mediat 1991;4(2):211-24.

217. Ogilvie BM, Askenase PW, Rose ME. Basophils and eosinophils in three strains of rats and in athymic (nude) rats following infection with the nematodes Nippostrongylus brasiliensis or Trichinella spiralis. Immunology 1980;39(3):385-9.

218. Ogilvie BM, Mackenzie CD, Love RJ. Lymphocytes and eosinophils in the immune response of rats to initial and subsequent infections with Nippostrongylus brasiliensis. Am J Trop Med Hyg 1977;26(6 Pt 2):61-7.

219. Wallace JL, Chapman K, McKnight W. Limited anti-inflammatory efficacy of cyclo-oxygenase-2 inhibition in carrageenan-airpouch inflammation. Br J Pharmacol 1999;126(5):1200-4.

220. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126(1):131-8.

221. Cartwright CA, McRoberts JA, Mandel KG, Dharmsathaphorn K. Synergistic action of cyclic adenosine monophosphate- and calcium- mediated

chloride secretion in a colonic epithelial cell line. J Clin Invest 1985;76(5):1837-42.

222. Mitchell TJ, Ketley JM, Burdon DW, Candy DC, Stephen J. The effects of Clostridium difficile crude toxins and purified toxin A on stripped rabbit ileal mucosa in Ussing chambers. J Med Microbiol 1987;23(3):199-204.

223. Moore R, Pothoulakis C, LaMont JT, Carlson S, Madara JL. C. difficile toxin A increases intestinal permeability and induces Cl- secretion. Am J Physiol 1990;259(2 Pt 1):G165-G172

224. Moore R, Pothoulakis C, LaMont JT, Carlson S, Madara JL. C. difficile toxin A increases intestinal permeability and induces Cl- secretion. Am J Physiol 1990;259(2 Pt 1):G165-G172

225. Bermeyer HU, Bernt E. Bermeyer HU, editors.New York: Academic Press; 1974;Lactate dehydrogenase: UV-assay with pyruvate and NADH. p. 574-9.

226. Castagliuolo I, LaMont JT, Letourneau R, Kelly C, O'Keane JC, Jaffer A, Theoharides TC, Pothoulakis C. Neuronal involvement in the intestinal effects of Clostridium difficile toxin A and Vibrio cholerae enterotoxin in rat ileum [see comments]. Gastroenterology 1994;107(3):657-65.

227. Schwartz D, Mendonca M, Schwartz I, Xia Y, Satriano J, Wilson CB, Blantz RC. Inhibition of constitutive nitric oxide synthase (NOS) by nitric oxide

generated by inducible NOS after lipopolysaccharide administration provokes renal dysfunction in rats. J Clin Invest 1997;100(2):439-48.

228. Pothoulakis C, Sullivan R, Melnick DA, Triadafilopoulos G, Gadenne AS, Meshulam T, LaMont JT. Clostridium difficile toxin A stimulates intracellular calcium release and chemotactic response in human granulocytes. J Clin Invest 1988;81(6):1741-5.

229. Ovary Z. Ackrody JF, editors.CIOMS Symposium. Oxford: Blackwell Press; 2000;Passive cutaneous anaphylaxis. p. 259-70.

230. Scudamore CL, Thornton EM, McMillan L, Newlands GF, Miller HR. Release of the mucosal mast cell granule chymase, rat mast cell protease-II, during anaphylaxis is associated with the rapid development of paracellular permeability to macromolecules in rat jejunum. J Exp Med 1995;182(6):1871-81.

231. Katayama M, Xu D, Specian RD, Deitch EA. Role of bacterial adherence and the mucus barrier on bacterial translocation: effects of protein malnutrition and endotoxin in rats. Ann Surg 1997;225(3):317-26.

232. Katayama M. Xu D, Specian RD, Deitch EA. Role of bacterial adherence and the mucus barrier on bacterial translocation: effects of protein malnutrition and endotoxin in rats. Ann Surg 1997;225(3):317-26.

233. Davies NM, Roseth AG, Appleyard CB, McKnight W, Del Soldato P, Calignano A, Cirino G, Wallace JL. NO-naproxen vs. naproxen: ulcerogenic,

analgesic and anti-inflammatory effects. Aliment Pharmacol Ther 1997;11(1):69-79.

234. Kristinsson J, Roseth AG, Sundset A, Nygaard K, Loberg EM, Paulsen JE, Aadland E, Fagerhol MK. Granulocyte marker protein is increased in stools from rats with azoxymethane-induced colon cancer. Scand J Gastroenterol 1999;34(12):1216-23.

235. Roseth AG, Fagerhol MK, Aadland E, Schjonsby H. Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study. Scand J Gastroenterol 1992;27(9):793-8.

236. Hughes S, Warhurst G, Turnberg LA, Higgs NB, Giugliano LG, Drasar BS. Clostridium difficile toxin-induced intestinal secretion in rabbit ileum in vitro. Gut 1983;24(2):94-8.

237. Kone BC, Higham S. Nitric oxide inhibits transcription of the Na+-K+-ATPase alpha1-subunit gene in an MTAL cell line. Am J Physiol 1999;276(4 Pt 2):F614-F621

238. Gwee KA, Graham JC, McKendrick MW, Collins SM, Marshall JS, Walters SJ, Read NW. Psychometric scores and persistence of irritable bowel after infectious diarrhoea [see comments]. Lancet 1996;347(8995):150-3.

239. Howard M, Jilling T, DuVall M, Frizzell RA. cAMP-regulated trafficking of epitope-tagged CFTR. Kidney Int 1996;49(6):1642-8.

240. Tousson A, Fuller CM, Benos DJ. Apical recruitment of CFTR in T-84 cells is dependent on cAMP and microtubules but not Ca2+ or microfilaments. J Cell Sci 1996;109 (Pt 6):1325-34.

241. Sundaram U, Knickelbein RG, Dobbins JW. Mechanism of intestinal secretion: effect of cyclic AMP on rabbit ileal crypt and villus cells. Proc Natl Acad Sci U S A 1991;88(14):6249-53.

242. Stewart CP, Turnberg LA. A microelectrode study of responses to secretagogues by epithelial cells on villus and crypt of rat small intestine. Am J Physiol 1989;257(3 Pt 1):G334-G343

243.Mitchell TJ, Ketley JM, Burdon DW, Candy DC, Stephen J. The effects of Clostridium difficile crude toxins and purified toxin A on stripped rabbit ileal mucosa in Ussing chambers. J Med Microbiol 1987;23(3):199-204.

Appendix

THIS DISSERTATION IS A COMPILATION OF THE FOLLOWING MANUSCRIPTS:

Asfaha S, Bell CJ, Wallace JL, MacNaughton WK. Prolonged colonic epithelial hyporesponsiveness after colitis: role of inducible nitric oxide synthase. Am. J. Physiol. 276(39): G703-G710. 1999.

Asfaha S, MacNaughton WK, Appleyard CB, Chadee K, Wallace JL. Persistent epithelial dysfunction and bacterial translocation after resolution of intestinal inflammation. Am. J. Physiol. Submitted.